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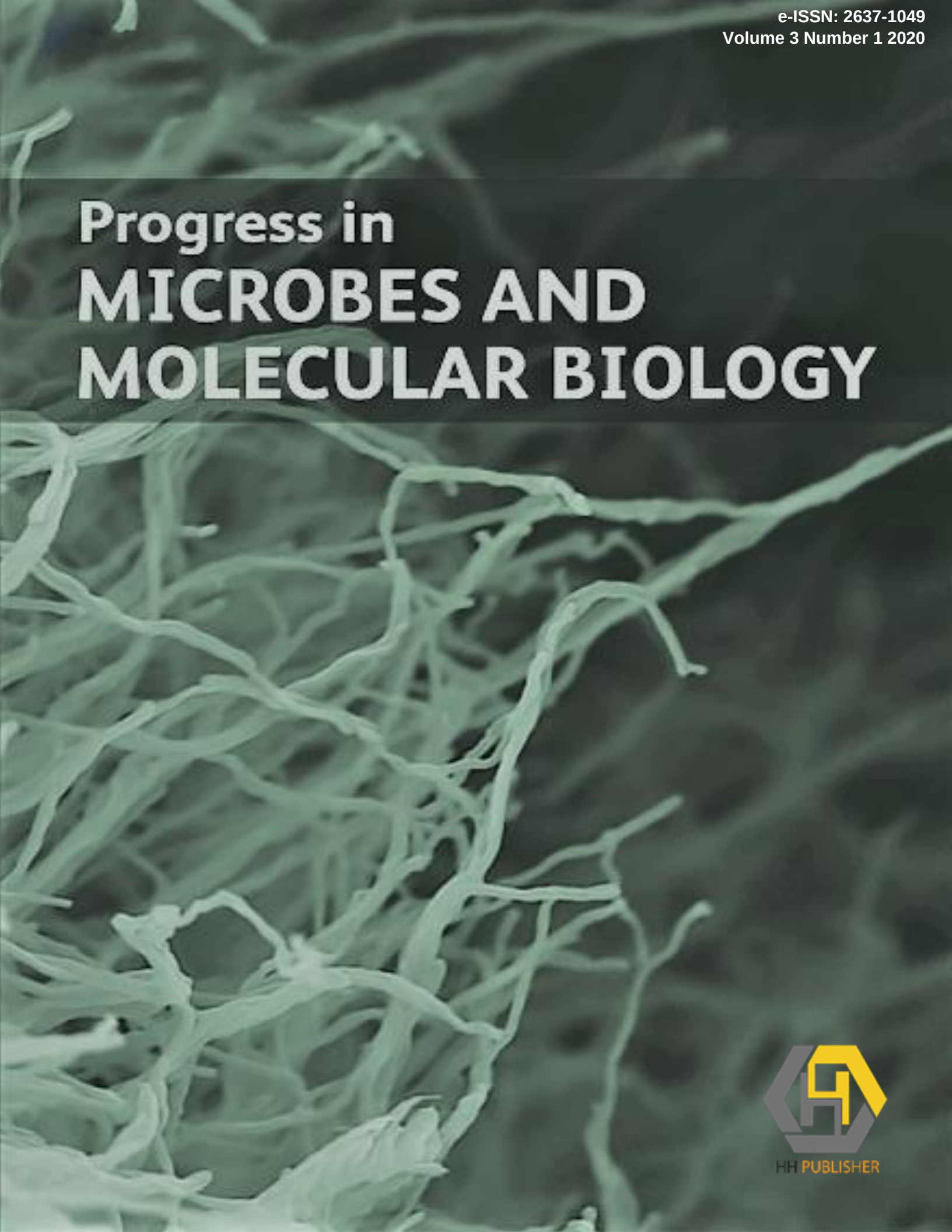

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Editorial Note

Progress in Microbes and Molecular Biology

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PMMB COVID-19 Bulletin: Spain (18th April 2020)

Hooi-Leng Ser[1*], Vengadesh Letchumanan[1], Jodi Woan-Fei Law[1], Loh Teng-Hern Tan[1], Nurul-Syakima Ab Mutalib[2], Learn-Han Lee[1*]

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Abstract: The growing threat of pandemics around the world has contributed to rising uncertainty during various periods of age throughout history, with notable instances such as the Bubonic Plague in the sixth century caused by the pathogen *Yersinia pestis*, and more recently Middle East Respiratory Syndrome (MERS) caused by the coronavirus MERS-CoV in 2012. Fast forward to the last month of 2019, the WHO China Country Office was informed of pneumonia cases with unknown etiology, which later on determined as a new type of coronavirus known as SARS-CoV-2. Zooming into European countries, Spain is no stranger to coronavirus-related pandemics, given that this country was the first to publicly report the 1918 flu pandemic caused by the H1N1 coronavirus. Unfortunately enough, Spain is once again not spared from the current ordeal, recording the highest confirmed cases of COVID-19 (diseased caused by SARS-CoV-2) in Europe, with cumulative cases of 190,839 and 20,002 deaths. The Spanish government has taken counter-measures for the management of COVID-19, consisting of the announcement of "state-of-emergency" and subsequent extension to control movements, setting up research grants to expedite drug and vaccine development in addition to introduction of the use of artificial intelligence as an official channel to provide advice and enquires about COVID-19. These are all part of a collective effort worldwide in alleviating dwindling healthcare resources in hopes of starving the virus of hosts by introducing travel restrictions and movement control as well as giving more time for a cure to be engineered. Through the advancement in technologies, improved trade, heightened human mobility and as well as the spurning of social media, we are able to closely monitor the latest progress of this pandemic; working together to remove the gloomy clouds before us will eventually lead to a solution of this pandemic.

Keywords: COVID-19; novel coronavirus; Spain; pandemic; PMMB

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Main text

As a member of the European Union since January 1986, the population in Spain was reported to reach nearly 47 million with healthy life years (at birth) of 68 years for both male and female in 2018 based on report by World Bank and Eurostat[1,2]. Looking at the total of death (n = 418,516) in 2016, 80.5 % of them occurred in those above age of 70 years or older, while only 3.5 % of these cases were related to communicable diseases (n = 14,847; 95% uncertainty interval = 13,208–16,482)[3]. As a matter of fact, the health reporting system in Spain has evolved much since the infamous health crisis during the World War I, known as the "Spanish Flu" or "La Grippe" and later on 1918 (H1N1) influenza pandemic[4–8]. The estimated worldwide mortality caused by this pandemic ranged from 20 to 100 million deaths. Even though Spain was the first country to publicly report this pandemic in the past, the true source of this pandemic remains a mystery till this date[6,7].

Fast forward to more than 100 years later, another

pandemic is befalling humanity again; on 11th March 2020, the Director-General of World Health Organization (WHO) Dr Tedros Adhanom Ghebreyesus announced the disease COVID-19 to be the latest pandemic, caused by the novel coronavirus, SARS-CoV-2 as the number of cases increased drastically — as high as 13-fold compared to China where first few cases had been reported in December 2019[9–12]. Taking a view at the European countries, Spain was not spared from this novel coronavirus and the first case was reported on 31st January involving a German tourist in the Canary Islands, who has been in contact with people who had travelled to China (Figure 1)[10,11,13]. It was at this juncture the health authorities begun to set an official test protocol to have those experiencing breathing difficulties, fever and with travel history to China's Hubei province over the past 15 days screened. These criteria were later broadened to include those with obvious symptoms and a history of travel to "hot-spots" such as northern Italy. Within two months of the first case, the Spanish government has taken a lot of counter-measures in trying to reduce the widespread, including school shutdowns and later on flight control along with gatherings (of more than 1,000 people) at closed venues in hardest-hit hit areas. By the second week of March, the European countries have reported more than 5,000 cases, with Spain ranking second in terms of most badly affected countries[11,13,14]. On the 14th March, the Spanish government used a Royal Decree (463/2020) to declare a "state-of-emergency", starting on 15th March which only allowed people to drive alone to perform basic needs activities such as procuring food or medication, attending health centres and financial institutions, returning to one's primary residence and caring for vulnerable people[15,16]. Another exception was those who are working in essential services. However, despite all of these measures, the number of confirmed cases in Spain continued to rise, leading to the subsequent announcement of extension on the "state-of-emergency" till midnight of 12th April and non-essential workers were ordered to remain indoors[17]. Along with these major announcements, the Spanish health ministry has also been constantly updating the general public regarding information on the disease along with advice on personal hygiene and preventive measures. The incorporation of artificial intelligence in management of the pandemic in Spain has also been deemed to be an important step to provide official response and inspire confidence in the public[18]. The conversational assistant known as Hispabot-Covid19 is a "chatbot service" set up by the government, allowing individuals to obtain immediate response about COVID-19 via Whatsapp app, regardless of time and place.

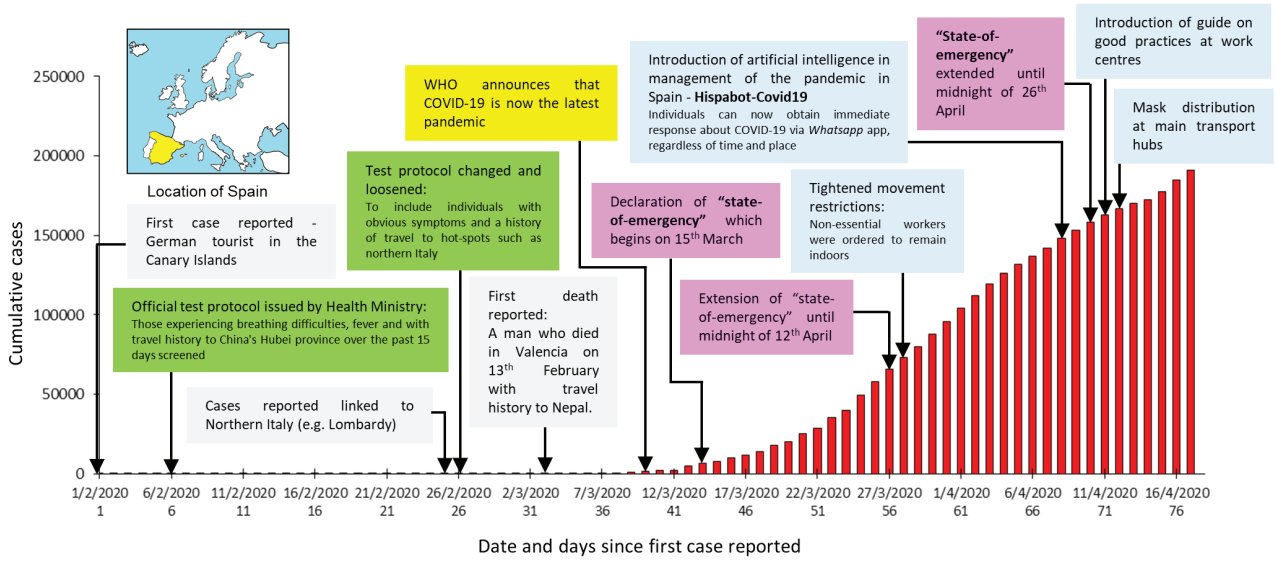

Figure 1. Graph of confirmed cases and important events including actions taken by the Spanish government for the management of COVID-19 (Data on number of confirmed cases retrieved from Coronavirus Resource Center, John Hopkins University of Medicine).

In a televised address to the nation, the Spanish Prime Minister Pedro Sanchez pointed out that the "lockdown" has begun to show positive results noted by the "the start of the decrease in the epidemic"[19,20]. Shortly after the approval of a Royal Decree to extend the state of emergency until midnight of 26th April by the Extraordinary Council of Ministers, the government published "a comprehensive guide on good practices in work centres to prevent the spread of COVID-19, coinciding with the return of their work force, on Monday (13th April) or Tuesday (14th April), encompassing all those workers in non-essential activities that cannot work from home"[21,22]. Besides promoting the "essential distancing" of 2 metres, the guideline recommends "travelling to work by means that do not involve more than two people being in the same place" and encourages individuals to take the appropriate hygiene measures including avoiding more than one person using a row of seats for those travelling in a car or in a vehicle for hire and for those commute via public transport are recommended to use a non-medical face mask. In the same announcement, the government has also emphasized that an individual who has symptoms or have been in close contact with people affected by the virus are recommended not to go to the work centre until he/she is confirmed not to be at risk or a risk to others, and also encouraged individuals to contact the COVID-19 hotline (available in each region) and refer to the published list of 10 actions (https://www.mscbs.gob.es/

profesionales/saludPublica/ccayes/alertasActual/nCov-China/documentos/20200325_Decalogo_como_actuar_COVID19.pdf) in the event of displaying symptoms.

Apart from ensuring supply of materials such as personal protective equipment, detection kits and ventilators to regional health authorities, the government has currently appointed several logistical points around the country to distribute a total of 10 million face masks at main public transport hubs "to those people who need to travel to work on those means of transport where it is more complex to respect the safety distance"[23–25]. Having that said, the Ministry of Health still recommends working from home as the top priority, while the State law enforcement officers will be performing corresponding checks at suburban and underground stations and at transport hubs to make sure that only authorized traveller are commuting under the Royal Decree on the state of emergency. The Spanish Minister of Health Salvador Illa has also pointed out in early April that the government would not make the use of face masks compulsory, unless the public can gain access to them easily and the ministry is still studying the measure before rolling it out[26,27].

The COVID-19 pandemic is indiscriminate, posing as an "unseen threat" to every nation in the world. As of 18th April, the total number of confirmed cases in Spain is recorded at 190,839 and death cases were reported to be 20,002 according to the data reported by Coronavirus Resource Center, John Hopkins University of Medicine[13, 14]. The Spanish government has recently expressed their support to the work of WHO to fight this crisis. As the Public Research Organization of the government, the Carlos III Health Institute has approved another four new research projects to tackle SARS-CoV-2 and is now supporting 15 research projects under the COVID-19 Fund with a total allocation of 5 million euros[28]. For instance, one of the newly financed project carried out by University of Seville, Institute of Biomedicine of Seville and Group of Technical Specialists in the Deactivation of Explosive Artefacts of the National Police is looking at building optical visualisation system to detect residues of virus, by using cameras that cater for different light spectrum. On the other hand, the Institute for Biomedical Research and Innovation of Cadiz is exploring the potential use of nano-sensors as diagnostic tool to identify immunoglobulin antibodies (IgG) which are produced by the host upon exposure to the virus. Another two latest research projects are more focused at therapeutic interventions — (a) carrying evaluation on approved medication(s) which could be effective against COVID-19 via supercomputing, and (b) looking into possible modification of a Spanish-produced vaccine, known as MTBVAC (which is being developed against tuberculosis) to be used in the battle against COVID-19. It is particularly exciting for the vaccine development project carried out the University of Zaragoza as the invention is already in in its final pre-clinical phase. In the event that the vaccine is capable of generating non-specific immunity against SARS-CoV-2, this could mean a vital breakthrough that permits next phase of clinical trial in humans.

Tackling a pandemic is surely not an easy task. Based on the protocols established by the WHO, every nation can be a "Noah", setting up surveillance system for diseases and notifying the WHO in a timely manner to ensure adequate preparedness plans[29–31]. With leaders over the world indicating that "failure is not as option", it is indeed dire times now as the spread of the virus cripples cities around and crashes the economies worldwide[32,33]. It may not be saving astronauts or rescuing animals as in the movies but instead focusing on real life which is redefining the earth's inhabitant way of life as we write. With every nation joining hands together in this battle against the "unseen enemy", it will just be a matter of time for the scientists and clinicians to discover the panacea — be it vaccine or drug to tackle this life-threatening coronavirus.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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Editorial Note

Progress in Microbes and Molecular Biology

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PMMB COVID-19 Bulletin: United Kingdom (22nd April 2020)

Loh Teng-Hern Tan[1*], Vengadesh Letchumanan[1], Hooi-Leng Ser[1], Jodi Woan-Fei Law[1], Nurul-Syakima Ab Mutalib[2], Learn-Han Lee[1*]

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Abstract: COVID-19 has greatly impacted the world and posed an enormous public health threat. The United Kingdom is hit harder by the COVID-19 crisis than any other European countries, besides Italy, Spain and France. The UK government has come under heavy criticism for its response to COVID-19, with lack of preparedness, shortages of personal protective equipment and COVID-19 testing. Despite the lockdown is in place to slow the spread of COVID-19, UK death toll continues to surge. As of 21st April 2020, more than 120,000 confirmed COVID-19 cases and 16,000 deaths had been recorded in UK.

Keywords: Novel coronavirus; SAR-CoV-2; COVID-19; United Kingdom; PMMB.

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Main text

On the late December 2019, the novel coronavirus SAR-CoV-2, identified as the cause of an outbreak of severe pneumonia in China, has quickly spread to all corners of the world. By 21st April, the number of reported confirmed cases of COVID-19 has exceeded 2.3 million globally[1,2]. COVID-19 has greatly impacted the world and posed an enormous public health threat which has not been seen in a respiratory virus ever since the 1918 H1N1 influenza pandemic[3]. After the assessment of the alarming levels of spread and severity by WHO, COVID-19 is characterized as a pandemic with more than 118,000 cases in 114 countries, and 4291 deaths recorded on 11th March 2020. SAR-CoV-2 becomes the first coronavirus which causes a pandemic. On 13th March, Europe became the epicentre of the pandemic[4] with more reported cases and deaths than the rest of the world combined, excluding China, reported by Dr. Tedros Adhanom Ghebreyesus (Director-General of WHO).

Since the beginning of the outbreak at the end of January, the world has been alarmed by the clear message from China and the scientific modelling study[5] that the COVID-19 was on the trajectory to become a global epidemic. However, within the month of February, the absence of isolation, quarantine and control tracing had further exacerbated the situation in United Kingdom (UK) leading to the upsurge of COVID-19 confirmed cases and number of critically ill patients overwhelm the healthcare system of the country. The SARS-COV-2 virus does not discriminate between social classes, nationalities, ethnicities or ideologies, as shown by the rapid domino effect of infections in UK government officials, even the Prime Minister, Boris Johnson succumbed to COVID-19 and was admitted to intensive care on 6th April 2020[6]. As of 21st April 2020, more than 120,000 confirmed COVID-19 cases and 16,000 deaths had been recorded in UK[1].

The first case of COVID-19 in UK was confirmed on 31st January 2020 (Figure 1), consisted of two members of a family of Chinese nationals staying in a hotel in York. They were identified in the community and

transferred to the regional Infectious Disease Unit at Hull University Teaching Hospitals directly from their hotel[7]. The index case (A), who is a 50-year-old healthy female, entered UK on 23rd January 2020 without any symptoms. However, she developed symptoms of fever, sore throat, dry cough and malaise on the third day of arrival. Meanwhile, a previously healthy 23-year-old male (case B) returned to UK from Hubei province on 6th January 2020, who had a close contact with case A on 28th January 2020, developed myalgia and dry cough. Promptly, case B sought advice from the National Health Service (NHS) and followed by admission to hospital due to being possibly at risk of COVID-19[8]. Although the first cases in UK was identified without any clear case definitions, the decision to test was performed purely because of high clinical suspicion and information about the distribution of infection. However, this show that case definitions should evolve rapidly with any newly emerging infection[7]

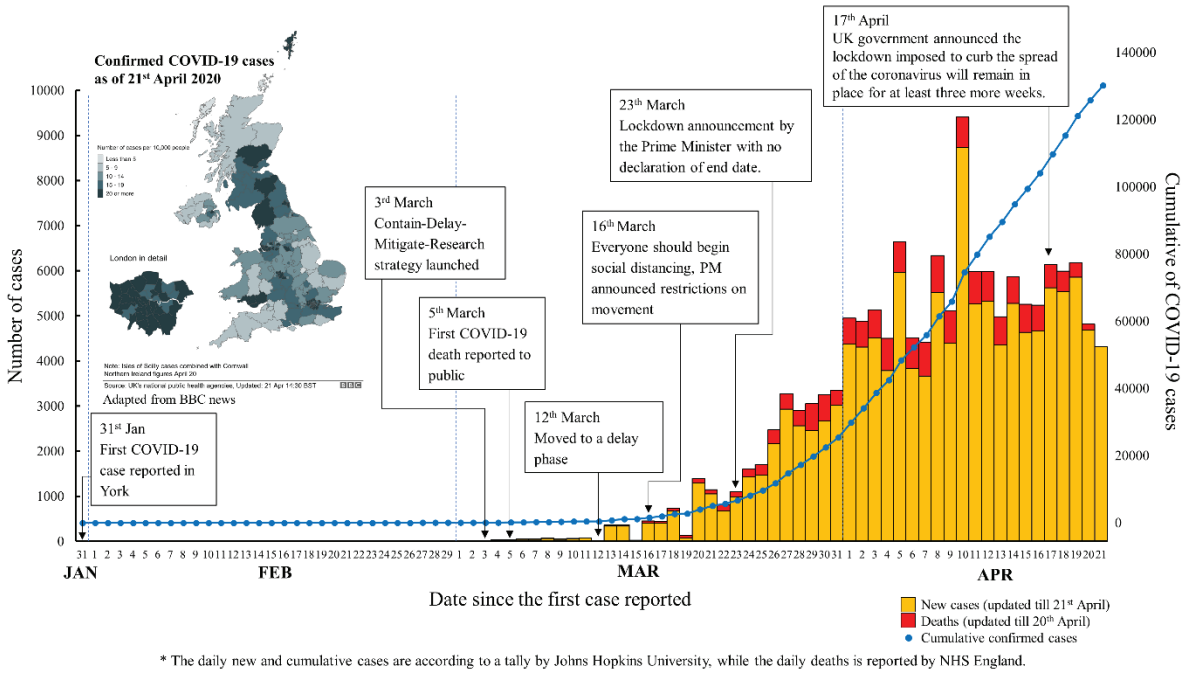

Figure 1. Illustrations of confirmed COVID-19 cases and deaths and the brief response plan taken by the UK government. The stacked bar graph shows the daily confirmed cases and deaths since the first case reported in UK while the line graph shows the cumulative confirmed cases. A heat map shows the distribution of confirmed cases across UK as of 21st April 2020.

As the COVID-19 pandemic progresses, the global community started implementing a broad range of measures depending on their own capacity in the respect of healthcare system, financial status, socio-political networks and self-sufficiency[9]. Countries like Singapore and South Korea have achieved relative control over the virus because of widespread testing, contact tracing and social distancing measures. The WHO-China Joint Mission on Coronavirus Disease also successfully demonstrated that immediate and decisive public health responses are critical to hinder or delay hundreds of thousands of cases in China[10]. Meanwhile, the UK government faces mounting criticism and rigorous scrutiny of its actions in preparations for and response to COVID-19. For instance, "When this is all over, the NHS England board should resign in their entirety," quoted by the Editor-in-Chief Richard Horton of *The Lancet*, expressing his disappointment over the inefficient response taken by the UK Government amid the COVID-19 pandemic[11]. He also commented that the failure of Contain-Delay-Mitigate-Research strategy, which was launched on 3rd March 2020 by the UK government as the preparedness plan for tackling COVID-19, was attributed to the relatively lower testing capacity in UK. Richard Horton also expressed his concern over the new plan of UK government shifting to Suppress-Shield-Treat-Palliate may be too late to deal with the consequence of this pandemic with the lack of preparedness, putting the lives of front-line staff and patients at stake[11].

The UK government launched action plans for tackling COVID-19 which includes four phases: contain, delay, research and mitigate[12]. The initial containment measures aimed to prevent COVID-19 from spreading by implementing approaches include the early detection, isolation and care of the infected patients and coupled with contact tracing and screening. These approaches have seen to be effective in country like China which implemented one of the so-called 'draconian' and controversial containment approaches by almost forcing the entire population to stay home. Although many argued that these extreme measures are unlikely to be replicated in Europe, which curtail human rights and cripple economies, Italy government eventually had resorted to impose the national lockdown measures on 9th March 2020 as a consequence of the overstraining of the country's healthcare system[13].

Case finding, contact tracing and testing and strict quarantine are the imperative measures in public health to control infectious diseases[14]. However, contact tracing started in the UK but stopped early in the epidemic, due to the questioning on its effectiveness. The reasons of

discontinuation of control tracing, which is against the WHO recommendation, have not been explained by the UK government. It seems to be linked to the shift from "contain" to "delay" by the UK government's action plan on 12th March 2020, when control tracing was substituted instead of coupling with other control measures[14]. Despite facing the criticisms on the slow decision made to move to delay from contain phase, the delay phase aims to slow the spread and push the peak of cases towards the spring and summer months to reduce pressure on the already overwhelmed NHS, in order to give time the researchers to comprehend the virus and potentially reduce the transmission given there is a seasonal element – as happens with influenza[7].

A modelling study by the researchers at Imperial College London has forced the UK government to shift their response plans for COVID-19, showing that the UK's heath service would soon be overwhelmed with severe cases of COVID-19 and resulted more than 500,000 deaths if no alternatives are taken by the government[15]. Almost immediately, the Prime Minister Boris Johnson announced new stringent restrictions on the movement of populations on 16th March 2020[16]. 'Urging everyone to practice social distancing and avoiding contact with others, households quarantine for 14 days if develops symptoms of COVID-19 and isolation of the high risk group (pregnant women and aged over 70) for 12 weeks', represented a critical factor in jolting the UK government into changing its policy which had previously only told people with symptoms to isolate at home for a week and suggested people over 70 may have to self-isolate[16]. Moreover, the study also suggests that population-wide social distancing applied to the population has the largest impact to reduce onward transmission. In addition, the combination of population-wide social distancing with other measures, such as self-isolation of cases and closure of school and university, which require the minimum policy has the potential to suppress transmission below the threshold of R=1 and effectively reduce the case incidence[15]. On 23rd March 2020, the Prime Minister officially announced to place UK in a state of lockdown, whereby all non-essential businesses are to close with immediate effect and residents are only allowed to go out to shop for essential items. The UK government's decision to impose this restrictions came under the increasing pressure to curb the spread of the virus and the joint efforts by the public health specialist, epidemiologists, scientist and doctors urging the government with the evidence on the best strategy to flatten the peak of COVID-19 outbreaks and to widen COVID-19 testing[17]. Initially, there were even reports on whether it is worthwhile to impose restriction, while also reports on "zero prospect" of lockdown of the city London[18]. Nevertheless, this enforced social distancing is absolutely crucial to save lives, to protect vulnerable in society and to ensure the healthcare system can cope and care for patients. According to another modelling study, a lockdown scenario of 8 days (17th March 2020) earlier than on the 24th March 2020 would have reduced the deaths from over 81,000 to below 19,000 by the end of a 12 week lockdown[19].

Apart from the lockdown intended to ease the pressure on hospitals, redeployment of clinical staff, recalling of retired doctors, newly graduated medical students and many clinicians may also be asked to practise outside their defined areas of expertise in responding to the unprecedented hospital demand[20]. In this challenging time, the situation in the healthcare setting is further compounded by the apparent increased risk of infection among healthcare workers, the understaffed service because of illness or self-isolation as well as shortages of personal protective equipment (PPE) in the past month. Despite the government has repeatedly assured the healthcare workers that millions of units of PPE have been delivered to the frontline[21], a survey by British Medical Association (BMA) showing that more than half of doctors working in high risk environment with either shortages or no supply of adequate face masks and no access to eye protection[22].

In addition to unpreparedness of the government and shortages of personal protective equipment, UK is also falling short on testing. Testing has regarded as one of the most effective strategies to curb with the rapid spreading COVID-19, thereby it offers the authorities the opportunity to isolate the infected patients and stem the spread of COVID-19. 'Test, test, test' is the mantra of the WHO and other countries in response to the pandemic[13]. Meanwhile, tests for healthcare workers are only becoming available in UK. South Korea has been among the most aggressive when it comes to testing, in contrary to the early stages in UK - where the government officials had even talked up a theory of allowing the disease to spread while protect the vulnerable in the society. As of 20th March 2020, there were only around 957 tests per million in UK as compared to countries like South Korea (6182 tests per million), Italy (3019 tests per million) and Australia (4294 test per million) [23]. On 2nd April 2020, reports showed that only 2,000 NHS frontline workers out of about half a million have been tested for coronavirus. On the same day, the health secretary, Matt Hancock, pledged that there will be 100,000 tests for coronavirus available daily by end of the month of April across UK [24].

At the time of writing, various countries are wondering when and how to ease coronavirus lockdowns as curves flatten and COVID-19 cases start to fall in some European countries, including Denmark, Germany, Switzerland and Austria[25]. The WHO has also warned that lifting the lockdowns should be done slowly and only when additional capacity in the healthcare system is in place to isolate cases and contact-tracing[26]. Meanwhile, the UK will probably have to maintain some level of social distancing for another 18 months or until a vaccine for the novel coronavirus is available, Neil Ferguson said, an epidemiologist and government adviser from Imperial College, who has helped shape the government's response to the pandemic. Furthermore, the health secretary also commented on 16th April that it was still too early to lift the lockdown[27].

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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Editorial Note

Progress in Microbes and Molecular Biology

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Efficacy of chlorine dioxide as a disinfectant

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Abstract: Chlorine dioxide plays a significant role in the industrial settings as disinfectants due to its broad antimicrobial property. Despite commonly use as germicide, chlorine dioxide demonstrates a good safety profile, rendering its suitability for use at water treatment and food preparation zones. Protein denaturation including envelope proteins is the major mechanism of chlorine dioxide to inactivate microorganisms even at low concentrations. Adverse reactions are not widely reported due to the typical use at a low concentration. The effectiveness of chlorine dioxide against various microorganisms, in both liquid and gaseous forms, over a wide range of pH and at an extremely low concentration has confirmed chlorine dioxide as a vital and versatile disinfectant.

Keywords: Disinfectants; Chlorine dioxide; SARS-CoV; Anti-microbial

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Introduction

Chlorine dioxide (ClO_2), a strong oxidant, has a long-standing significant role in the industrial settings as disinfectants, especially at water purification and food preparation areas, due to its wide spectrum antimicrobial property [1]. The significance of its antimicrobial role is further enhanced during the outbreak of *Bacillus anthracis* in 2001, when fumigation of vaporous hydrogen peroxide and ClO_2 was used to destroy *B. anthracis* spores, along with a combination of HEPA vacuuming, cleaning and bleach application. In this article, we revisit the efficacy and safety of ClO_2 as a suitable sanitizing agent during the current COVID-19 pandemic crisis.

Recently, it is shown that ClO_2 is a potent disinfectant in preventing infectious disease outbreaks due to its oxidative properties in eliminating microbes [2]. In gaseous form, ClO_2 is able to eliminate culturable bacteria and detoxify bioterrorism agents such as *Bacillus* spores. Moreover, ClO_2, when used as a disinfectant on surfaces, has been reported to exhibit antimicrobial properties against various kinds of microbes efficiently even in wet environments. A concentration of 700-1100 ppm of ClO_2 is also a feasible alternative in replacing glutaraldehyde-based disinfectants [3, 4]. Aside from its possibility as a potent disinfectant, ClO_2 has been reported to be a disinfectant without causing side effects due to its rapid action and safe antimicrobial properties [5]. Furthermore, ClO_2 can be used a keratolytic compound with anti-inflammatory properties but is non-toxic to human tissue [6]. In addition, this chemical compound has a history of being used in water purification and disinfection process during food preparation due to its wide spectrum of antimicrobial effects. ClO_2 is also found to be more effective than hydrogen peroxide as bleaching agent of teeth [7].

A very recent review on dermatologic reactions to various types of disinfectants used to reduce the risk of coronavirus infection indicated that ClO_2 is safe, even with prolonged skin contact [8]. ClO_2 solution is also closely examined for its potential use to inactivate viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [9] and human papillomavirus (HPV) [10]. Interestingly, ClO_2 solution was also used to sterilize recycled KN95s or surgical face masks during critical shortage of such supplies [2].

Mechanisms behind the efficacy of ClO_2 against microbes

A study revealed that due to interactions between ClO_2 and biological thiol groups of amino acids, bacteria are unable to develop resistance against ClO_2 [5].

ClO_2-based disinfectants have been shown to be effective in eliminating *B. anthracis* spores in solutions and less toxic than sodium hypochlorite in an *in vitro* study with human skin keratinocytes [11]. Besides, another study has shown that virus inactivation by ClO_2 is achieved through denaturation of viral envelope proteins, thereby able to prevent aerosol-induced influenza virus infection at low concentrations [12].

The potential role of ClO_2 in completely inactivating porcine reproductive and respiratory syndrome virus (PRRSV) was demonstrated through the action of degrading the genome and proteins of the virus [4]. This study also confirmed that the expression of inflammatory cytokines induced by this virus can be reduced by ClO_2. This is further supported by studies reporting protein-denaturing activities due to covalent oxidative modification of cysteine, tryptophan and tyrosine residues of model proteins (bovine serum albumin and G6PD of *Saccharomyces cerevisiae*) as the mechanism behind the efficacy of ClO_2 against microbes [13,14]. Furthermore, 0.03 ppm of ClO_2 has been indicated to prevent aerosol-induced influenza A virus by denaturing the envelope proteins of the virus [12]. The mechanism of norovirus inactivation by ClO_2 is attained through degradation of viral protein, including viral genomic RNA and disruption of viral strucutre [15]. In addition, an observation on ClO_2-reduced lysozyme activities showed the potential role of ClO_2 in denaturation and degradation of protein using Raman spectroscopy and gel electrophoresis [16].

Revisit the literature

The wide spectrum of antimicrobial properties of ClO_2 is supported by its ability to inactivate various kinds of microbes including Gram-positive and Gram-negative bacteria, enveloped and non-enveloped viruses in low concentrations (as low as 0.05 ppm) and wet states [17]. In a quantitative bactericidal suspension test, it is demonstrated that vegetative forms of bacteria such as *Staphylococcus aureus* and *Escherichia coli* can be killed in the 100 mg/L of ClO_2 solution [18]. Furthermore, ClO_2 concentration at as low as 0.03 ppm is effective against aerosol-induced influenza virus infection in a study with mice models [12]. However, concentrations of ClO_2 equal to or more than 0.6 mg/L are required for a complete inactivation of viruses such as hepatitis A viruses, Norwalk and Norwalk-like viruses [19]. A 3-log reduction in murine norovirus 1 (MNV-1) was found when stainless steel contact surfaces were treated with ClO_2 gas at 2 mg/L for 5 minutes and 2.5 mg/L for 2 minutes while a complete virus inactivation was shown in a 1-minute treatment with 4 mg/L of ClO_2 gas [15]. Although free residues of chlorine over a concentration of 2.19 mg/L of ClO_2 in wastewater do not entirely inactivating *E. coli* and f2 phage, it is able to completely inactivate SARS-CoV [20].

Low-concentration ClO_2 gas (mean 0.05 ppmv, 0.14 mg/ m^3) treatment in an area with a high humidity is useful in decreasing the risk of infection by norovirus without side effects [17]. Furthermore, propidium monoazide (PMAxx)-viability RTqPCR assay revealed that ClO_2 is effective to a certain level in inactivating genogroup I and II Human norovirus (HuNoV) strains on contaminated food [21]. Laboratory investigations also showed that the counts of natural or inoculated microbes including bacteria, yeast and mold can be reduced effectively in the range of 1-5 log by using 3-100 ppm of ClO_2 solution [22]. Elimination of *B. subtilis* spores by ClO_2 was found due to damages to its membrane but no DNA damage [23]. Also, a study on the disinfection of wastewater revealed that ClO_2 is capable of inactivating bacteria such as coliforms although not as effective as chlorine of the same dosage [20].

Concluding Remarks

The broad-range activity against microorganisms, effectiveness over a wide pH range (pH 4.0-10.0), rapid action together with the versatility of using it in either liquid or gaseous state have conferred ClO_2 as an important disinfectant for daily use in the past two decades. In comparison to other disinfectants, ClO_2 offers a higher antimicrobial performance at an extremely low concentration with no microbial resistance developed at present.

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Conflicts of Interest

The authors declare that there is no conflict of interest in this work.

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Authors' Contributions

Methodology, Investigation, Writing. SY, CFG, LCM; Validation, Writing — review & editing. SY, YCL, CFG, VK, LCM.

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Original Research Article

Progress in Microbes and Molecular Biology

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In-depth characterization of miRNome in papillary thyroid cancer with BRAF V600E mutation

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Abstract: MicroRNAs (miRNAs) are small non-coding RNAs, which play a critical regulatory role in papillary thyroid carcinoma (PTC). BRAF V600E is a hotspot mutation occurring in a majority of PTC cases and is proposed to be associated with poor clinical outcomes. The relationship between BRAF V600E status and miRNA expression in PTC has not been comprehensively studied. In this study, we aimed to identify the differentially expressed miRNAs in PTCs with and without BRAF V600E in an unbiased manner. Five fresh frozen thyroid cancer tissues paired with their respective adjacent normal tissues from PTC patients were subjected to BRAF V600E genotyping using Sanger sequencing and small RNA deep sequencing (miRNAseq). MiRNAs differentially expressed between BRAF V600E-positive and BRAF V600E-negative PTC tissues were validated in silico using The Cancer Genome Atlas (TCGA) THCA datasets containing 420 samples. MiRNA target prediction and pathway enrichment analysis were performed to identify biological pathways altered in this cancer. We identified 174 differentially expressed miRNAs; 80 were significantly over-expressed, while 94 were under-expressed (adj. p-value < 0.1; log2 fold change ≤ -1 or ≥ 1). Fifteen miRNAs were significantly differentially expressed only in BRAF V600E-positive PTC, and eight of these were validated in TCGA THCA dataset (hsa-miR-212, -132, -135b-3p/5p, -200b, -200a-3p/5p, -27a-3p/5p, -29a and -1296). Subsequent analysis revealed significant enrichment of cancer-related pathways including proteoglycans in cancer, ECM-receptor interaction and MAPK pathways in BRAF V600E-positive PTC. Using the miRNAseq and in silico validation using TCGA THCA study, we identified eight miRNAs that were differentially expressed in PTC tissues with BRAF V600E. This study also complemented the existing knowledge about deregulated miRNAs in PTC development.

Keywords: microRNA; papillary thyroid cancer; BRAF V600E, next-generation sequencing.

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Introduction

The incidence rate of thyroid cancer had risen significantly in many countries worldwide in the past decade. In 2012, there were approximately 230,000 and 70,000 new cases of thyroid cancer among women and men, respectively [1,2]. According to the United States Cancer Statistics 2019, 52,070 new thyroid cancer cases with 2,170 deaths were estimated by 2019 in both sexes [3]. Most thyroid cancers originated from follicular epithelial cells, which were further divided into well-differentiated papillary thyroid carcinoma and follicular carcinoma, poorly differentiated carcinoma and anaplastic carcinoma [4–6]. Papillary thyroid carcinoma (PTC) is the most prevalent histological type that contributed to 85 to 90% of reported cases [7]. Several of its clinicopathological features are correlated to poor prognosis in PTC patients, which include the male gender, larger tumour size, older age, extrathyroidal extension, thyroid capsule invasion,

lymph node metastasis as well as *BRAF* mutation [8]. Although PTC patients had a high survival rate [1,9], these prognostic factors had been shown to affect the overall survival among PTC patients [10].

MiRNAs are small non-coding RNAs that comprise of 19 to 22 nucleotides, which negatively regulate gene expression. Each miRNA can take part in many cellular pathways, and thus, miRNAs are involved in many different diseases [11] including thyroid cancers [12–15]. In addition, it was reported that different histopathological types of thyroid tumours have discrete miRNA profiles [16]. miR-146b [17,18], miR-221 and miR-222 are among the commonly upregulated miRNAs in papillary thyroid carcinoma [16,19–23]. These non-coding RNAs are promising biomarkers to identify aggressive PTC cases [17,24].

BRAF is a serine-threonine kinase that is activated by RAS binding and protein recruitment to the cell membrane [25,26]. Activation of MEK along with the MAPK signalling pathway is activated by BRAF phosphorylation [27]. The most frequent genetic changes in PTC are point mutations of *BRAF* which are observed in 35 to 70% of PTC cases [26,28]. More than 95% of *BRAF* mutations detected in thyroid cancers are thymine to adenine transversion at position 1799 (T1799A), resulting in the substitution of valine by glutamate at residue 600 (V600E) [29–31]. Various studies had shown that the *BRAF* V600E mutation was associated with lymph node metastasis, therefore, it had received the attention as a diagnostic and prognostic molecular marker in recent years to improve the diagnosis and identify individuals at increased risk of PTC recurrence [30,32].

In the past few years, the evolution of next-generation sequencing technologies has allowed global expression profiling of miRNAs [33] and the discovery of novel human miRNAs [14,34]. In this study, we aimed to identify the differentially expressed miRNAs in PTCs with *BRAF* V600E using small RNA sequencing. To achieve this objective, five fresh-frozen tumour tissues paired with their normal adjacent thyroid tissues were included in the study. The mutational analysis of *BRAF V600E* gene and differentially expressed miRNAs were performed using Sanger sequencing and small RNA sequencing, respectively. The results were validated with an *in silico* approach using the dataset obtained from The Cancer Genome Atlas (TCGA) THCA.

Materials and Methods

Clinical Specimens

Five pairs of tumour-adjacent normal fresh frozen tissues were collected from patients diagnosed with PTC from the UKM Medical Centre (UKMMC). This study was approved by the Universiti Kebangsaan Malaysia Research Ethics Committee (UKMREC; UKM 1.5.3.5/244/UMBI-2015-002). Informed consent was obtained from all the study participants. The tissues were dissected, snap-frozen and stored in liquid nitrogen. All samples were cryosectioned and stained using haematoxylin and eosin and the percentage of tumour cells and normal cells contents were assessed by a pathologist. Only tumour samples with at least 80% cancerous cells and normal adjacent thyroid tissues with less than 20% necrosis were selected for further analysis.

Nucleic Acid Isolation

All fresh frozen tissues were subjected to nucleic acid extraction using Allprep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The integrity of RNA was assessed using Agilent BioAnalyzer 2100 (Agilent Technologies, CA, U.S.), while the quality of DNA was assessed using 1% agarose gel. The quantity and purity of RNA and DNA were assessed using Qubit 2.0 fluorometer (Thermo Scientific, MA, U.S.) and Nanodrop 2000c Spectrometer (Thermo Scientific, MA, U.S.), respectively.

BRAF V600E Genotyping

PCR amplification of genomic regions of interest was performed using *BRAF* V600E forward primer 5'-TGCTTGCTCTGATAGGAAAATG-3' and *BRAF* V600E reverse primer 5'-AGCATCTCAGGGCCAAAAAT-3' [35]. Amplification was performed in a reaction volume of 25 µl containing 50 ng DNA template, 10 µM each primer (Integrated DNA Technologies, IA, U.S.),10x PCR Gold Buffer without $MgCl_2$, dNTP Mix (10 mM), $MgCl_2$ solution (25 mM) , AmpliTaq Gold® (5U/µl) (Applied Biosystems, CA, U.S.) and nuclease-free water. PCR conditions were as follows; 95°C for 4 minutes; 35 cycles of 95°C for 45 seconds, 50°C for 30 seconds and 72°C for 1 minute; 72°C for 5 minutes; and held at 4°C. PCR products were visualized by electrophoresis on 1.5% agarose gel with an expected size of ~ 228 bp. PCR purification was conducted using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) as per manufacturer's instruction. Subsequently, DNA sequencing was performed using ABI Prism 3130xl Genetic Analyzer (Applied Biosystem, CA, USA).

Library Preparation and miRNA Sequencing

RNA samples from tumour samples and their adjacent normal tissues were processed into libraries using TruSeq Small RNA Sample Prep Kit (Illumina, CA, USA). Briefly, 3′ and 5′ adapters were sequentially ligated to the ends of small RNAs fractionated from 2 µg of total RNA, and reverse transcribed to generate cDNA. The cDNA was amplified using a common primer complementary to the 3′ adapter, and a primer containing 1 of 48 index sequences. Samples were size-selected (140–160 bp fragments) on a 6% polyacrylamide gel, purified, quantified and pooled for multiplexed sequencing. The resulting pooled libraries were normalized to 2 nM and were hybridized to oligonucleotide-coated single-read flow cells for cluster generation using HiSeq® Rapid SR Cluster Kit v2 on Hiseq 2500 (Illumina, CA, USA). Subsequently, the clustered pooled miRNA libraries were sequenced on the HiSeq 2500 for 50 sequencing cycles using HiSeq® Rapid SBS Kit v2 (50 Cycle) (Illumina, CA, USA).

Bioinformatics and Statistical Analyses

Pre-processing of data was executed in BaseSpace software (Illumina, CA, USA), and FASTQ files were generated. miRNA Analysis app version 1.0.0 was used for determination of differentially expressed miRNAs using the workflow described by Cordero *et al*, 2012 [25]. Briefly, the pipeline includes 3' end adapter removal using Cutadapt, annotation to miRBase v21, mapping using SHRIMP aligner and differential analysis of miRNAs using DESeq2. Benjamini and Hochberg's [36] correction was applied to ensure a false discovery rate (FDR) less than 0.1 and absolute log2 fold change ≤ -1 or ≥ 1 were considered for further analysis. Heatmaps were created using GeneE from the Broad Institute (http://www.broadinstitute.org/cancer/software/GENE-E). MiRNA target prediction via DIANA-TarBase v7.0 [37] and pathway enrichment analysis was performed using DIANA-miRPath v3.0 [38]. Other statistical analyses were performed using GraphPad Prism 6 unless stated otherwise.

In silico Validation

We used the TCGA-generated level 3 miRNA sequencing data from THCA project [24]. These data were accessed from 'https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm' on April 13, 2016. The normalised expression (reads per million or RPM) of all miRNAs was log2-transformed and used for fold change calculation. We then performed the Students' unpaired t-test with Benjamini Hochberg false discovery rate (FDR) multiple testing correction and log2 fold change calculation using Bioconductor version 3.1 (BiocInstaller 1.18.2) [39] in R version 3.2.0 (R Development Core Team, 2008).

Results

Demographic Data

All patients in the small discovery set were women with a mean age of 51.6 years. Majority of the tumours were located in the right lobe of the thyroid and all tumours were larger than 1 cm. All of the patients had lymph node metastasis at diagnosis. Validation cohort from TCGA THCA studies included 229 *BRAF* V600E-positive PTC, 132 *BRAF* V600E-negative PTC and 59 unpaired normal thyroid samples.

miRNAseq Analysis

With the rapid run mode using HiSeq Rapid SBS Kit v2, we achieved an average of 5.6 M reads per sample with Q30 (3,779,968 to 8,308,285 reads in each sample). The percentage of mapped reads in normal samples were significantly lower than in tumour samples (Supplementary Table 1). Figure 1 illustrated the quality control statistics of miRNAseq experiment for *BRAF* V600E-positive versus adjacent normal samples. In Figure 1(A), approximately 45 to 75% of the reads were identified as isomiR (known precursor) and 25 to 55% were the already known mature miRNAs. Figure 1(B-D) illustrated the Principle Component Analysis (PCA) plots of miRNAs family, mature miRNAs and precursor miRNAs expression profiles. PCA analysis revealed that samples formed distinct clusters, withall tumour and normal samples clustering based on their respective groups.

Differentially Expressed miRNAs

There were 174 miRNAs significantly differentially expressed in PTC *BRAF* V600E-positive versus their normal adjacent thyroid tissues. Eighty miRNAs were upregulated, while 94 miRNAs were downregulated (log2 fold change ≤ -1 or ≥ 1; FDR p-value < 0.1). The volcano plot (Figure 2) illustrated the significantly differentially expressed miRNAs. In addition, the unsupervised hierarchical clustering analysis and heatmap illustrated in Figure 3, clearly demonstrated the difference of the miRNA expression profiles between tumour and normal samples.

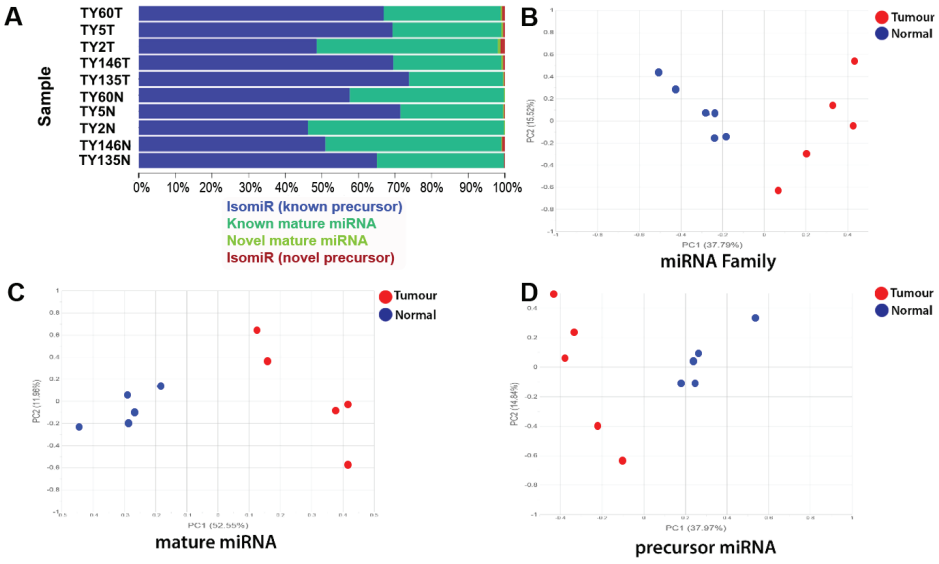

Figure 1. Quality control statistics of miRNAseq experiment. (A) Distribution of miRNA sequences among the subcategories in *BRAF* V600E PTC and their adjacent normal. (B-D) Principle Component Analysis (PCA) plots of miRNAs family, mature miRNA and precursor miRNAs. The plots showed that global miRNAs family, mature miRNAs and precursor miRNAs expression pattern clearly differentiate the samples according to respective group

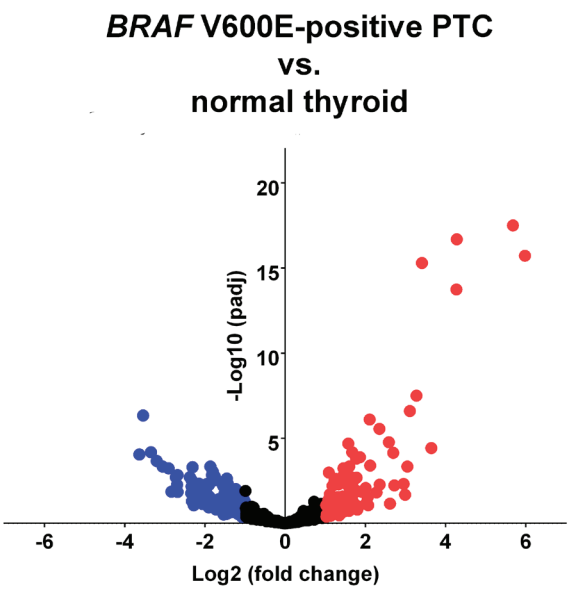

Figure 2. MiRNAs with statistical significance after a t-test are shown in red and blue on a volcano plot above. Those with no significance are shown in black. The red dots represent significantly upregulated miRNAs while blue dots represented significantly downregulated miRNAs.

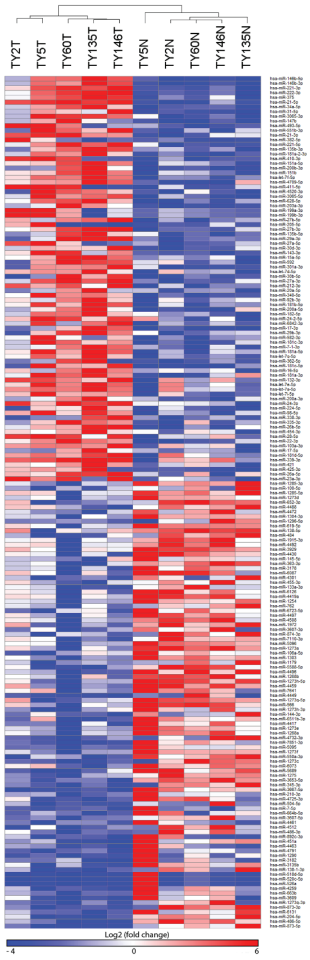

Figure 3. Hierarchical clustering and heatmap representation of differentially expressed miRNAs in discovery set. The list of differentially expressed miRNAs were filtered using FDR-adjusted p value <0.1, absolute log2 fold change ≥1 or ≤-1. In *BRAF* V600E-positve PTC versus normal-adjacent tissues, miRNAseq revealed 174 differentially expressed miRNAs. Ninety-four (94) were under-expressed while 80 were over-expressed.

Pathway Enrichment Analysis of the Deregulated miRNAs

A single miRNA might play specific roles in the pathogenesis of PTC; however, the miRNA pathway as a whole might also be of importance. Therefore, DIANA-mirPath [37], a web-based computational tool, was used to identify pathways that were potentially altered by the expression of multiple miRNAs, and to incorporate miRNAs into molecular pathways. Based on the 80 upregulated miRNAs, there were 44 significantly enriched pathways identified by the meta-analysis algorithm using "Pathway union" option (p-value < 0.05) (Figure 4A). Thirty-eight out of the 80 upregulated miRNAs were involved in the proteoglycans in the cancer pathway, targeting 176 genes. Figure 4B illustrated the hierarchical clustering analysis and the heatmap derived from the 44 significantly enriched pathways. The number of miRNAs and experimentally validated predicted targets involved in each pathway were tabulated in Table 1.

We also performed the same analysis on 94 significantly downregulated miRNAs. Only five significantly enriched pathways were identified: fatty acid biosynthesis, fatty acid metabolism, ECM-receptor interaction, fatty acid elongation and proteoglycans in cancer pathway with FDR adj. p-value < 1E-325, 2.57E-07, 7.70E-05 and 0.000352, respectively.

TCGA THCA in silico Validation

To validate the observed significance of the 174 miRNAs in *BRAF* V600E-positive PTC as compared to their normal adjacent thyroid tissues, we analysed the expression of these miRNAs in TCGA THCA dataset. Comparison between *BRAF* V600E-positive PTC versus normal thyroid tissues revealed 123 significantly upregulated and 319 downregulated miRNAs (Supplementary Table 2). To identify miRNAs which were deregulated only in *BRAF* V600E-positive PTC, the intersection of the differentially expressed miRNAs was performed (Figure 5). Since TCGA THCA dataset did not comprehensively annotate the miRNAs based on 3p and 5p arm, the intersection was performed without taking into account the arm annotation. From the intersection, 15 miRNAs were deregulated in *BRAF* V600E-positive PTC versus normal thyroid tissues in both and TCGA THCA datasets and the current study. From these 15 miRNAs, eight miRNAs were in concordance with TCGA THCA data expression levels which included hsa-miR-212, hsa-miR-132, hsa-miR-135b-3p and 5p, hsa-miR-200b, hsa-miR-200a-3p and 5p, hsa-miR-27a-3p and 5p, hsa-miR-29a and hsa-miR-1296 (log2 fold change 1.59, 1.43, 2.36, 1.72, 2.12, 1.31, 1.52, 1.60, 1.69 and -1.12; adj. p-value < 0.1), respectively (Figure 6).

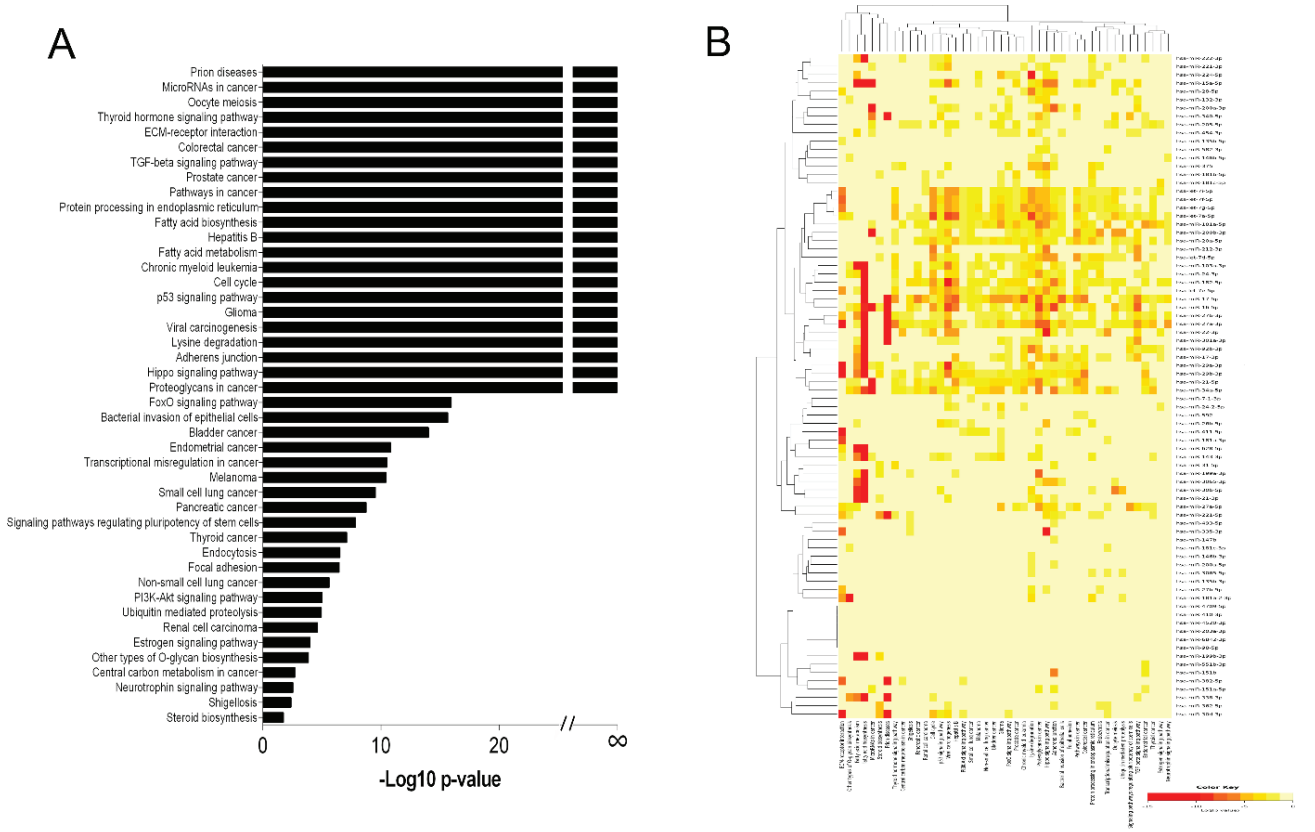

Figure 4. Pathway enrichment analysis of 80 significantly upregulated miRNAs in *BRAF* V600E-positive PTC and miRNAs versus pathway heatmap (clustering based on significance levels) using the DIANA miRPath v3.0 interface. (A) A list of 44 significant KEGG targeted pathways with their log10 p-value. (B) Hierarchical cluster and heatmap. Dark red indicate lower significant values. The attached dendograms on both axes represent the hierarchical clustering results for miRNAs and pathways respectively. MiRNAs are clustered together by exhibiting similar pathway targeting patterns and pathways are clustered together by related miRNAs.

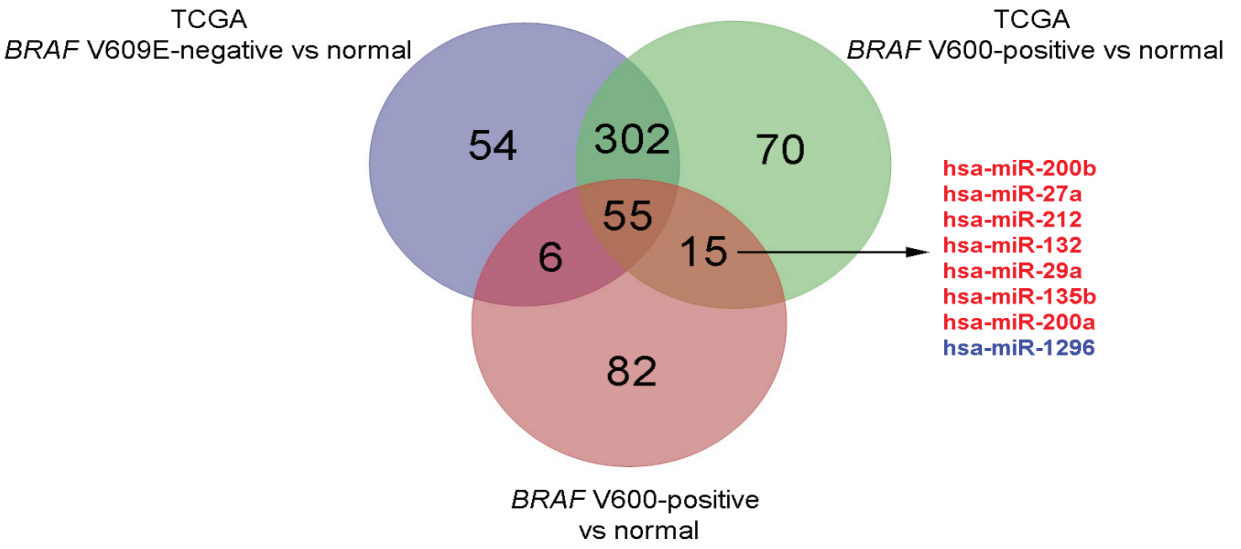

Figure 5. Venn diagram of the differentially expressed miRNAs in discovery and in silico validation dataset. From the intersection, there were 15 miRNAs that were differentially expressed in *BRAF* V600E-positive PTC versus normal. From these 15 miRNAs, eight deregulated miRNAs from discovery data were in concordance with TCGA THCA data (seven were upregulated and one was downregulated).

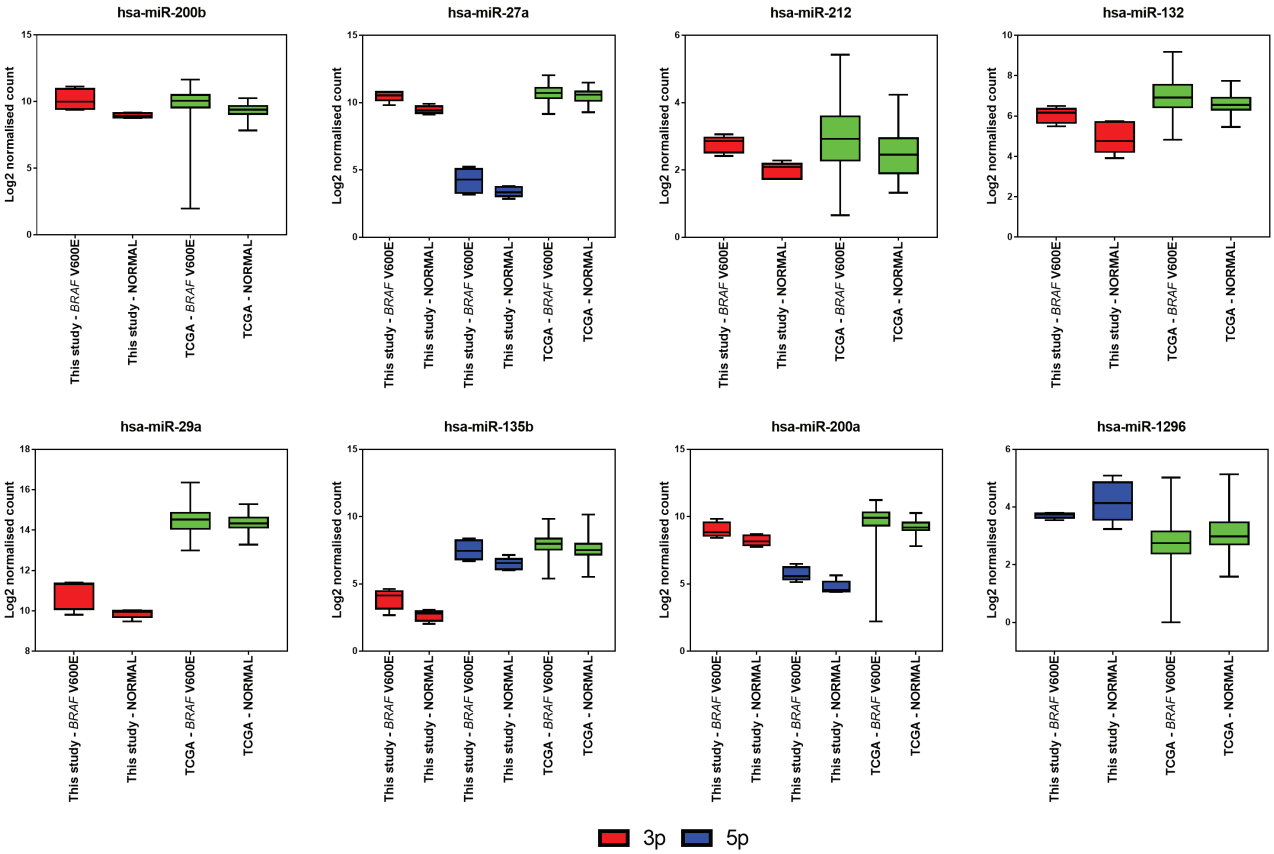

Figure 6. Box plot of eight concordance miRNAs. All miRNAs were significantly expressed (adjusted p-value <0.1; log2 fold change ≤-1 or ≥1).

Table 1. Number of miRNAs and experimentally validated targets involved in each of the 44 enriched pathways.

KEGG pathway	Adj p-value	No. of genes	No. of miRNAs
Prion diseases	<1E-325	18	8
MicroRNAs in cancer	<1E-325	140	12
Oocyte meiosis	<1E-325	83	19
TGF-beta signaling pathway	<1E-325	62	19
Thyroid hormone signaling pathway	<1E-325	95	20
ECM-receptor interaction	<1E-325	50	21
Colorectal cancer	<1E-325	59	21
Prostate cancer	<1E-325	81	21
Fatty acid biosynthesis	<1E-325	7	23
Pathways in cancer	<1E-325	303	23
Fatty acid metabolism	<1E-325	36	24
Cell cycle	<1E-325	109	24
Protein processing in endoplasmic reticulum	<1E-325	138	24
Hepatitis B	<1E-325	118	25
Chronic myeloid leukemia	<1E-325	70	27
Glioma	<1E-325	58	28
p53 signaling pathway	<1E-325	66	29
Viral carcinogenesis	<1E-325	177	31
Lysine degradation	<1E-325	39	33
Hippo signaling pathway	<1E-325	119	35
Adherens junction	<1E-325	66	37
Proteoglycans in cancer	<1E-325	173	38
FoxO signaling pathway	1.75E-14	104	22
Bacterial invasion of epithelial cells	2.16E-14	65	21
Bladder cancer	3.91E-13	34	18
Endometrial cancer	4.77E-12	47	18
Small cell lung cancer	2.19E-10	72	17
Transcriptional misregulation in cancer	5.84E-10	133	17
Melanoma	9.28E-10	56	20
Signaling pathways regulating pluripotency of stem cells	7.09E-09	98	14
Thyroid cancer	1.00E-08	26	16
Endocytosis	1.43E-08	159	16
Pancreatic cancer	2.29E-07	62	13
Focal adhesion	6.68E-07	142	12
PI3K-Akt signaling pathway	1.00E-06	182	14
Ubiquitin mediated proteolysis	2.39E-06	105	14
Non-small cell lung cancer	5.32E-06	48	14
Renal cell carcinoma	1.47E-05	58	13
Estrogen signaling pathway	2.93E-05	60	8
Other types of O-glycan biosynthesis	0.000125	21	11
Central carbon metabolism in cancer	0.000955	50	9
Neurotrophin signaling pathway	0.001194	75	8
Shigellosis	0.002684	50	10
Steroid biosynthesis	0.009698	13	12

Discussion

There was various type of *BRAF* mutations reported for malignant tumours including PTC such as *BRAF* V600E, *BRAF* V600D, *BRAF* V600Q, *BRAF* V600V and *BRAF* V600L [40,41]. For our study, we only focused on *BRAF* V600E mutation as it was the most common *BRAF* mutation in PTC, which comprised more than 90% of cases [42]. Many studies had demonstrated an association of this *BRAF* mutation with the aggressive clinicopathological characteristics of PTC such as extrathyroidal invasion, lymph node metastasis and recurrence of PTC [8,43]. It was suggested that the involvement of *BRAF* V600E mutation in the activation of RAS/RAF/MAPK pathway could result in higher deregulation of miRNA expression [44].

While previously published studies utilized microarray and real-time PCR, we used small RNA deep sequencing to determine the deregulation of miRNAs in *BRAF* V600E-positive PTC patients in an unbiased manner. There were more downregulated miRNAs in the tumour samples as compared to their adjacent normal thyroid tissues (80 upregulated versus 94 downregulated miRNAs). Deregulation of hsa-miR-146b-5p, hsa-miR-146b-3p, hsa-miR-222-3p, hsa-miR-221-3p, hsa-miR-204-5p and hsa-miR-7-5p were further reconfirmed in this study, signifying that dysregulation of these miRNA was common in PTC versus normal thyroid tissues. The association between *BRAF* V600E status and miRNA expression in PTC had been controversial. A large-scale analysis of TGCA data had demonstrated that the *BRAF* V600E mutation was one of the key drivers of PTC [24]. Other studies had shown that downregulation of hsa-miR-7-5p and hsa-miR-204-5p in PTC were associated with the *BRAF* V600E mutation [23,44]. There were however contradictory findings that showed that *BRAF* V600E mutation is not related with the aggressiveness of PTC and thus, cannot serve as prognosis marker for PTC [45–47].

MiR-200a and miR-200b were the members of miR-200 family. Upregulation of these two miRNAs was observed in PTC [48], follicular thyroid carcinoma (FTC) and follicular adenoma [16], while being downregulated in anaplastic thyroid carcinoma [49]. It was suggested that miR-200b downregulates the tumour suppressor genes [48]. These miRNAs were shown to play a crucial role in tumour cell metastasis progression or epithelial-mesenchymal transition (EMT) [50] and inhibit angiogenesis [51]. Hsa-miR-200a and hsa-miR-200b were also upregulated in thyroid cell-derived cell lines and tissues with *BRAF* V600E [16,48]. In this study, miR-200a and miR-200b were upregulated in *BRAF* V600E-positive cases in both discovery and TCGA THCA datasets, suggesting their relation to *BRAF* V600E mutation.

Among the significantly enriched pathways in *BRAF* V600E-positive PTC were proteoglycans in cancer, cell cycle pathway and ECM-receptor interaction pathway. There were 173 genes with 38 miRNAs in proteoglycans in cancer, 109 genes with 24 miRNAs in cell cycle pathways and 50 genes with 21 miRNAs involved in the ECM-receptor interaction pathway. Proteoglycans (PGs) are key molecular constituents of the ECM and cell surfaces and play important roles in integrating signals from growth factors, chemokines and integrins, cell to cell interactions as well as matrix adhesion [52,53]. PGs act in a context-dependent manner; some have pro- and anti-angiogenic activities, while others can directly stimulate cancer growth by controlling key signalling pathways [52,54,55]. Aberrant accumulation of PGs in human thyroid cancer was first reported in 1984 [56]. Using the glycoproteomics approach, Arcinas and colleagues studied the expression of proteoglycans in various thyroid cancer cell lines. Two transmembrane heparan sulfate proteoglycans, syndecan-1 and syndecan-4, were uniquely expressed in FTC-133 and XTC-1 respectively. In addition, a GPI-anchored proteoglycan, glypican-1, was identified in three thyroid cancer cell lines (FTC-133, XTC-1 and DRO-1). The extracellular heparan sulfate proteoglycan basement membrane-specific core protein or perlecan was only detected in ARO, a dedifferentiated thyroid cancer cell line [57].

Numerous studies support the important role of proteoglycans as miRNA targets in cancer progression [58,59]. Deregulation of miRNAs results in atypical expression patterns of proteoglycans and their biosynthetic enzymes, thus leading to abnormal cell proliferation, apoptosis, adhesion, migration, invasiveness and epithelial-to-mesenchymal transition [60–62]. Therapeutic strategies targeting the microRNA–proteoglycan are emerging for cancers such as in melanoma and medulloblastoma [60,63,64]. While the relationship between miRNAs expression and ECM-receptor interaction pathway in regards to *BRAF* V600E has been reported [65,66], there is currently no published evidence linking miRNA regulation to proteoglycans in thyroid cancers, especially in the *BRAF* V600E-positive PTC.

In order to validate our results in a larger dataset, we re-analysed TCGA THCA dataset containing 229 *BRAF* V600E-positive PTC, 132 *BRAF* V600E-negative PTC and 59 normal thyroid tissues. From the intersection of our discovery data with the TCGA data, 15 miRNAs were significantly deregulated in *BRAF* V600E-positive as compared to normal thyroid tissues and eight miRNAs were in concordance in term of expression levels. The remaining seven miRNAs showed the opposite trend of expression. The discrepancies could be due to the fact that TCGA THCA datasets used were from unmatched samples. Secondly, at the time of the data retrieval, TCGA THCA had not yet annotated the miRNAs according to their 3p and 5p arm and thus, the expression level between these two arms could not be differentiated. Nevertheless, we were able to reconfirm the expression of commonly deregulated miRNAs in PTCs and provide a new list of miRNAs related to *BRAF* V600E.

Conclusion

In conclusion, our study illustrated the interplay between *BRAF* V600E status and differentially expressed miRNAs in PTC. This information would add to the understanding of the molecular mechanisms of miRNAs in *BRAF* V600E-positive PTC. Although these findings were needed to be validated in larger sample size, they

could serve as a basis for the identification of a potential diagnostic or prognostic biomarker of PTC. In addition, functional studies to clarify further the mechanisms of miRNA regulation in *BRAF* V600E-positive PTC were warranted.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

A Mohamad Yusof, NS Ab Mutalib involved in the specimen collections, library preparation and sequencing, data analyses, acquisition of data and drafting the manuscript. FYF Tieng performed the TCGA analyses. S. Saidin performed the *BRAF* V600E genotyping. I. Mohamed Rose assessed tumour percentage of the tissues. SN Abdullah Suhaimi and R Muhammad were thyroid surgeons involved in specimen retrieval. I Ismail and R Jamal provided critical review on the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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Microbial community diversity in the soil of Barrientos Island estimated by RAPD and Biolog Ecoplate methods

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Abstract: The diversity of soil microbial communities at Barrientos Island with different soil characteristics were evaluated using PCR-based method random amplified polymorphic DNA (RAPD) and community level physiological profiles (CLPP) of Biolog Ecoplate. The soils were selected from 17 different locations around Barrientos Island inhabited by different breeders. Shannon-Weaver index and multivariate analysis were performed to characterize variations of soil microbial communities. Both RAPD and CLPP methods exhibited that most soils with different type of rookery and characteristics could possibly affect the DNA sequence diversity and soil microbial diversity. The abandoned type of rookery had the highest Shannon-Weaver index as exhibited by soil sample 445 (3.4 for RAPD) and 450 (3.09 for CLPP). Higher coefficients of DNA sequence similarity were found in soil samples colonized by similar breeders, like soil 442 and 446 (both were active Chinstrap rookery) shared highest similarity in DNA sequences (73.53). The cluster analysis of RAPD profiles by UPGMA and principle component analysis (PCA) of Biolog Ecoplate exhibited similar influence of type of rookery and soil condition towards soil microbial community diversity. The results may suggest that the change in microbial community DNA composition is accompanied with the change in microbial functional properties.

Keywords: Microbial; diversity; random amplified polymorphic DNA (RAPD); Biolog; soil; Barrientos Island

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Introduction

The soil microbial community is the most important composition of the soil ecosystem[1]. They are involved in many ecosystem processes such as nutrient transformation, litter decomposition, plant health maintenance and soil organic matter formation[2]. Soil microbiological parameters, such as microbial biomass carbon and basal respiration have been suggested and used as possible indicators of soil quality[3–5].

Recently, soil microbial community structure is frequently used as indicators for soil quality and fertility[1]. However, the use of cultivation-based method to study soil microbial community are proven to be challenging as only a small fraction of microorganisms (less than 10%) are culturable[6,7]. The limitation of culturable methods resulted in difficulty to understand the shift in the complex microbial community of soils[8]. These limitations could be overcome by using Biolog system and various molecular methods such as RAPD, PCR-DGGE, RFLP and others[1,9–14]. The advancement of molecular methods has enabled various advancement and discovery in the study of microbial genomics[15–28].

The RAPD analysis uses arbitrary short primers that amplify the intervening portion of the genome, and creating variably sized amplicons[29] that could be applied to study soil microbial community structure[9] and bacteria genome[30,31]. RAPD has become a popular DNA-based method as it is rapid, simple and able to provide meaningful information about soil microbial community than isolate-based methods[32]. The Biolog system is used to assess community level physiological profiles (CLPP) of a soil sample. It is a means of investigating the functional diversity of soils as they could reflect how the soil microbial communities could utilize a range of carbon substrates[33]. In this study, RAPD and Biolog Ecoplate

methods were used to investigate the influence of soil characteristics like type of rookery on soil microbial community diversity.

Material and Methods

Environmental Sampling

In 2007, during the XI Ecuadorian Antarctic Expedition to the Research Station "Pedro Vicente Maldonado", Greenwich Island, South Shetland Islands, sampling for analysis of microbial communities from the soil was carried out at Barrientos Island (coordinates: S 62° 24' 18.7" to S 62° 24' 32.4" and W 59° 44' 13.2" to W 59° 45' 39.3"). Top soil samples of upper 20 cm layer (after removing the top 2–3 cm) were collected from 17 different sites within Barrientos Island. These sites have various interesting fauna and flora activities (Table 1). Soils were sampled into sterile plastic bags using an aseptic metal trowel, and kept in the dark for transport to Malaysia. Soils were subsequently stored at -20°C, with an aliquot stored at -80°C for molecular analysis like RAPD. While a portion of each soil sample was stored in 4°C and analyzed by Biolog Ecoplate assay.

Table 1: Soil samples characteristics.

Soil reference	Type of rookery / nest	Soil condition
442	Active Chinstrap penguin	Guano
443	Abandoned Gentoo penguin	Guano
444	Abandoned Gentoo penguin	No guano
445	Abandoned Gentoo penguin	No guano
446	Active Chinstrap penguin	Guano
447	Active Chinstrap penguin	No guano
448	Active penguin	No guano
449	Active Chinstrap penguin	Guano
450	Abandoned Penguin	Guano
451	Active Gentoo penguin (resting-deleted) area	Guano
452	Abandoned Penguin	Guano
453	Penguin resting area	Guano
455	Active Gentoo penguin	Guano
456	Abandoned Penguin	No guano
457	Active Gentoo penguin	No guano
458	Seal colony	Guano
460	Giant Petrel nest	No guano

Soil DNA Extraction and Purification

To minimize possible contaminants, all post-sampling manipulations were performed in a UV-sterilized laminar box hood, using sterile glass vials. Total soil DNA was extracted and purified from 1g dry weight of soil using GF-1 Soil Sample Extraction kit (Vivantis, Selangor, Malaysia). The kit uses a specially-treated silica-based material fixed into a column to efficiently bind DNA in the presence of high salt. This kit applies the principle of a mini-column spin technology and the use of optimized buffers to ensure that only DNA is isolated while cellular protein, humic acid and other low molecular weight impurities are removed during the subsequent washing stages. (Cat. No: GF-SD-025). DNA yield and quality were assessed by 0.8% (w/v) agarose gel electrophoresis following by DNA quantification using a Biophotometer (Eppendorf, Hamburg, Germany) and ratio A_{260}/A_{280} was measured. Pure DNA has an A_{260}/A_{280} ratio of 1.7–1.9.

PCR Amplifications and Fragment Visualization

RAPD arbitrary primer OPO 05 (5'-CCCAGTCACT-3'), OPO 06 (5'-CCACGGGAAG-3') and OPO 18 (5'-CTCGCTATCC-3') was used for amplification of soil DNA by PCR using the Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The PCR reaction mixture consisted of ~10 ng of soil bacteria DNA, 2.0 µl of 10X optimized PCR buffer with 20 mM $MgCl_2$, 2.0 µl of 10 mM dNTPs, 1 unit of Taq polymerase (Intron Biotechnology, South Korea) and 0.5 µl of 100 nM primer and sterile ultrapure water was added to final volume of 20 µl. The cycling parameters were 4 min at 94°C for pre-denaturation, 45 cycles each of 1 min at 94°C for denaturation, 1 min at 36°C for annealing, 1 min at 72°C for extension and 7 min at 72°C for final extension. The PCR amplification products were resolved by electrophoresis in 1.5% agarose gel (Promega, Madison, WI.), which was stained with ethidium bromide (0.5 µg ml^{-1}) and viewed under a gel documentation system (Alpha Imager, Alpha Innotech, California).

Data Analysis of RAPD Fingerprints

Three arbitrary primers were used to amplify microbial community DNA from 17 soil samples. Since primer sequences were random and non-selective to soil DNA samples, amplification for one primer was equal to one random sampling from the whole microbial DNA sequences[32]. The number of RAPD fragments was considered to represent the RAPD fragment richness (*S*) of the whole DNA sequences. The calculations above rely on an assumption that each RAPD fragment contributes equally to the microbial diversity[32].

Since RAPD fragments amplified in all 17 soil samples contributed to the diversity of DNA sequences differently as compared to those fragments amplified in only one, two, and three samples, it was necessary to make a modification for another diversity calculation. A fragment amplified in all 17 soil samples had the smallest contribution to the diversity because of no polymorphism, and therefore scored 0 for the diversity. A fragment amplified in only one sample had the biggest contribution and scored 1. The other fragments, amplified in two or three samples, counted 2/3 or 1/3, respectively. This modification (modified richness, i.e., modified S), in fact, enlarged the contribution of the characteristic sequence to the DNA sequence diversity. The richness and modified richness of soil microbial community DNA sequences reflect to cer-

tain extent the diversity of soil microbial community DNA sequences, but do not indicate the relative abundance of soil microbial community DNA sequences. Shannon-Weaver index is developed to measure the species diversity of the community by integrating species richness and abundance. Her, we used Shannon-Weaver index as a measure of soil microbial community DNA sequence diversity by molecular marker. Diversity of soil microbial community DNA sequences was estimated using the equation below:

$$D\text{sh} = -\sum_{I=1}^{S} Pi\ln Pi = -\sum_{I=1}^{S} (Ni / N)\ln(Ni / N)$$

where *D*sh is Shannon-Weaver index, *Pi* is the percent of the *i*th RAPD fragment gray degree to each DNA sample, *Ni* is the net gray degree quantity (subtracted by the background gray degree of a gel) of the *i*th RAPD fragment in each DNA sample, *N* is the total net gray degree quantity of all RAPD fragments examined in each DNA sample, and S is the number of RAPD fragments in each DNA sample. The range of *D*sh is between 0 and ln(S). By merging the data of RAPD fragment net gray degrees from each primer into a single dataset, a cumulative diversity of Shannon-Weaver index was calculated for each sample using the same equation above.

RAPD-based Cluster Analysis

By using BioNumerics version 6.0 gel analysis software (Applied Maths, Kortrijk, Belgium), the position of the markers in RAPD gels were normalized from lane-to-lane and gel-to-gel variation. This normalization enables comparison of banding patterns originating from different RAPD gels, provided there was a high degree of gel reproducibility based on migration of standards. Then a binary matrix was constructed for each microbial community based on the presence and absence of bands. Jaccard's coefficient (a similarity measurement) was used to calculate the matrix and the data were subjected to clustering based on the unweighted pair group method using arithmetic averages (UPGMA) to identify samples that generated patterns similar to each other [34]. Results were displayed in dendrogram form to illustrate the relationship between microbial communities from different soil.

Biolog EcoPlate Inoculation and Incubation

One hundred and fifty ml of sediment slurry from each sample was placed in sterile 400 ml beakers. Physiological saline solution was added to bring the volume to 200 ml. The resultant slurry was sonicated in a water bath for 5 min before 15 ml of the supernatant was extracted and centrifuged at 500 g for 3 min. A 150 ml aliquot of the centrifuged supernatant was then used to inoculate a microtitre Biolog EcoPlate. Biolog EcoPlates have 96 wells that contain 31 unique carbon compounds, in addition a control of distilled water repeated in triplicate. When bacteria use one of these carbon sources, tetrazolium dye is reduced by bacterial respiration and accumulates as insoluble formazin. These results in the well turning from clear to darker shades of purple depending on the amount of formazin produced[35]. Each plate was cultivated at 25°C for 168 h, and the optical density at both 590 nm (color development plus turbidity) and 750 nm (turbidity only) was read every 24 h[36].

Biolog Data Analysis

The final values used to represent the activity in each well were the 590 nm values minus the 750 nm values after being corrected for readings in the control well at these wavelengths. Well optical density values that were negative or under 0.06 were set to zero[36]. Average well color development (AWCD) was calculated as described by Garland and colleagues, i.e. AWCD (590–750 nm) = $\Sigma(C_{590-750})/31$, where 31 represents the number of carbon sources used in Biolog EcoPlate. The final values of each well at 168 h were used to calculate the Shannon's diversity index (*H*), where $H = -\Sigma(Pi\ln Pi)$, *Pi* is the proportional optical density value of each well, and $Pi = C_{590-750}/\Sigma\ (C_{590-750})$. Normalized data were analyzed by principal component analysis.

Results

RAPD Analysis

In the present study, 3 arbitrary primers were used to amplify soil microbial DNA. The RAPD pattern generated by arbitrary primers OPO 05, 06 and 18 for 17 soil samples were shown in Figure 1 as gel photo. Three primers generated a total of 416 RAPD fragments with OPO 05, OPO 06 and OPO 18 generated 130, 142 and 144 fragments, respectively. The number of fragments scored per primer ranged from 1 to 11, 5 to 11 and 3 to 12 for primer OPO 05, 06 and 18, respectively. Eight of the total fragments (1.9%) were polymorphic. The ratio of polymorphic fragments in each primer was 3.1% (OPO 05), 1.4% (OPO 06) and 1.4% (OPO 18), respectively (Table 2).

Table 2: Three primer amplified outputs to microbial community DNA from 17 soil samples.

Primers	Amplified Fragment	Non-polymorphic Amplified fragments	Polymorphic fragments	Ratio of polymorphic fragments to total fragments
OPO5	130	126	4	3.1%
OPO6	142	140	2	1.4%
OPO18	144	142	2	1.4%
Total	416	408	8	1.9%

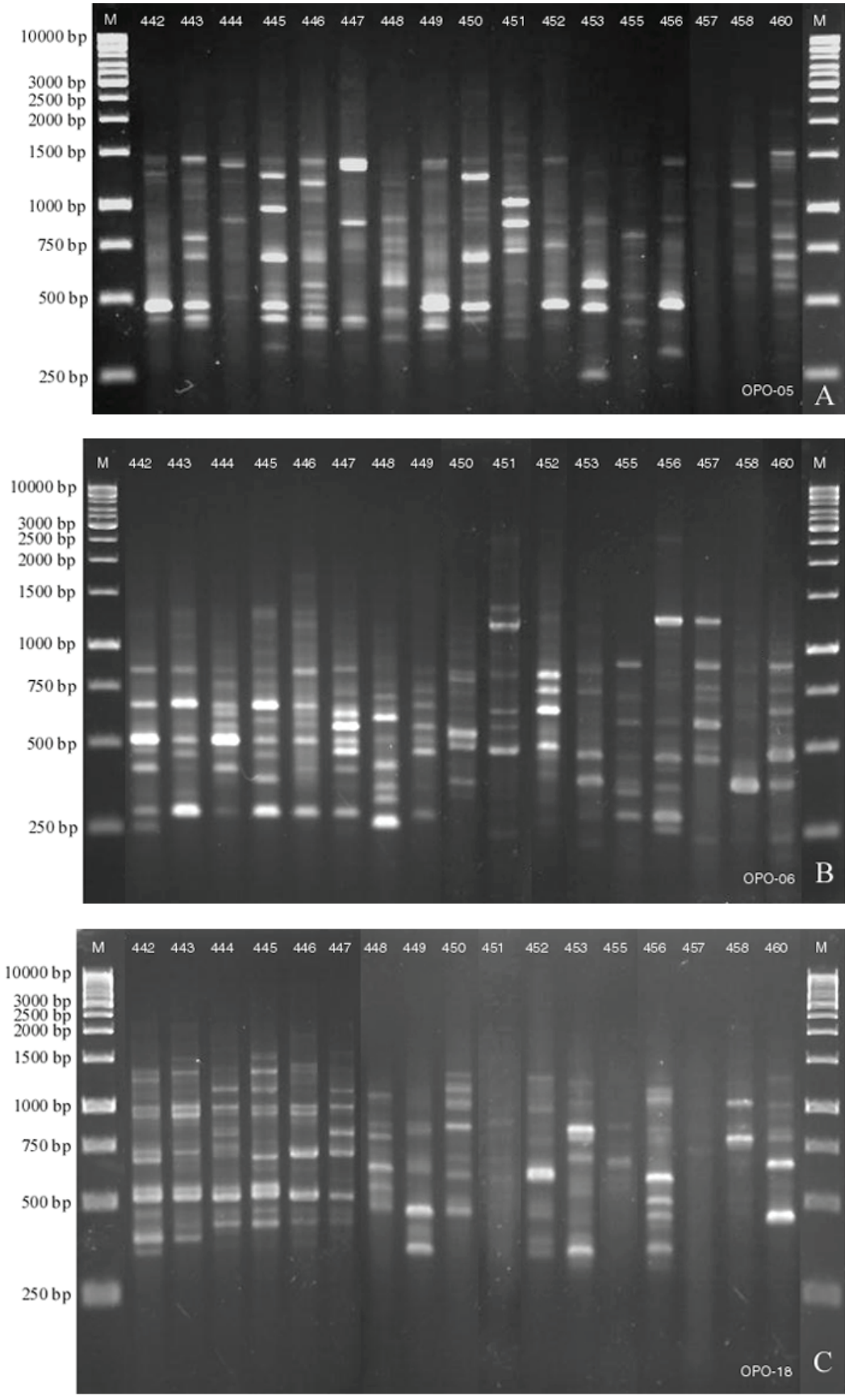

Figure 1: RAPD fingerprint for 17 soil samples for OPO 05 (A), OPO 08 (B) and OPO 18 (C). Lane "M" contain DNA molecular weight markers. The numbers indicate the soil sample number as in Table 1.

RAPD analysis — Diversity of Soil Microbial Communities DNA sequence

RAPD fragment richness (*S*) in DNA for 17 soils microbial communities in Barrientos Island are shown in Table 3. Average RAPD fragments richness for sample 445 and 446 were both 10.7, respectively, which were the highest richness among all soils from Barrientos Island. Soil sample 443, 456 and 444 were the subsequent samples with high average richness value of 10, 9.3 and 9, respectively. Whereas soil 457 contain the lowest richness, with average only 4 RAPD fragments per primer. Three soils comprised of relatively low richness, which is 457, 458 and 455 with 4, 4.3 and 5.3 RAPD fragments per primer, respectively.

The modified richness (modified *S*) for 17 soils showed that sample 445 had the highest value for average of modified richness (7.09); as soil 457 had the lowest value (2.44) (Table 4). By assigning a standard value of 1 for modified richness for the soil with the highest diversity (445), soils with high richness value were 445, 445 and 443 with 1, 0.95 and 0.91, respectively. The Shannon-Weaver indices (*D*sh) for microbial community at RAPD are shown in Table 5. The cumulative diversities of Shannon-Weaver indices for 445 were the highest (3.40), whereas 457 was the lowest (1.16). Coefficient of DNA sequence similarity indicated differences between soils at DNA level (Table 6). Coefficient of DNA sequence similarity was the highest (73.53) between sample 442 and 446. The similarity coefficient between sample 447 (active Chinstrap penguin rookery) and sample 458 (seal colony rookery) was the lowest (6.07).

Table 3: Richness (S) for soil microbial community in 17 soil samples at DNA level.

Primer	Soil samples																
	442	**443**	**444**	**445**	**446**	**447**	**448**	**449**	**450**	**451**	**452**	**453**	**455**	**456**	**457**	**458**	**460**
OPO5	7	10	6	9	11	7	9	9	9	10	5	7	5	6	1	4	9
OPO6	7	9	9	11	11	9	9	8	8	6	9	6	7	11	8	5	9
OPO18	12	11	12	12	10	7	7	7	8	5	7	10	4	11	3	4	8
Total	26	30	27	32	32	23	25	24	25	21	21	23	16	28	12	13	26
Average	8.7	10	9	10.7	10.7	7.7	8.3	8	8.3	7	7	7.7	5.3	9.3	4	4.3	8.7

Table 4: Modified Richness (Modified S) for soil microbial community in 17 soil samples at DNA level.

Primer	Soil samples																
	442	**443**	**444**	**445**	**446**	**447**	**448**	**449**	**450**	**451**	**452**	**453**	**455**	**456**	**457**	**458**	**460**
OPO5	3.50	5.88	3.31	5.69	6.44	3.31	6.56	5.19	6.38	6.56	2.88	4.13	3.63	4.06	0.69	2.63	6.5
OPO6	3.56	5.81	4.56	6.88	7.00	5.00	6.06	4.44	4.63	3.56	6.06	2.94	4.75	7.69	5.19	3.13	5.88
OPO18	7.88	7.69	8.25	8.69	6.81	4.50	4.56	3.56	4.88	3.13	5.00	6.69	2.50	7.25	1.44	2.13	4.94
Average Modified *S*	4.98	6.46	5.37	7.09	6.75	4.27	5.73	4.40	5.30	4.42	4.65	4.59	3.63	6.33	2.44	2.63	5.77
Relative Value	0.70	0.91	0.76	1	0.95	0.60	0.81	0.62	0.75	0.62	0.66	0.65	0.51	0.89	0.34	0.37	0.81

Table 5: Shannon-Weaver diversity indices (Dsh) of microbial community in 17 soil samples from random amplified polymorphic DNA (RAPD) and community level physiological profiles (CLPP) analysis.

Methods	Soil samples																
	442	**443**	**444**	**445**	**446**	**447**	**448**	**449**	**450**	**451**	**452**	**453**	**455**	**456**	**457**	**458**	**460**
RAPD	2.38	3.10	2.58	3.40	3.23	2.04	2.75	2.11	2.55	2.11	2.24	2.21	1.73	3.03	1.16	1.26	2.75
CLPP	2.58	2.63	2.01	1.65	2.32	1.75	1.74	2.84	3.09	2.81	2.90	2.55	2.86	2.49	2.46	3.04	2.54

Table 6: Jaccard's average similarity coefficient of 17 soil samples generated by UPGMA analysis.

Soil samples	442	443	444	445	446	447	448	449	450	451	452	453	455	456	457	458	460
442	100.00																
443	58.29	100.00															
444	47.11	49.37	100.00														
445	58.89	64.33	48.89	100.00													
446	73.53	67.71	61.15	61.62	100.00												
447	50.38	56.67	72.75	40.71	60.88	100.00											
448	29.02	35.36	22.93	18.86	33.90	23.65	100.00										
449	46.95	49.03	51.82	35.33	53.29	58.19	33.37	100.00									
450	43.11	44.02	47.76	41.61	38.93	45.38	18.59	31.50	100.00								
451	35.10	36.31	30.63	23.97	28.26	24.85	35.45	33.33	30.80	100.00							
452	46.67	47.62	33.06	35.51	48.11	55.61	14.65	46.83	36.45	23.69	100.00						
453	32.97	34.17	21.01	32.29	31.77	27.15	37.88	33.33	35.72	25.97	23.49	100.00					
455	24.78	21.72	33.75	32.92	26.43	22.09	14.61	21.37	20.79	42.03	23.23	23.71	100.00				
456	45.87	28.69	20.64	37.19	33.99	27.39	26.15	44.45	33.79	27.67	49.75	25.71	30.47	100.00			
457	20.45	22.82	39.97	35.95	33.43	22.45	12.97	17.63	34.29	34.70	26.39	23.99	26.67	22.45	100.00		
458	15.79	23.23	17.25	17.50	24.91	6.07**	30.91	6.67	16.24	13.89	6.67	29.29	9.53	19.40	44.45	100.00	
460	25.00	29.87	40.27	38.89	34.76	40.51	28.29	13.06	51.36	21.77	37.78	26.67	17.99	30.28	37.68	37.68	100.00

RAPD analysis — UPGMA analysis of RAPD profiles

RAPD profiles were subjected to clustering based on unweighted pair group method using arithmetic averages (UPGMA) to identify soil samples that generated patterns similar to each other. UPGMA of 17 soil samples using composite analysis of OPO 05, 06 and 18 revealed 8 clusters (Figure 2) that clade together. Within the 8 clusters produced, 5 clusters (I, III, IV, VI, VII) comprised soil samples from similar type of rookery in each respective cluster. However, some soil samples from different type of rookery were clustered together, i.e cluster II, V and VIII. Cluster I comprised of 2 abandoned penguin rookeries (452 and 456). Cluster II consisted of 1 abandoned penguin rookery (444) and 2 active Chinstrap rookeries (447 and 449). Cluster III contained 2 active Chinstrap rookeries (442 and 446) at high similarity level of 73.5% (Table 6). Cluster IV comprised 2 abandoned penguin rookeries (443 and 445) while cluster V contained 2 rookeries from abandoned penguin rookery (450) and petrel nest (460). Cluster VI comprised of 2 penguin resting rookeries (448 and 453) whereas cluster VII consisted of 2 active Gentoo rookeries (451 and 455). Lastly cluster VIII comprised of active Gentoo (457) and seal colony (460) type of rookeries.

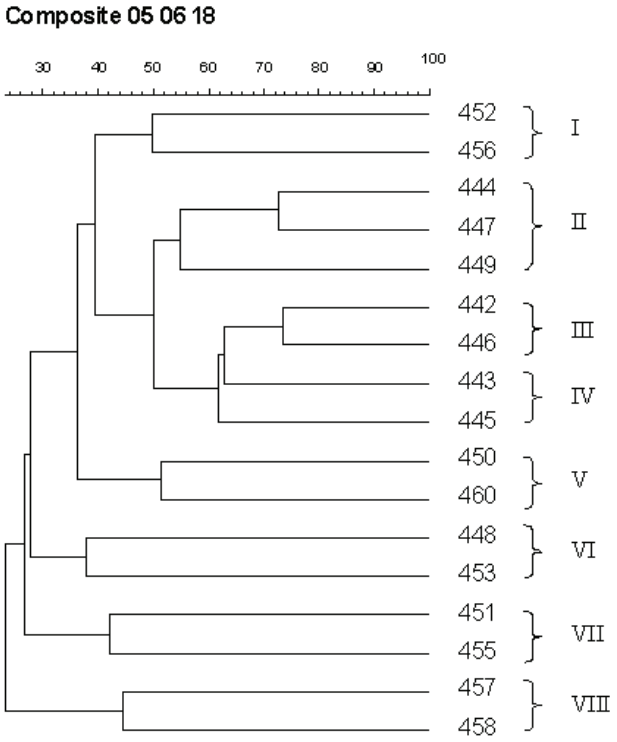

Figure 2. Dendrogram derived from RAPD composite analysis of arbitrary primer OPO 05, 06 and 18. A total of 8 clusters were formed from 17 soil samples used in this study.

Biolog analysis — Average well color development

AWCD of Biolog Ecoplates is an important index for elevated diversity of soil microbial biomass function. The values represent the changes of soil bacterial community activity in utilizing catabolic diversity in different type of rookery. The AWCD generally followed the same pattern with incubation time, but the pattern varied for different soil samples (Figure 3). In general, the AWCD value was the highest in sample 458 (active seal rookery), and lowest in sample 444 (abandoned penguin rookery). The AWCD values represent the metabolic activity of soil microbial community in using the carbon sources, thus proposed that the effect of rookery activities have influence on soil bacteria community metabolic function.

Biolog analysis — Shannon's diversity

Differences in the Shannon-Weaver index of the soil bacterial community of different rookery after incubating for 168 h in Biolog Ecoplate were observed (Table 5). The Shannon indices were significantly highest in sample 450, a soil with penguin abandoned type of rookery and soil surface covered by lots of guano. While sample 445 and 448 with low Shannon indices of 1.65 and 1.74, respectively showed no guano at that area.

Biolog analysis — Principle component analysis

The principle component analysis was conducted to better understand differences in carbon utilization by soil microorganism. The PCA plot shows that carbon substrate utilization profiles were able to clearly separate soil samples to group according to type of rookery (Figure 4). Five significant groups were formed (A, B, C, D, E), each according to certain type of rookeries. Group A and B comprised of 3 (443, 444, 445) and 2 (452, 456) abandoned rookeries, respectively. Group C consisted of soil samples from active Chinstrap penguin rookeries (442, 446, 447, 448). Group D comprised of penguin areas (453, 457) while group E consisted of Gentoo penguin active rookeries (451, 455).

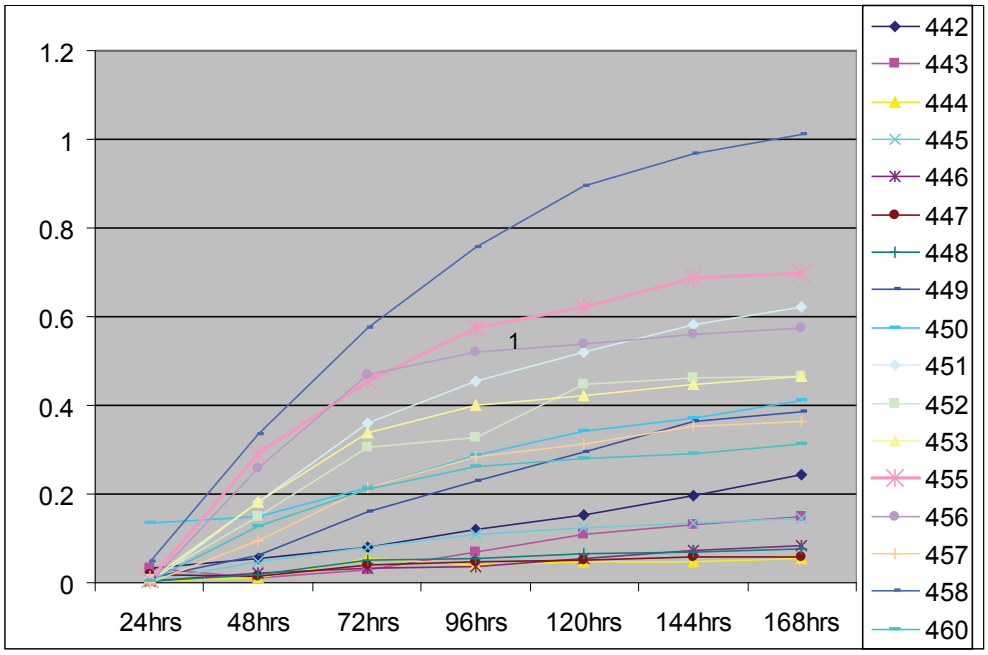

Figure 3. Difference in AWCD of soil bacterial community over time for 17 different soil samples.

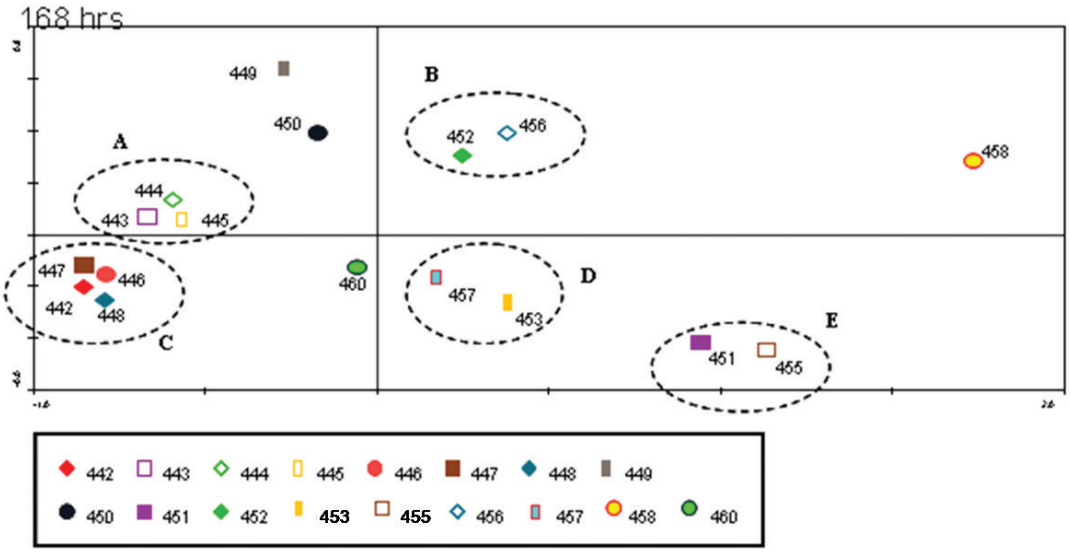

Figure 4. Principle component analysis (PCA) of Biolog EcoPlate data from different soil samples of Barrientos Island.

Discussion

The potential microbial diversity as an indicator of soil quality is impeded due to the difficulties in measuring[9]. Microbial population in soils are very large, with more than 10^9 organisms per gram of soil[37,38]. It is also very diverse with more than 10^4 species per gram of soil[39]. Only 1–10% of these microbes can be isolated and studied in pure culture, therefore, various microbial methods are emerging as useful tools to study microbial communities in soils[9,40].

The difference among microbial species is fundamentally signified in their genetic diversity at DNA sequence and their metabolic function. Therefore, molecular method like RAPD and substrate utilization pattern (Biolog Ecoplate) can be used to study soil microbial community diversity[9]. PCR-based technique like the RAPD has become a popular method to assess soil microbial community as it is simple, rapid and sensitive means to identify small variations between similar genomes[41,42]. The Biolog system was initially developed for bacterial identification. Later the system has found to be useful to characterize soil microbial diversity from various different environments like soil, sediments, freshwater and seawater[35]. The substrate utilization patterns of the Biolog system have been used to provide "fingerprints" of microbial community structure[43,44] and also as an indication of metabolic potentials[45,46]. The multivariate analysis of the utilization pattern of different carbon substrates generated from the 96-well Biolog Ecoplates enables the classification of microbial community functional diversity. The substrate utilization patterns in this study successfully differentiated most of the soil samples according to different type of rookery.

In this study, the RAPD analysis demonstrated that different type of rookery activities could considerably affect soil microbial communities. As 80% (4/5) of highest RAPD fragment rich samples were from abandoned penguin rookeries (445, 443, 444, 456) (Table 1, Table 3). Compared to soil samples with active breeders' type of rookeries (i.e. 457, 458, 455) that exhibited average richness range from 4–5.3, soil samples of abandoned penguin rookeries (443, 444, 445, 450, 452, 456) showed much higher average richness with value range from 7–10.7 (Table 3). The Shannon-Weaver indices (*D*sh) for the microbial community at RAPD in the 17 soils showed the average Shannon indices for abandoned penguin rookeries (443, 444, 445, 450, 452, 456) were significantly higher than that of rookeries with active breeders (i.e. 457, 458, 455). This observation is indeed interesting and warrant further study to understand these patterns. For Biolog Ecoplate analysis of Shannon-Weaver indices, 77% (442, 443, 446, 449, 450, 451, 452, 453, 455, 458) of soil samples with high Shannon index (>2.3) (Table 5) were soil samples covered with guano, these could possibly infer that catabolic diversity of the soil bacterial community could be increased with the existence of organic manure like guano [1]. Soil samples namely 445 (3.4 for RAPD) and 450 (3.09 for CLPP) which exhibited highest Shannon index for RAPD and CLPP were both from abandoned type of rookeries. Soil samples like 443, 450 and 452 all shared high Shannon index in both RAPD and Biolog methods used. Overall the correlation of Shannon index between RAPD and Biolog is not really strong, this could be due to the variance of targeted microbes in both methods, as Biolog method only reveals fast growth bacteria activity only.

In regards to coefficient of DNA sequence similarity, results indicated the possibility of different samples colonized by different breeders caused a shift in the species composition of soil microbial communities. As for most of the soil samples colonized by similar breeders, comparatively higher coefficients of DNA sequence similarity were found, like sample 442 and 446 (both active Chinstrap rookery) shared highest similarity in DNA sequences (73.53) (Table 6).

The AWCD analysis of Biolog Ecoplates revealed changes of soil bacterial community activity in utilizing catabolic diversity in different type of rookery. From 10 soil samples with highest AWCD values, 7 were from active breeders' rookeries and only 3 were from abandoned type of rookeries. The average AWCD value for all the 9 active breeders rookeries (442, 446, 447, 449, 451, 455, 457, 458, 460) were 0.4203, which is significantly higher than average AWCD of 6 abandoned rookeries (443, 444, 445, 450, 452, 456) with value of 0.2998. Therefore, this suggested that the metabolic activity of soil bacteria was higher in active breeders' rookeries and lower in abandoned type of rookeries. As a result, this observation showed that the effects of rookery activities have influence on soil bacteria community metabolic function.

The cluster analysis of RAPD profiles by UPGMA showed great similarity in DNA profiles for microbial communities that shared similar type of rookery. Total 63% (5/8) of the clusters formed were from similar type of rookery in each respective cluster (Figure 2). These results suggest that there was a systematic change in the sequence diversity associated with different type of rookery at sampling sites. The PCA results of Ecoplate showed that carbon substrate utilization profiles were able to clearly separate most soil samples (76%) to group according to type of rookery (Figure 4). Sample 458 and 460, inhabited by seal and petrel respectively, both were evidently separated from the rest of the samples which were inhabited by penguins. This observation from CLPP method revealed that there is positive influence of type of rookery and soil condition towards soil microbial community diversity.

In generally, combination of molecular methods with other tools can be used to improve our understanding of the effect of different soil characteristics to soil microbial diversity. In this study, the genetic diversity of microbial populations by RAPD genetic fingerprinting and metabolic diversity using Biolog substrate utilization assays were used to investigate the effect of different type of rookery activities and characteristics on soil microbial populations. Both RAPD and CLPP method revealed the similar influence of type of rookery and soil condition towards soil microbial community diversity. The results may suggest that the change in microbial community DNA composition is accompanied with the change in microbial functional properties[9]. Nonetheless, both the methods have limitations in determining soil microbial community. The RAPD method may be affected by effects of PCR bias, like the size of random primer, sensitivity to reaction conditions and the possibility of co-migration[47,48]Applications of random amplified polymorphic DNA (RAPD. The Biolog system assesses the metabolic diversity of culturable bacteria only. It is a system that could indicate activity of the fast growth bacteria or eutrophic bacteria only. Therefore, microorganisms like slow-growing bacteria, fungi and uncultured bacteria activity are expected to have minimal influence on the microbial metabolite profile[49,50]. So, only a part of soil microbial characteristics was discovered by the Biolog Ecoplate method.

As a conclusion, it is necessary to incorporate comprehensive approaches at diverse level, including traditional, metabolic and molecular level to understand more precisely about the changes in the diversity of microbial communities[32,51].

Author Contributions

The research and manuscript writing were performed by LH-L, NS-AM and VL. L-HL and Y-KC founded the research project.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Original Research Article

Progress in Microbes and Molecular Biology

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Methicillin-resistant *Staphylococcus aureus* (MRSA) on dispensing counters of community pharmacies in Klang Valley

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Abstract: *Staphylococcus aureus* has been causing contamination and infection in the hospital and community settings. Methicillin-resistant *Staphylococcus aureus* (MRSA) was first discovered in the 1960s and epidemics of MRSA were reported soon after the usage of methicillin. The incidence rate of MRSA infections has been increasing for the past 50 years, and community-associated infection may be slowly replacing hospital-associated MRSA strains. This study aimed to investigate the prevalence of MRSA on the dispensing counters of community pharmacies under different settings - community pharmacies in shopping malls and high streets in Klang Valley. With verbal consent, swab samples were collected from dispensing counters of 23 community pharmacies using sterile cotton buds moistened with sterile sodium chloride (NaCl) solution. Samples were spread on nutrient agars and *Brilliance* MRSA 2 selection agars and incubated at 37ºC. The numbers of colony were documented and statistically analysed using Microsoft Excel and Statistical Package for the Social Sciences (SPSS) Statistics. The results showed that the prevalence of MRSA on the dispensing counters was 22% (5 out of 23), and the difference in MRSA contamination between community pharmacies in shopping mall and high street setting was insignificant ($p > 0.05$). This study serves as the pioneer study of its kind in Klang Valley. All healthcare professionals and individuals are strongly advised to practise a good level of hygiene to avoid MRSA cross contamination.

Keywords: MRSA; pharmacy; dispensing counter; cross-contamination; Klang Valley

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Introduction

Asia has one of the highest incidence rates of MRSA infections in the world[1- 3]. Besides MRSA, vancomycin-intermediate *S. aureus* (VISA) strains and vancomycin-resistant *S. aureus* (VRSA) strains are also reported in several Asia countries[4]. From 1986 to 2007, the prevalence rate of MRSA infections in Malaysia has increased from 17% to 44.1%[5]. On average, MRSA patients in intensive care units are hospitalised three times longer and have five times greater risk of death compared to other patients in the intensive care units[6]. In addition, there have been reports in which patients infected with MRSA have a mortality rate of 34% within 30 days compared to 27% in patients infected with non-antibiotic resistant *S. aureus*[7]. Researchers have been working persistently to search for potential compounds to inhibit MRSA, while some studies exhibited good potential but further works are needed before these discoveries could be developed as marketable drugs[8–11].

Hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) differ in terms of their molecular and clinical epidemiology. HA-MRSA infection is linked to serious, invasive diseases, such as skin and soft tissue infections (SSTIs), pneumonia and bloodstream infection (BSI) in hospitalised patients[12]. On the other hand, CA-MRSA infection may slowly replace HA-MRSA[13] with cross contamination between hospital and community settings[14,15]. CA-MRSA is defined as MRSA infection which happens without any recent hospital exposure, and is usually associated with SSTIs in healthy, young individuals[16]. In addition, uncommon cases of skin infections, for instance, necrotizing fasciitis has been reported to be associated with CA-MRSA[17]. Furthermore, CA-MRSA strains also cause other invasive infections; for example, urinary tract infections, osteomyelitis, bacteremia, pneumonia and septic shock. However, each of these infections only accounts for less than 4% of all CA-MRSA infections[18]. With the statistics and adverse effects of MRSA discussed above, this study aims to

study the prevalence of MRSA in selected community pharmacies in Klang Valley, Malaysia. Dispensing counters of these pharmacies will be the study site of this project.

Material and Methods

Agar Preparation and Procurement

Nutrient agars (Oxoid) were prepared according to the manufacturer's instructions. *Brilliance* MRSA 2 pre-made agar plates were purchased from Thermo Fisher Scientific and stored in dark at 4°C until used.

Study Sites

Twenty-three (23) community pharmacies were randomly identified for sample collection in Klang Valley. All pharmacies were either located in shopping complexes, or at high-street setting.

Sample Inoculation

All samples collected were inoculated onto nutrient agars and *Brilliance* MRSA 2 agars and incubated at 37°C for 12-16 hours. The numbers of visible colony were documented and data were analyzed. The results were presented in colonies forming unit per mL (CFU/mL). All procedures were conducted under aseptic conditions.

Ethical Clearance

This study was conducted under the approval of SEGi Ethics Committee, with ethics approval number of SEGi/RIMC/FOP/28/2018.

Results

Study Sites

Figure 1 indicates the pharmacies visited over the course of the sample collection period in this study. The blue pointers indicate the absence of MRSA and the red pointers indicate the presence of MRSA. In Kuala Lumpur city, three community pharmacies were found to have MRSA on their dispensing counters. Meanwhile, in Damansara and Petaling Jaya area, one community pharmacy in each area was found to have MRSA on the dispensing counter.

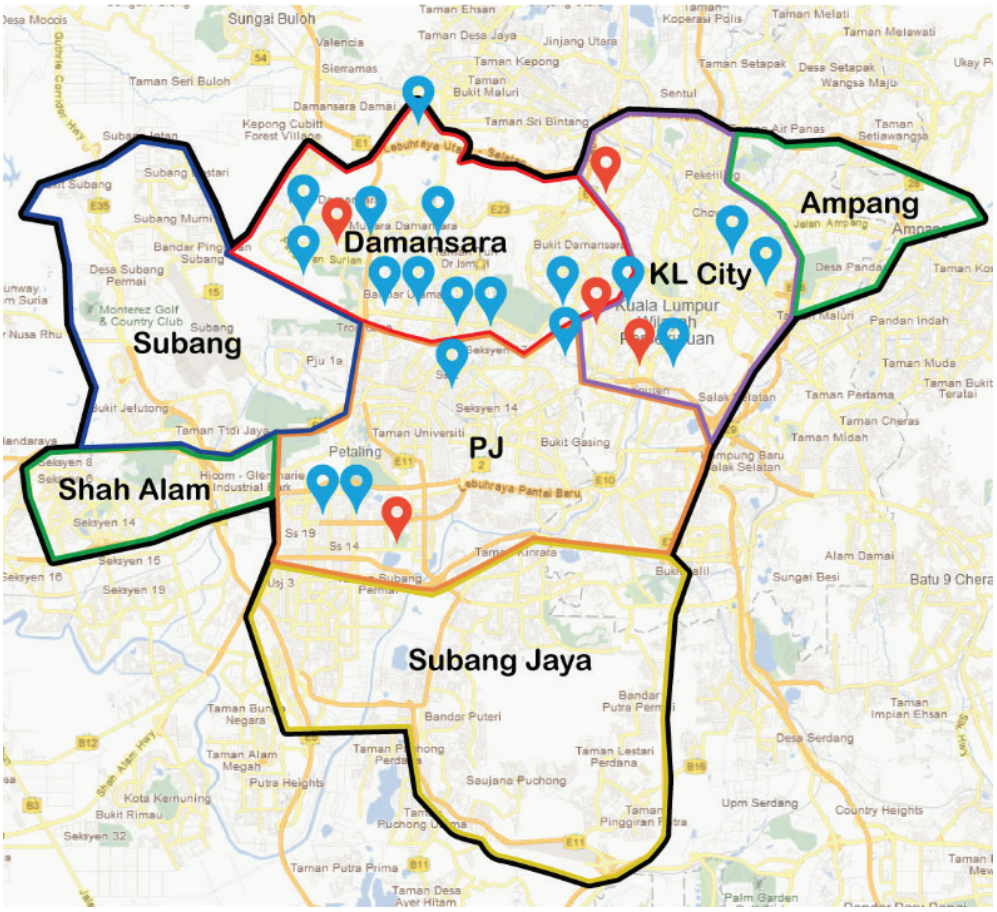

Figure 1. Selected community pharmacists for this study. (Blue pointers) Community pharmacies without MRSA detected on dispensing counter; (Red pointers) Community pharmacies with MRSA detected on dispensing counter.

Prevalence of Total Microorganism and MRSA Counts

Based on the graph in Figure 2, different levels of microorganism prevalence were detected on all 23 dispensing counters of community pharmacies. MRSA was detected on five dispensing counters out of 23 community pharmacies in Klang Valley.

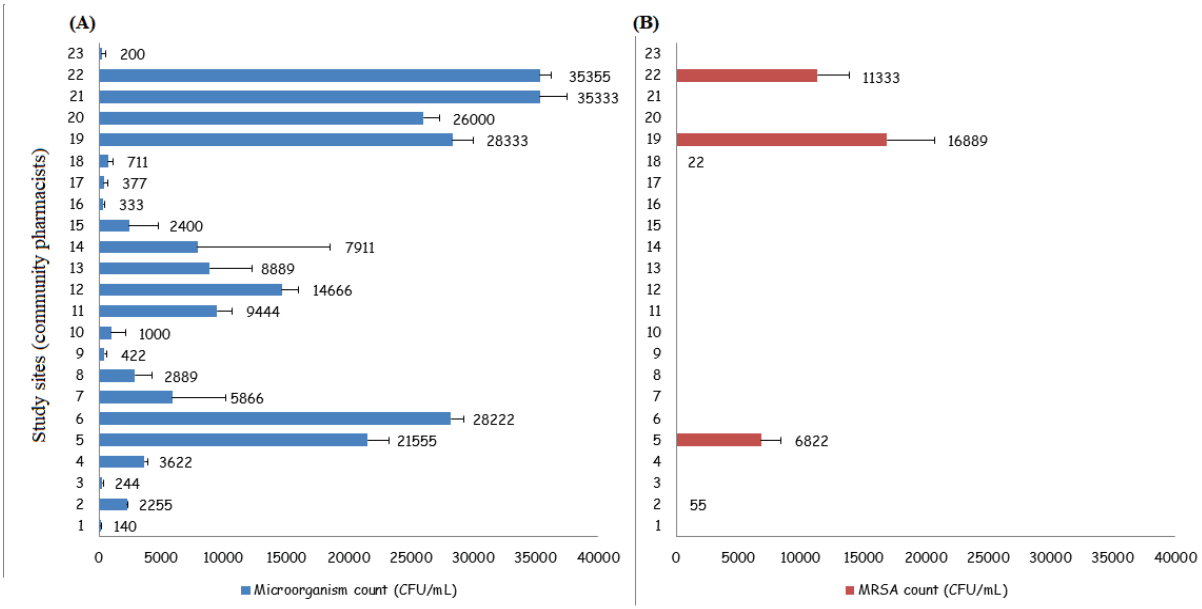

Figure 2. Microorganism count on 25 cm^2 of dispensing counters of selected community pharmacies. (A) Total microorganism count on nutrient agars; (B) Total MRSA count on *Brilliance* MRSA 2 agars.

MRSA Contamination between Community Pharmacies in Shopping Mall and High Street Settings

Figure 3 shows MRSA was detected on two dispensing counters of community pharmacies in high street. Three dispensing counters of community pharmacies in shopping mall setting were detected with MRSA.

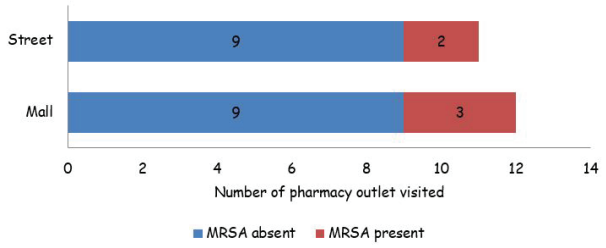

Figure 3. No significant difference in MRSA contamination between community pharmacies in shopping mall and high street settings.

Table 1 shows that the nature of community pharmacy settings (shopping mall or high street) did not significantly correlate with the presence of MRSA contamination on dispensing counter ($P > 0.05$).

Table 1. Independent T-test on difference in MRSA contamination between community pharmacies in shopping mall and high street settings.

		t-test for Equality of Means
		Sig. (2-tailed)
Location	Equal variances assumed	.708

Discussion

A comparable study was conducted at Yamaguchi University Hospital, Japan in 2013. The study assessed the presence of MRSA contamination on the surfaces of bed side rails, overbed tables and curtains in the rooms of 24 inpatients that were infected with MRSA infections. The prevalence of MRSA contamination on the surfaces of bed side rails and overbed tables was 31.6% (6 out of 19) and 25% (6 out of 24) respectively. The total number of bedside rails examined was 19 instead of 24 because 5 of the patients were using beds without side rails. However, there was an absence of MRSA contamination on the surfaces of all 24 curtain samples[19].

Three other similar studies that evaluated the prevalence of MRSA contamination on the surfaces of curtains have presented different results. The prevalence of MRSA contamination was reported to be 28% (14 out of 50)[20], 15.5% (31 out of 200)[21], and 92% (12 of 13 samples)[22]. The method used for MRSA detection may have been different for each study to which it leads to differences in results. Therefore, further studies are needed to answer the inconsistencies in these results.

In this study, the prevalence of MRSA on the dispensing counters of community pharmacies in Klang Valley was found to be 22% (5 out of 23). The number might be worrying since community pharmacies are easily accessible by the public. Although the data collected could be improved, the cleanliness of dispensing counters should be prioritized since patients normally receive their medications from pharmacists over the dispensing counters. As a result, the transmission of MRSA to other healthy populations might occur through the platform of dispensing counters.

In addition, we also found that the occurrence of MRSA contamination between different community pharmacy settings (shopping mall or high street) was not statistically significant. The location of pharmacies whether in a shopping mall or at high street, did not significantly reflect the hygiene of the dispensing counter. We would like to highlight other factors that may affect the cleanliness of pharmacy, for instance, the responsibility of pharmacist and staff members. In addition, increasing awareness of public to consult community pharmacists might also lead to cross

contamination of antibiotic-resistant microorganism[23], with several research studies reported evidence of microorganism transmissions between environmental surfaces and patients[24].

Based on the Good Dispensing Practices by World Health Organization (WHO), a clean, organized and safe working environment provides a basis for good practice. Dispensing environments of a community pharmacy should be clean, hygienic and uncontaminated since most of the pharmaceutical products are for internal use. The dispensing environment of a community pharmacy includes work surfaces, staff members, physical surroundings, shelves, counters and so on. In addition, staff members should maintain good personal hygiene and wear clean clothing if they are involved in dispensing. The staff members should also avoid skin contact with pharmaceutical products during dispensing to prevent any contamination. Maintaining a clean working environment in the community pharmacy requires daily cleaning of floors and working surfaces, daily removal of waste and a regular routine of shelves cleaning. It is also essential to clean the equipment used after handling any pharmaceutical products[25].

Private healthcare sector such as community pharmacy is in growing need in developing countries. These pharmacies are always the primary source of healthcare for the public due to convenience, close in proximity, reasonable price, responsiveness and flexibility in operating hours[26]. Therefore, community pharmacy should be kept at good level of hygiene to prevent cross contamination of multidrug-resistant microorganism.

Conclusion

MRSA contamination has been detected on dispensing counters of community pharmacies in Klang Valley. The prevalence of MRSA on the dispensing counters was 22% (5 out of 23). In addition, this study also showed that the presence of MRSA contamination was independent from the location and setting of community pharmacies.

Authors Contribution

The research and manuscript writing were performed by Y-LC. H-WT founded the research project.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Original Research Article

Progress in Microbes and Molecular Biology

HH PUBLISHER

Landscape Of *HOXA* Genes Methylation in Colorectal Cancer

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Abstract: Colorectal cancer (CRC) is among the most common cancers worldwide and the second leading cause of cancer-related death in Malaysia. The *HOXA* gene cluster is a family of Homeobox A genes encoding transcriptional regulators that play vital roles in cancer susceptibility and progression. Dysregulated *HOXA* expression influences various aspects of carcinogenesis processes. Therefore, this study aims to elucidate the methylation landscape of *HOXA* genes in CRC. Twelve pairs of CRC — adjacent normal tissues were subjected to Infinium DNA MethyEPIC array. Differentially methylated regions were identified using the ChAMP Bioconductor and methylation levels of *HOXA* genes were manually curated. We identified 100 significantly differentially methylated probes annotated to *HOXA* genes. *HOXA3* has the highest number of differentially methylated probes (n=27), followed by *HOXA2* (n=20) and *HOXA4* (n=14). The majority (43%) of the probes were located at the transcription start site (TSS) 200, which is one of the gene promoters. In respect to CpG islands (CGI), the probes were equally located in the island and shore regions (47% each) while a minor percentage was in the shelf (6%). Our work gave a comprehensive assessment of the DNA methylation pattern of *HOXA* genes and provide the first evidence of *HOXA2, HOXA3* and *HOXA4* differential methylation in Malaysian CRC. The new knowledge from this study can be utilized to further increase our understanding of CRC methylomics, particularly on the homeobox A genes. The prognostic and diagnostic roles of the differentially methylated *HOXA* genes warrant future investigations.

Keywords: Homeobox A genes; colorectal cancer; DNA methylation; *HOXA2*; *HOXA-AS3*

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Introduction

Cancer is a continuous global burden and colorectal cancer (CRC) placed as the fourth most frequently diagnosed cancer worldwide[1] and second in Malaysia[2,3]. Cancer occurs through the accumulation of multiple genetics and epigenetics changes[4]. Somatic mutation in *APC*, *BRAF*, *KRAS*, *PIK3CA* and *TP53*[5–8] are identified in CRC at varying frequencies and are perceived as the drivers of CRC formation. Although there are many efforts on investigating the molecular alterations involved in CRC pathogenesis, the existing knowledge remains inadequate for an early diagnosis and prognosis assessment. Therefore, further understanding of epigenetic components involved in CRC carcinogenesis is highly sought after and will unravel new genes which can be utilized as the diagnostic, prognostic and predictive biomarkers to prevent CRC-related mortality.

Epigenetics mechanism can be generally categorized into histone modification and DNA methylation[9], with the latter being the most widely investigated. The clinical application of DNA methylation markers to determine at-risk patient populations, improve diagnostic criteria, and provide prognostic factors to guide treatment decisions are becoming increasingly relevant. This

is because DNA methylation can be reversed, thereby providing alternative treatment options for patients with methylation phenotype. Clinically, DNA methylation has also been demonstrated to have significant utility owing to its stability and relative ease of testing[9]. For instance, *SEPT9* gene is the first US Food and Drug Administration (FDA)–approved diagnostic assay for CRC screening (https://www.epiprocolon.com). However, the clinical utility of the methylated *SEPT9* assay is still limited owing to patients' heterogeneity factor which includes various demographic characteristics and pathological features[10]. The search for a good diagnostic, prognostic and predictive DNA methylation markers in CRC is an active area of research.

Homeobox genes play a role as the master regulators of morphogenesis and are aberrantly expressed in cancer[11]. These genes possess a highly conserved DNA sequence that code for the homeodomain proteins, which act as transcription factors that bind specifically to the DNA motifs and regulate the genes involved in cellular processes including adhesion, proliferation and differentiation[11]. Over 200 homeobox genes have been discovered in the human genome and are divided into four *HOX* gene clusters namely *HOXA*, *HOXB*, *HOXC*, and *HOXD*, positioned at the chromosome 7p15, 17q21.2, 12q13, and 2q31 loci, respectively. Of these, there are 11 *HOXA* genes[12] (Table 1).

Table 1. The list of 11 HOXA genes under HOXL subclass homeoboxes [11].

HGNC ID (gene)	**Approved symbol**	**Approved name**
HGNC:5099	*HOXA1*	Homeobox A1
HGNC:5103	*HOXA2*	Homeobox A2
HGNC:5104	*HOXA3*	Homeobox A3
HGNC:5105	*HOXA4*	Homeobox A4
HGNC:5106	*HOXA5*	Homeobox A5
HGNC:5107	*HOXA6*	Homeobox A6
HGNC:5108	*HOXA7*	Homeobox A7
HGNC:5109	*HOXA9*	Homeobox A9
HGNC:5100	*HOXA10*	Homeobox A10
HGNC:5101	*HOXA11*	Homeobox A11
HGNC:5102	*HOXA13*	Homeobox A13

Dysregulated homeobox gene expression is a frequent in cancer and one of the mechanisms causing such dysregulation is DNA methylation. The *HOXA* cluster is often methylated in the non–small cell lung cancer (NSCLC)[13,14], while *HOXA5* and *HOXA11* promoter methylation diminishes their tumour-suppressive function through transcriptional silencing[15,16]. *HOXA11* is hypermethylated in gastric cancer tissues and is significantly associated with TNM III and IV patients[17]. Hypomethylation *HOXA10* and *HOXA11* able to discriminate ovarian cancer tissue from the normal tissue[18]. *HOXA9* was reported to be hypermethylated in half of the ovarian cancer patients and is significantly associated with endometrioid histological subtype[19]. In bladder cancer, *HOXA9* promoter methylation has been linked with cisplatin chemotherapy-resistant and metastatic bladder cancer, and reversing the DNA methylation using decitabine sensitized the cancer cells to cisplatin[20]. Yet, little is known about *HOXA* methylome in CRC. Only recently, Li and colleagues reported the DNA methylation status of three HOXA genes, which are *HOXA2*, *HOXA5* and *HOXA6* in CRC using targeted bisulfite sequencing assay[21]. Nevertheless, a comprehensive, unbiased methylome profile of *HOXA* genes has not been described. Therefore, this study aims to investigate the methylation landscape of *HOXA* genes in CRC context.

Material and Methods

Clinical specimens

The archived 12 pairs of tumour-adjacent normal fresh frozen colon tissues (n=24) from CRC patients were retrieved from the UMBI-HCTM Biobank. The tissues were collected according to the procedures approved by UKM Research Ethics Committee. As a quality control procedure, all tissues were cryosectioned, followed by haematoxylin and eosin staining for the pathologist to determine the percentage of tumour cells and normal cells contents. Only tumour samples with ≥ 80% cancerous cells and normal adjacent colon tissues with ≤ 20% necrosis were selected for DNA extraction using Allprep DNA/RNA/miRNA Universal Kit (Qiagen, USA) according to the manufacturer's instructions. Then, the integrity of DNA was assessed using agarose gel electrophoresis while the quantity and purity were evaluated using Nanodrop 2000c Spectrometer (Thermo Fisher Scientific, USA).

Bisulfite Conversion and Methylation Microarray

Five hundred nanogram (500 ng) of DNA was subjected to bisulfite conversion to change all unmethylated cytosine to uracil using the EZ DNA methylation — Gold kit (Zymo Research, USA) according to the manufacturer's protocol. The effectiveness of bisulfite conversion was determined using Universal Methylated DNA Standard & Control Primers (Zymo Research, USA) according to the manufacturer's protocol. The Infinium DNA MethylationEPIC assay, covering 850,000 CpG dinucleotides spread over the whole genome, was performed according to the manufacturer's specifications (Illumina, Inc.).

Methylation Microarray Data Analysis

The raw idat files obtained from methylation microarray were subsequently analyzed using GenomeStudio V1.9.0 and CHAMP Bioconductor packages[22]. Filters were applied to all datasets where CpG sites with detection p-values ≥ 0.01 in one or more samples were omitted from further analysis. To reduce the technical biases intrinsic to the probe design, the raw intensities were SWAN-normalized prior to statistical analysis[23]. β-values were then extracted and subjected to further statistical analysis.

Expression of *HOXA* genes from The Cancer Genome Atlas (TCGA) study

The mRNA expression data of selected *HOXA* genes in CRC were retrieved using web-based FireBrowse Gene Expression Viewer tool (https://gdac.broadinstitute.org/)

from Broad Institute. This tool provides access to results of various omics analyses involving more than 14,000 cancer cases, from 38 types of cancers based on TCGA data version 2016_01_28.

Statistical Analysis

A T statistic from the limma Bioconductor package was used to determine the differentially methylated CpG sites[24,25]. The CpG sites were further filtered at an adjusted p-value < 0.05 to identify significant differentially methylated *HOXA* genes. To substantiate the specificity and accuracy of the differentially methylated probes, the discriminative performance of the probes was evaluated by receiver operating characteristic (ROC) curves, and the area under the ROC curve (AUC), specificity, and sensitivity at the optimal cut-offs were determined using GraphPad Prism V8 (GraphPad Software, Inc., USA).

Results

Locations of differentially methylated loci in *HOXA* genes

We analysed the differential methylation status of 12 CRC tissue samples with the 12 adjacent cancer-free colonic tissue samples and only differentially methylated regions with adjusted p-value < 0.05 were reported. From the list of differentially methylated probes, we further filtered for *HOXA* genes. Here, we found that there are 100 CRC-associated differentially methylated probes in 11 *HOXA* genes, noncoding *HOXA-AS3* and *HOXA10-HOXA9* readthrough. *HOXA3* has the highest number of differentially methylated probes (n=27), followed by *HOXA2* (n=20) and *HOXA4* (n=14) (Figure 1A). The majority (43%) of the probes were located at the transcription start site (TSS) 200 (Figure 1B), which is one of the gene promoters. In respect to CpG islands (CGI), the probes were equally located in the island region and shore regions (47% each) while a minor percentage was in the shelf (6%) (Figure 1C).

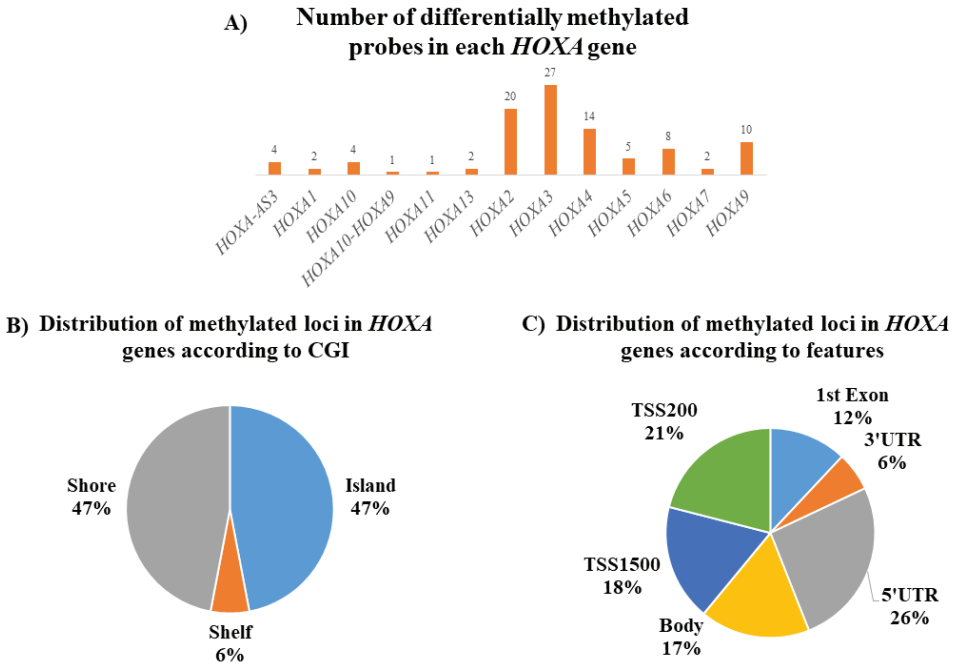

Figure 1. Differentially methylated HOXA genes in CRC. (A) The number of differentially methylated probes in each HOXA genes. (B) Distribution of methylated loci in HOXA genes with respect to features. (C) Distribution of methylated loci in HOXA genes with respect to CGI.

The genomic and gene-related regions of the significant differentially methylated *HOXA* genes were distributed differently. Generally, 53 probes (in seven genes) were hypermethylated compared to 47 loci (eight genes) that were hypomethylated. The largest portion of hypomethylated sites (55.3%) were in the shore and subsequently decreased in other categories (island 42.6% and shelf 2.1%). In contrast, more than half (50.9%) of the significantly hypermethylated loci of *HOXA* genes were on the island, followed by the shore (39.6%), and shelf (9.4%). None of the loci was identified in opensea region. Meanwhile, most of the significantly hypomethylated loci were in the 5'UTR (46.8%), followed by TSS200 (14.9%), gene body (12.8%), and 8.5% in each 1st exon, 3'UTR and TSS1500. Meanwhile, around a quarter (26.4%) of the significant hypermethylated loci were located in TSS200 and TSS1500, while the rest were mainly found in the gene body (20.8%), 1st exon (15.1%), and, to a lesser extent, in the 5' and 3' UTR (7.5% and 3.8%, respectively).

Differentially methylated HOXA genes

All of the 11 *HOXA* genes are significantly differentially methylated. Due to the power of comprehensive contents in the microarray platform, we also identified significant hypomethylation of the noncoding *HOXA-AS3* and *HOXA10-HOXA9* readthrough. High resolution, probe-level analyses revealed hypomethylation of 20 loci in *HOXA3*, with the remaining seven loci were hypermethylated. In *HOXA2* and *HOXA9*, all the probes were hypermethylated, while probes in *HOXA4* were hypomethylated. *HOXA6* exhibited eight hypermethylated loci and only one was hypomethylated. The summary of hypo- and hypermethylated probes in each gene were summarized in Table 2.

Table 2. The number of hypermethylated and hypomethylated probes in the *HOXA* genes.

Genes	Number of hypermethylated probes	Genes	Number of hypomethylated probes
HOXA1	2	*HOXA-AS3*	4
HOXA2	20	*HOXA10*	4
HOXA3	7	*HOXA10-HOXA9*	1
HOXA5	5	*HOXA11*	1
HOXA6	7	*HOXA13*	2
HOXA7	2	*HOXA3*	20
HOXA9	10	*HOXA4*	14
		HOXA6	1

The 100 significant probes with the methylation changes (Δβ) are illustrated in Table 3. It is worth mentioning that the loci in *HOXA2* were the locations with the highest methylation changes; these probes were hypermethylated in CRC as compared to the normal colon. On the other hand, the loci in *HOXA3* were the most hypomethylated probes in CRC as compared to the normal colon.

Table 3. The 100 significant differentially methylated probes in *HOXA* genes.

Genes	Probes	Adjusted p-value	Δβ	Feature	CGI
HOXA2	cg06055873	2.66E-05	0.368	1st Exon	shore
HOXA2	cg24058604	1.35E-04	0.362	TSS200	shore
HOXA2	cg05921905	2.49E-04	0.362	TSS200	shore
HOXA2	cg04737131	3.75E-04	0.357	TSS1500	shore
HOXA2	cg17353412	6.30E-06	0.357	1st Exon	shore
HOXA2	cg20747380	2.23E-05	0.356	1st Exon	shore
HOXA2	cg02979457	3.64E-04	0.326	TSS200	shore
HOXA2	cg22943986	1.19E-03	0.322	TSS1500	shore
HOXA2	cg06786372	5.16E-03	0.319	Body	shore
HOXA2	cg26069745	1.55E-04	0.317	1st Exon	shore
HOXA2	cg06769202	8.92E-04	0.312	TSS200	shore
HOXA2	cg09871315	1.95E-03	0.293	TSS1500	shore
HOXA2	cg23979631	2.88E-04	0.291	TSS200	shore
HOXA5	cg03744763	7.31E-03	0.286	TSS1500	island
HOXA1	cg07450037	1.02E-03	0.282	Body	shore
HOXA2	cg20087093	2.13E-03	0.276	TSS1500	shore
HOXA2	cg02803819	7.12E-03	0.258	Body	shelf
HOXA9	cg12600174	1.19E-02	0.249	TSS200	island
HOXA3	cg27539480	4.52E-03	0.244	3'UTR	shore
HOXA2	cg23206851	6.94E-03	0.243	TSS1500	shore
HOXA3	cg02627455	5.21E-03	0.240	5'UTR	shelf
HOXA2	cg13661519	4.09E-03	0.240	Body	shelf
HOXA3	cg07153966	2.74E-02	0.234	Body	island
HOXA9	cg21001184	1.42E-02	0.228	TSS200	island
HOXA9	cg03217995	3.19E-02	0.226	Body	shore
HOXA2	cg00188704	3.46E-02	0.222	Body	shelf
HOXA1	cg03116258	4.38E-04	0.220	1st Exon	shore
HOXA5	cg14882265	4.94E-02	0.211	TSS1500	island
HOXA2	cg02225599	1.51E-02	0.204	TSS1500	island
HOXA3	cg14216068	9.16E-03	0.194	3'UTR	island
HOXA3	cg09591524	3.06E-02	0.186	5'UTR	island
HOXA6	cg14044640	3.34E-02	0.184	TSS200	island
HOXA5	cg03368099	2.16E-02	0.183	TSS1500	island
HOXA7	cg20725013	2.91E-02	0.182	Body	shore
HOXA3	cg02439266	1.46E-02	0.182	5'UTR	island
HOXA5	cg01748892	3.58E-03	0.180	TSS1500	island

HOXA3	cg12305431	1.79E-02	0.175	5'UTR	shelf
HOXA5	cg13694927	1.20E-02	0.168	TSS1500	island
HOXA9	cg26476852	4.68E-02	0.167	1st Exon	island
HOXA9	cg20399871	2.10E-02	0.164	1st Exon	island
HOXA6	cg03529432	4.98E-02	0.161	TSS200	island
HOXA9	cg16104915	1.81E-02	0.154	TSS200	island
HOXA6	cg22469274	4.68E-02	0.154	TSS200	island
HOXA6	cg09936824	3.98E-02	0.153	TSS1500	island
HOXA6	cg19183743	4.34E-02	0.150	TSS1500	shore
HOXA7	cg21778348	3.36E-02	0.149	Body	island
HOXA9	cg16913789	4.62E-02	0.146	Body	island
HOXA2	cg01217984	1.10E-02	0.134	TSS1500	island
HOXA9	cg05065989	1.07E-02	0.129	TSS200	island
HOXA9	cg07778029	3.18E-02	0.125	1st Exon	island
HOXA6	cg04265576	2.56E-02	0.123	TSS200	island
HOXA9	cg03698009	4.16E-02	0.108	Body	island
HOXA6	cg12810523	4.84E-02	0.103	TSS200	island
HOXA10	cg08938793	4.30E-02	-0.041	3'UTR	shore
HOXA6	cg23590202	8.97E-04	-0.052	TSS1500	shore
HOXA13	cg01363170	4.33E-02	-0.056	3'UTR	shelf
HOXA-AS3	cg10374314	4.89E-02	-0.070	Body	shore
HOXA10	cg05092861	5.66E-03	-0.078	TSS200	shore
HOXA4	cg23884241	2.58E-02	-0.078	1st Exon	island
HOXA4	cg04317399	1.43E-02	-0.084	1st Exon	island
HOXA13	cg02366798	4.96E-02	-0.089	3'UTR	shore
HOXA4	cg03724423	2.08E-02	-0.096	TSS1500	shore
HOXA4	cg11410718	1.61E-02	-0.108	TSS200	island
HOXA4	cg07317062	2.15E-02	-0.114	5'UTR	island
HOXA10	cg01078824	2.94E-02	-0.118	TSS200	shore
HOXA4	cg19142026	1.84E-02	-0.137	5'UTR	island
HOXA4	cg17591595	4.32E-02	-0.151	TSS1500	shore
HOXA4	cg22997113	1.80E-02	-0.170	1st Exon	island
HOXA3	cg16406967	1.99E-02	-0.196	5'UTR	island
HOXA3	cg22798849	3.57E-02	-0.198	5'UTR	island
HOXA3	cg18680977	1.76E-02	-0.209	5'UTR	island
HOXA3	cg16748008	4.02E-02	-0.220	5'UTR	island
HOXA4	cg11532431	2.10E-02	-0.231	Body	island
HOXA11	cg05516617	8.55E-03	-0.231	3'UTR	shore
HOXA3	cg23403004	2.42E-04	-0.244	5'UTR	shore
HOXA3	cg04778178	2.48E-04	-0.254	5'UTR	island
HOXA3	cg16644023	4.88E-02	-0.255	5'UTR	shore
HOXA3	cg15982700	4.41E-02	-0.259	5'UTR	shore
HOXA3	cg23806243	2.21E-03	-0.260	5'UTR	shore
HOXA3	cg00318947	1.81E-02	-0.270	5'UTR	shore
HOXA4	cg00562553	1.01E-02	-0.272	1st Exon	island
HOXA4	cg20171892	5.24E-04	-0.273	Body	island
HOXA3	cg04351734	2.10E-02	-0.274	5'UTR	island
HOXA10	cg05517976	1.19E-02	-0.275	TSS200	shore
HOXA4	cg11227540	1.46E-02	-0.279	Body	shore
HOXA4	cg09799676	3.54E-03	-0.286	Body	island
HOXA3	cg21556281	1.04E-02	-0.291	5'UTR	shore
HOXA-AS3	cg14429861	7.94E-03	-0.299	TSS200	shore
HOXA3	cg18430152	7.29E-04	-0.305	5'UTR	island

HOXA10-HOXA9	cg22274074	1.72E-03	-0.307	TSS1500	shore
HOXA3	cg14072564	6.32E-04	-0.311	5'UTR	island
HOXA4	cg17132446	4.36E-03	-0.323	Body	shore
HOXA3	cg01820751	2.39E-03	-0.328	5'UTR	shore
HOXA-AS3	cg06188746	3.12E-03	-0.331	TSS200	shore
HOXA-AS3	cg18091117	3.05E-03	-0.331	TSS200	shore
HOXA3	cg03483713	4.12E-04	-0.346	5'UTR	shore
HOXA3	cg26297005	1.81E-03	-0.361	5'UTR	island
HOXA3	cg15725372	6.95E-04	-0.367	5'UTR	island
HOXA3	cg00431187	7.17E-04	-0.381	5'UTR	shore
HOXA3	cg09798023	2.84E-04	-0.390	5'UTR	shore

TSS: transcription start site

UTR: untranslated regions

Expression of *HOXA* genes and their correlation with methylation level

Using FireBrowse, gene expression of the *HOXA* genes were retrieved from COAD[5] and COADREAD[26] studies (Figure 2). The data were presented as log2 fold change. The expression of seven *HOXA* genes was downregulated (*HOXA1*, *HOXA2*, *HOXA4*, *HOXA5*, *HOXA6*, *HOXA7*, and *HOXA13*) while four of genes (*HOXA3*, *HOXA9*, *HOXA10*, *HOXA11*) were upregulated. The expression profiles of *HOXA1*, *HOXA2, HOXA5, HOXA6, HOXA7, HOXA10*, and *HOXA11* are inversely related to the methylation level as predicted, but not the *HOXA4*, *HOXA9* and *HOXA13*.

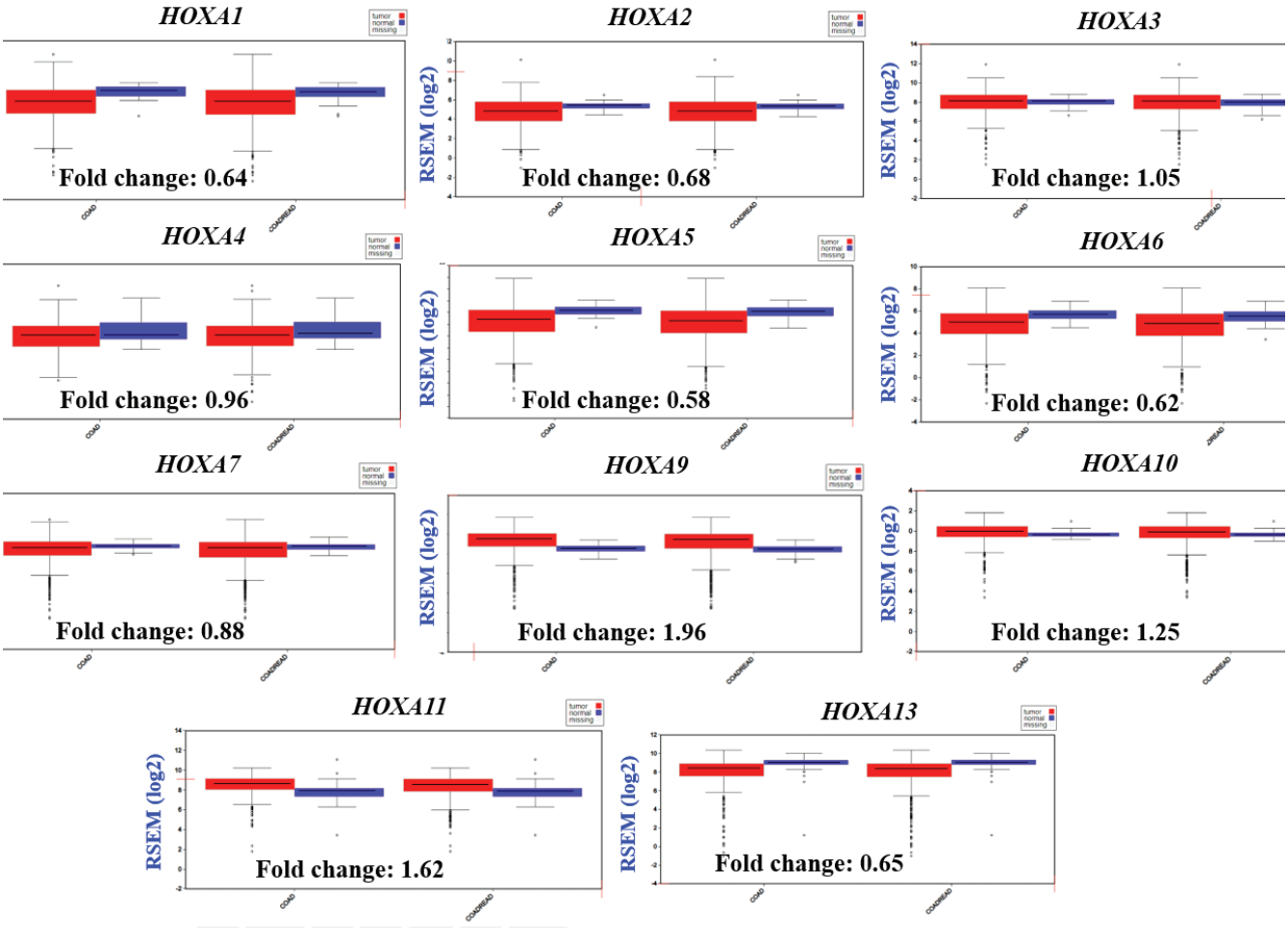

Figure 2. Gene expression of *HOXA* genes from TCGA COAD and COAREAD studies.

Receiver operating characteristics (ROC) curve analysis

Lastly, the sensitivity and specificity of the methylation levels were further assessed using receiver-operator curve (ROC) analysis. The methylation levels of 10 topmost hypermethylated CpG sites significantly differentiated the CRCs from the normal colonic tissues (*p*-value 0.0032 to 0.0002) (Table 4). The highest discriminative accuracy was demonstrated by *HOXA2* cg06055873 (AUC = 0.9514, confident interval = 0.8547 to 1.000, *p*-value = 0.0002). Other candidate probes also reached high diagnostic accuracy (Table 4; Figure 3).

Table 4. Receiver operating characteristics (ROC) curve analysis of the top 10 differentially methylated probes in *HOXA2* gene.

Gene_probe	Area	Std. Error	95% confidence interval	*P* value
HOXA2 cg06055873	0.9514	0.04935	0.8547 to 1.000	0.0002
HOXA2 cg24058604	0.9444	0.04489	0.8565 to 1.032	0.0002
HOXA2 cg05921905	0.875	0.08441	0.7096 to 1.04	0.0018
HOXA2 cg04737131	0.8958	0.07498	0.7489 to 1.043	0.001
HOXA2 cg17353412	0.9444	0.05546	0.8357 to 1.053	0.0002
HOXA2 cg20747380	0.9375	0.06155	0.8169 to 1.058	0.0003
HOXA2 cg02979457	0.8681	0.07918	0.7129 to 1.023	0.0022
HOXA2 cg22943986	0.8611	0.09216	0.6805 to 1.042	0.0027
HOXA2 cg06786372	0.8542	0.08306	0.6914 to 1.017	0.0032
HOXA2 cg26069745	0.9167	0.0691	0.7812 to 1.052	0.0005

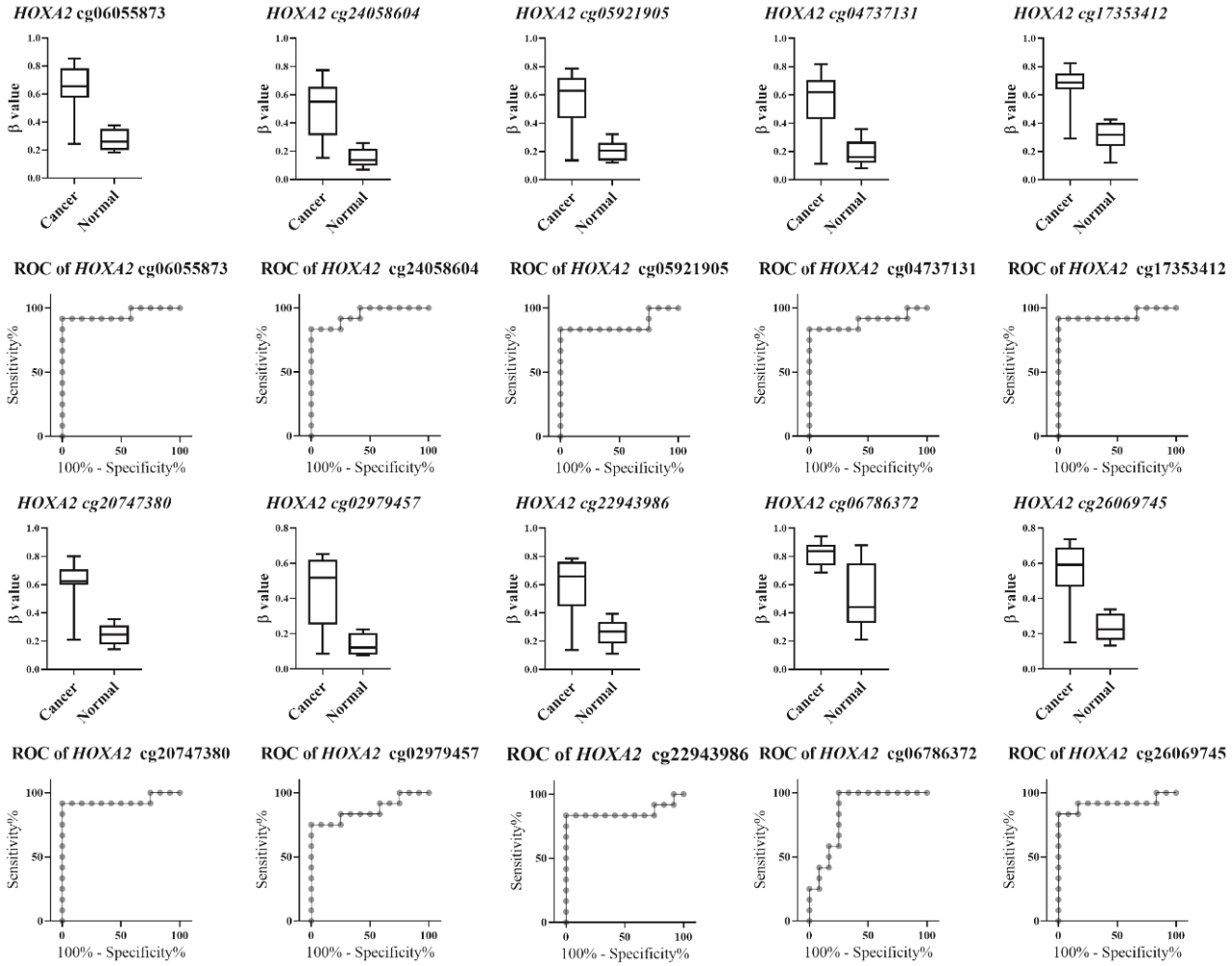

Figure 3. Box plot illustrating the comparison of β values and ROC curve-based evaluation of the diagnostic accuracy for the top 10 hypermethylated *HOXA2* probes in cancerous and normal tissues.

Discussion

In this study, we analyzed in greater detail the genome-wide methylation patterns of *HOXA* genes from 12 CRCs compared with their adjacent normal tissues. Interestingly, due to the high throughput nature of microarray platform, our analysis revealed that all of the 11 *HOXA* genes are significantly differentially methylated in CRCs. We are the first to report this new finding and shed new light on the possible role of these genes in colorectal carcinogenesis. Epigenetic alteration of *HOXA* genes has been widely studied in many cancers, especially non-small cell lung cancer (NSCLC)[27–29], yet, the investigation about this gene cluster in CRC is lacking. To date, there is only a handful of study which investigates the methylome of *HOXA* genes[21,30–32].

HOXA2 is the most significantly hypermethylated *HOX* gene in our study, and the methylated loci were mostly located in the promoter regions (TSS200 and TSS1500). Hypermethylation of this gene in CRC has also been recently reported in concordance with our finding[21]. In addition, the significant association between *HOXA2* methylation with age, node status (N), stage, metastasis (M), lymphovascular invasion, perineural invasion, as well as the number of lymph node was also demonstrated[21]. Another study has shown that *HOXA2* is hypermethylated in the rectal cancer mucosa compared to the nonmalignant rectal mucosa[30]. DNA methylation is known to be inversely correlated with mRNA expression; however, the published data on *HOXA2* expression is lacking and the aforementioned two studies did not investigate the gene expression levels. Therefore we attempted to investigate the mRNA expression using the TCGA CRC dataset and the finding is in agreement with our hypothesis. The expression of *HOXA2* is indeed downregulated in CRC compared to the normal tissues by 0.68 fold, and thus, could be explained by its hypermethylation status. Nevertheless, *HOXA2* promoter is also found to be hypermethylated in nasopharyngeal cancer, whereby it associates with low mRNA expression in the biopsies and cell lines[33]. Moreover, in a study involving 101 patients from stage I–III NSCLC, methylation of *HOXA2* was proposed to have prognostic significance in squamous cell carcinoma (SCC) subtypes patients[34]. Due to the limited number of patients and the lack of clinical information in our study, the association with clinical features were not established and warrant further investigation. In addition, ROC curves for *HOXA2* gene show exceptional diagnostic ability in differentiating CRC from the normal healthy tissue, especially in Stage I patients with the AUC = 0.9979 [21]. We also observed a similar trend, whereby the loci in *HOXA2* are the most significantly hypermethylated and exhibited high discriminative accuracy.

HOXA5 and *HOXA6* promoters were both hypermethylated in our study and this is supported by recent data by Li and colleagues[21]. The authors went on to demonstrate a significant association between *HOXA5* hypermethylation and age, tumour (T), metastasis (M), stage, and patients' tumour status, while *HOXA6* hypermethylation is correlated with age and presence of *KRAS* mutation. Similarly, with *HOXA2* genes, *HOXA5* and *HOXA6* have not been studied in detail in CRC. In other malignancies, such as NSCLC, *HOXA5* is hypermethylated[35] and a separate study showed that low *HOXA5* expression indicates unfavourable prognosis and reduces cell proliferation by via p21 expression[36]. Our TCGA analyses revealed downregulation of *HOXA5* and *HOXA6* by 0.58 and 0.62 fold, respectively. The relationship between downregulation of *HOXA5* and *HOXA6* with CRCs patients prognosis is the subject for further research. *HOXA5* also plays a role in haematopoietic differentiation, whereby *HOXA5* is hypermethylated in the development of acute myeloid leukaemia (AML)[37]. Additionally, *HOXA6* hypermethylation was reported in oral cancer [38] and more recently in meningiomas[39].

We observed the hypomethylation of *HOXA3* and *HOXA4* among our patients, which is in disagreement with other CRC studies [31, 32]. It is unclear what causes the discrepancies and it will be worthwhile to reconfirm this finding in a larger cohort. By looking at the gene expression of *HOXA3* and *HOXA4* in CRC, several studies partly support our findings. For instance, Zhang and colleagues[40] demonstrated that *HOXA3* expression is increased in both CRC tissues and cell lines. Their analysis of the relationship between *HOXA3* and tumour progression has revealed that elevated *HOXA3* expression is linked with poor survival rates in CRC. On the other hand, our finding on *HOXA4* hypomethylation is also partially supported by Bhatlekar and colleagues whereby *HOXA4* is found to be overexpressed in CRC[41]. They further demonstrated that overexpression of *HOXA4* encourages self-renewal, leading to the overabundance of colon cancer stem cell[42], which play an essential role in the metastasis and relapse of this disease. Taken together, *HOXA3* and *HOXA4* hypomethylation, as identified from our study, may play an important role in CRC.

To the best of our knowledge, hypomethylation of the long noncoding RNA *HOXA-AS3* and *HOXA10-HOXA9* readthrough has never been reported before. Therefore we are the first to notice such observation in CRC. *HOXA10-HOXA9* readthrough represents a naturally occurring read-through transcription between the *HOXA10* and neighbouring *HOXA9* and is a candidate for nonsense-mediated mRNA decay (NMD), which does not produce any protein product. Published literature regarding this readthrough is severely lacking. On the other hand, *HOXA-AS3* has been gaining more attention from cancer researchers. In lung cancer, *HOXA-AS3* expression was significantly increased and inhibition of *HOXA-AS3* impairs cancer cell proliferation, migration, and invasion[43]. *In vitro* experiment further supported its oncogenic role, where the A549-derived xenografts with silenced *HOXA-AS3* had significantly reduced tumour weights and volumes. These findings suggest the potential application of *HOXA-AS3* inhibition as an effective targeted therapy for lung cancer patients[43]. The authors also concluded that the upregulated *HOXA-AS3* expression was shown to be caused by histone acetylation, and the link between histone deacetylation and DNA methylation has been established[44]. Furthermore, *HOXA-AS3* confers resistance towards cisplatin treatment via interaction with *HOXA3*

in NSCLC[45]. In glioma, *HOXA-AS3* upregulation promotes tumour progression and predicts poor prognosis[46]. It is probable that the hypomethylation of *HOXA-AS3* in our CRC patients could lead to its increased expression. It will be interesting to validate this observation and investigate its function in CRC.

An interrogation *HOXA*-associated oncogenes or tumour suppressors as prospective mechanisms as predictive biomarkers may offer novel therapeutic strategies for treating cancers[47], including CRC. Yet, the obstacle lays in the fact that our knowledge and understanding of *HOXA* genes in the context of CRC are still insufficient. In this study, we further extended the understanding of CRC pathology by investigating the methylome landscape of *HOXA* genes. Nevertheless, our study is not without limitation. While our sample size is rather small, the hypo- and hypermethylation of the *HOXA* genes reported in this study are relevant to carcinogenesis as reported in several studies. For future study, validation of *HOXA* methylation changes in cancer tissues from a larger cohort is necessary, and the association with survival and other clinicopathological data is warranted. Furthermore, an integrated analysis with gene expression data will be of importance to further establish the correlation between *HOXA* methylation and gene regulation.

Conclusion

Using the latest methylation microarray platform, we report a detailed, unbiased landscape of *HOXA* genes methylome and discovered epigenetically regulated candidate genes in CRC carcinogenesis. Specifically, our results provide the primary evidence that aberrant methylation of *HOXA2, HOXA3* and *HOXA4* in Malaysian CRC. The new knowledge from this study can be utilized to further increase our understanding of CRC methylomics, particularly on the homeobox A genes. The prognostic and diagnostic roles of the differentially methylated *HOXA* genes warrant future investigations.

Author Contributions

MI and RB performed the lab experiments, data analysis and manuscript writing. LT-HT, L-HL and NS-AM provided vital guidance for the project and improvement of the writing. The project was conceptualised by NS-AM.

Conflict of interest

The authors declare that there is no conflict of interest in this work.

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Coliform contamination on faucet surface of water vending machines in Klang Valley

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Abstract: In Malaysia, water vending machine serves as an alternative to drinking water supply. However, the quality of drinking water obtained from water vending machines may vary due to microorganism contamination which caused by inadequate hygienic practices and routine maintenance of the machines. In this study, 100 water vending machines were randomly selected from 10 districts of Klang Valley. Sterile cotton swabs were used to collect swab samples from all selected subjects, with swab samples collected on the outer surface of water faucets. Samples were sent to laboratory for culture analyses using Nutrient agars (Oxoid) and HiCrome™ Coliform agars (HiMedia Laboratories). The results showed that none of the water vending machine was contaminated by faecal coliform, however, with close to 80% of the subjects were found contaminated by total coliform (eg. *Klebsiella, Enterobacter* or *Citrobacter* species). Although the presence of total coliform may not be deleterious to the health of end users, our findings highlights the need for authorities and water vending service providers to set an effective sanitation procedure in maintaining the hygienic level of water vending machines.

Keywords: Vending machine; microorganism; faecal coliform; water; hygienic; Klang Valley Malaysia

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Introduction

Food and water have never been safer in terms of the incidence of infectious illness. A variety of bacteria, viruses and even parasite are transmitted via food and water, thus causing a rise of cases in recent years[1–13,]. A growing population with limited resources of clean water has resulted in 4 billion cases of diarrhoea every year[14]. Waterborne diseases can be caused by various pathogenic agents, including *Escherichia, Enterobacter, Shigella, Klebsiella, Campylobacter*, *Cryptosporidium*, *Giardia, Salmonella* and enteric viruses[15]. Of this, *Salmonella, Vibrio, Escherichia*, *Campylobacter* and *Citrobacter* are among the causative agent of foodborne pathogens as well[16–31].

In Malaysia, quality of drinking water remains as one of the main concerns of consumers. A survey conducted by Aini *et al.* reported that odour, taste and colour were the major issues with water supply[32]in spite of these, quantity and quality of drinking water is still one of the main concerns of Malaysian consumers today. An exploratory study was undertaken to determine the level of awareness of respondents on water issues, assess their perception on drinking water quality, and identify measures undertaken by households to improve drinking water quality and to determine sustainable water practices. A cross-sectional research design, utilizing a survey was conducted among urban residents of Seremban town. Data showed that each household had a mean of five members, with an average household income of RM3788.00 (US$1000). Unaffordable water filtration systems have resulted in good sales of product water from vending machines[33], which serve as an alternative source of drinking water. Several factors such as hygienic practice of users, routine maintenance by owners, quality control by authorities are crucial in maintaining the quality of drinking water[34]the vending machine has contributed to a revolution in how we buy food and drink. Despite the very obvious benefits associated with this technology, vending machines have not always been welcome by the customers they are intended to serve. Although occasionally blamed for various nonspecific illnesses, there have been very few studies about the microbiology of food and drink served from such machines. The few studies that have been reported have found high total viable counts (TVC).

Faecal coliform is a rod-shaped anaerobic bacterium with no sporulation. Its members consist of various species of bacteria such as *Escherichia, Enterobacter, Klebsiella, Salmonella* and *Shigella.* They exist in the faecal materials and intestinal tract of humans or warm-blooded animals and enter the water bodies through

the waste products. However, they are usually non-pathogenic and can be used as an indicator organisms to indicate the presence of faecal material in water[33,35].

E.coli O157:H7, a pathogenic strain of *E. coli*, is pathogenic and causes gastroenteritis, bloody diarrhoea, urinary tract infection (UTI) and haemolytic-uremic syndrome (HUS)[36]. According to United States Environmental Protection Agency, the presence of *E. coli* serves as a good indicator of faecal coliform contamination to evaluate the microbiological quality of water. On the other hand, the presence of other coliforms such as *Klebsiella*, *Salmonella*, or *Shigella* in consumable products also increases the risk of health conditions such as urinary/respiratory infections[37], salmonellosis[16,38–40], and shigellosis[41], respectively.

According to National Water Quality Standards, Ministry of Health Malaysia, a Class I water quality should contain total coliform at the maximum of 100 CFU/mL, with faecal coliform at the maximum of 10 CFU/mL. In view of the importance of drinking water quality in relation to the health of users in public, this study aimed to test the presence of faecal coliform and total coliform contamination on the faucets of water vending machines. The results were also correlated to different districts, brands, status of maintenance and licence.

Material and Methods

Agar Media

Nutrient agar powder (Oxoid) and HiCrome™ Coliform agar (HiMedia Laboratories) were purchased and prepared according to manufacturer's instructions. All procedures in this section were conducted under aseptic conditions.

Sampling Sites

Ten water vending machines were randomly chosen from commercial areas of each of the 10 districts of Klang Valley, including Ampang, Cheras, Gombak, Kajang, Klang, Kuala Lumpur, Puchong, Petaling Jaya, Subang Jaya, and Shah Alam. These areas were selected for their higher population density. In total, 100 sampling sites were identified.

Sampling Sample

Sample collections were conducted in biological triplicates, using sterile cotton buds collecting microbiome on the outer surface of water faucets at 1 cycle anticlockwise. The cotton buds were then immediately kept in sterile 15 mL falcon tubes pre-filled with 1 mL of pre-sterilised 0.8% NaCl solution. Samples were sent to Biology Laboratories for downstream assays within 5 hours.

Detection of Total Microbial and Coliform

All samples were spread on either Nutrient agar or HiCrome™ Coliform agar. All agars were incubated (37 °C, 18 hours) and the numbers of colony were documented (in CFU/mL) and analysed. Meanwhile, the colour and morphology of colonies were also observed and recorded. Some colonies were selected for Gram staining for further characterisation.

Statistical Analysis

All results and data were analysed using Statistical Package for the Social Sciences (SPSS) software (version 23). Kruskal-Wallis tests and Mann-Whitney U Tests were used in these analyses. Statistical analyses were conducted based on total microbial counts, between brands, maintenance status, licence status of water vending machines.

Results

Sampling Sites

The sampling sites were presented in Figure 1, which was generated using Google My Maps.

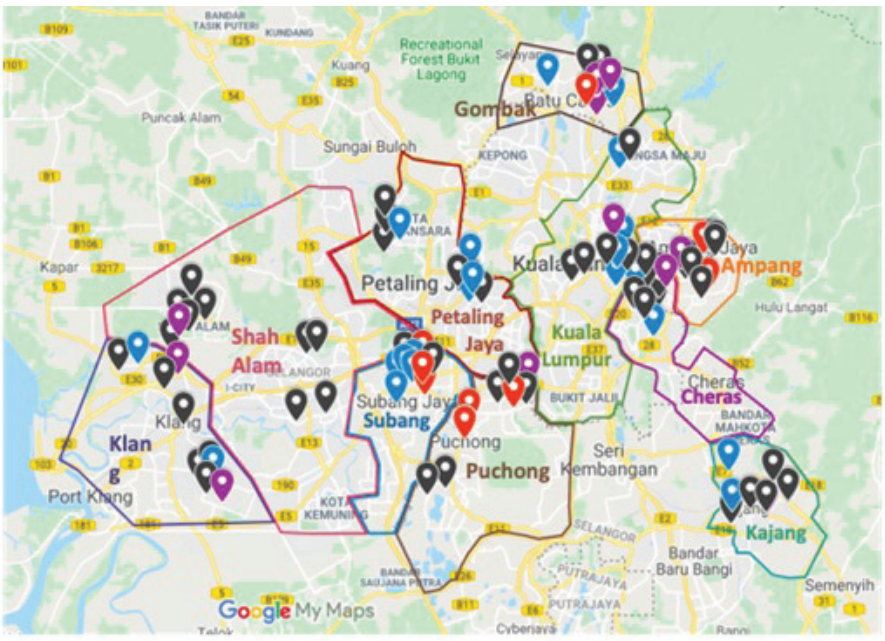

Figure 1. Sampling sites of this research project. Red pointers indicate the location of water vending machines detected with *Enterobacter* or *Citrobacter*; Black pointers indicate the presence of *Salmonella* or *Shigella*; Purple pointers indicate the presence of *Klebsiella*; Blue pointers indicate the absence of coliform contamination.

Total Microbial Counts of Various Districts in Klang Valley

Total microbiome on the faucet surface of water vending machines were measured using Nutrient agars and HiCrome™ Coliform agars (Figure 2).

Bacterial Colonies on HiCrome™ Coliform Agars

The number of bacterial colonies tabulated in Table 3 indicates the presence of *Klebsiella* species (light-pink colonies), *Enterobacter* or *Citrobacter* species (salmon-red colonies), or other Gram negative coliform bacterial species (opaque-white colonies).

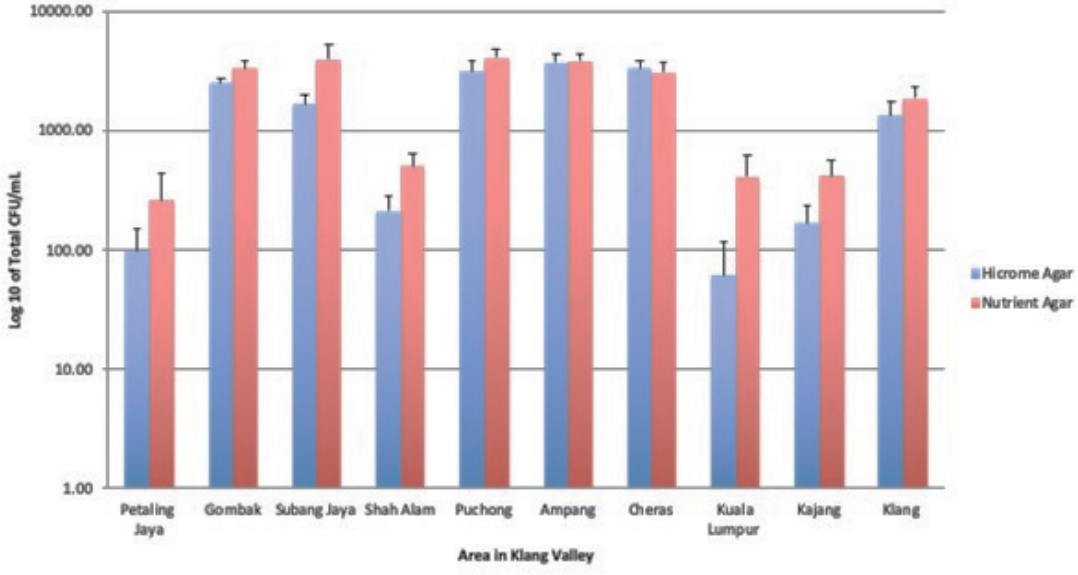

Figure 2. Average of total microbiome (measured in CFU/mL) of various samples collected from 10 districts in Klang Valley.

Table 1 illustrates the outcome of Kruskal Wallis test between different districts. The difference between total CFU/mL detected on HiCrome™ Coliform agars were significant ($P < 0.05$), while results presented by Nutrient agars showed the opposite ($P > 0.05$).

Table 1. Correlation between different districts of Klang Valley and total microbial counts detected on Nutrient agars and HiCrome™ Coliform agars.

	Asymptotic significance	***P*-value**
HiCrome Coliform agar with SLS (HA)	.015	< 0.05
Nutrient agar (NA)	.112	> 0.05

Correlation Between Microbial Counts and Maintenance Status of Water Vending Machines

Table 2 shows that the status of licence during sampling does not contribute significantly to the hygienic level of water vending machines ($P > 0.05$).

Table 2. Correlation between the status of licence during sampling and total microbial counts detected on Nutrient agars and HiCrome™ Coliform agars.

	Asymptotic significance	***P*-value**
HiCrome Coliform agar with SLS (HA)	.464	> 0.05
Nutrient agar (NA)	.179	> 0.05

Table 3. Total number of vending machines with colonies presented in light pink, salmon red, or opaque white.

District	**Total number of vending machines with:**		
	Light-pink colonies	Salmon-red colonies	Opaque-white colonies
Ampang	1	2	10
Cheras	1	0	8
Gombak	4	1	8
Kajang	1	0	8
Klang	2	0	8
Kuala Lumpur	1	0	6
Petaling Jaya	1	0	4
Puchong	3	2	10
Shah Alam	1	0	10
Subang Jaya	1	3	5
Total	**16**	**8**	**77**

Discussion

In 2018, the National Water Service Commission (SPAN) has confirmed the absence of *E. coli* from Malaysia's water supply, which was also claimed safe for direct consumption. Nevertheless, for the supplier or owner of water vending machines located in various business centres in Klang Valley,

they should maintain a routine maintenance to prevent the filter membranes in RO system become overgrown with microorganisms. The quality control of water vending machines by authorities or service providers is crucial in maintaining the health of end users.

Our study suggested that the level of contamination was not significantly associated with different districts of Klang Valley (Table 1). This conclusion was drawn from the *P*-value given using Nutrient agar as a general growth medium. The low *P*-value from HiCrome™ Coliform agars was deemed less reliable since the agars were supplied with sodium lauryl sulfate (SLS), which suppresses the growth of many microorganisms[42]. This finding was in accordance to the dynamic growth and strong population movement within Klang Valley[43,44].

During the study, we noticed that less than half (43%) of the 100 water vending machines received routine maintenance. Nevertheless, the status of maintenance was not found strongly associated with the hygienic level on the surface of water vending machine faucets. Our record shows that, in Shah Alam, 7 out of 10 selected water vending machines had undergone routine maintenance around a week before the day of sampling, whereas only 30–60% of water vending machines had clear indication of routine service from other areas. Nevertheless, statistical analyses have shown that the status of routine maintenance was not strongly associated with the presence of total coliform on the water faucet surface. The same trend was observed for the status of licence issued by respective authorities (Table 2), with only 9 out of 100 subjects were found labelled clearly with licence obtained from authorities.

Despite the standard operating procedure (SOP) set by authorities or service providers, faucets of water vending machines can still be contaminated due to physical contact with hands of user or exposure to fomites on water containers. Routine maintenance with effective sanitisation of all outer and inner surfaces is crucial[45]. We would like to highlight the stressing needs for proper sanitisation on the contact points between users and water vending machines for the persistent hygienic issues[46–48].

Although the presence of faecal coliform in water supply may not be harmful, but it is an indication of the presence of faeces[49]. Despite cases of water vending machines contaminated by faecal coliforms (*E. coli* O157:H7)[50], fortunately, none of the selected subjects in this study was found to harbour faecal coliform (Figure 1, Table 3). However, in accordance to other studies[51,52],the presence of other total coliform (eg. *Klebsiella*, *Enterobacter* or *Citrobacter* species) was reported in our findings.

Conclusion

In conclusion, our findings report the absence of faecal coliform from the faucet surface of all selected water vending machines in Klang Valley. However, the presence of other total coliforms highlights the importance of a proper and effective sanitisation by authorities and service providers — regardless of the area, licence and maintenance status of the machines.

Authors Contribution

The research and manuscript writing were performed by Y-MA. H-WT founded the research project.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Original Research Article

Progress in Microbes and Molecular Biology

HH PUBLISHER

Streptomyces sp. strain MUSC 5 from mangrove forest in Malaysia: Identification, antioxidant potential and chemical profiling of its methanolic extract

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Abstract: The present study explored the antioxidant potential of a *Streptomyces* sp. strain MUSC 5 from the mangrove forest soil in the Pahang State, Peninsular of Malaysia and determined the presence of biologically active chemical constituents contained in the methanolic extract. The 16S rRNA genomic DNA extraction, phylogenetic analysis and phenotyping methods were used to confirm identity of strain. The antioxidant potential of methanolic extract from *Streptomyces* sp. strain MUSC 5 was assessed using a number of antioxidants assays which included free radical scavenging assays, metal chelation and ferric reduction antioxidant power (FRAP) assay. Furthermore, Gas chromatography-Mass Spectrometry (GC-MS) was used to determine the presence of biologically active chemical compounds in methanolic extract of *Streptomyces* sp. strain MUSC 5. The strain was confirmed as belonging to *Streptomyces* genus. Antioxidant studies of the methanolic extract from *Streptomyces* sp. strain MUSC 5 revealed antioxidant activity of 24.97 ± 0.99 %, 22.95 ± 3.21% and 26.81 ± 1.05% against free radicals ABTS, DPPH and metal chelation, respectively. The result of FRAP assay was expressed in dose of 1-2 mg which was equivalent to 1.73- 2.15 microgram (µg) of ascorbic acid. GC-MS analysis carried out on the methanolic extract of *Streptomyces* sp. strain MUSC 5 detected the presence of 11 known compounds belonging to pyrrolopyrazine, esters, fatty acid esters, triterpene and an alkane group of compounds. The study supports the notion that *Streptomyces* from underexplored mangrove forest offer promising *Streptomyces* with antioxidant activity and could serve as important sources for new antioxidant agents.

Keywords: *Streptomyces*; Antioxidative; Mangrove; Radical scavenging

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Introduction

Oxidative stress is a pathological condition caused by presence of high levels of reactive oxygen species (ROS) with an insufficient amount of defensive antioxidants in the body originating from mitochondria, in some instances, can be acquired as air pollutant such as carbon monoxide[1]. To date, there is growing evidence linking oxidative stress caused by mitochondrial dysfunction with the progression of degenerative diseases in the

adult population[2]. The role of antioxidant is to supply the body with optimal antioxidants, thereby reducing unwanted effects of circulating ROS. Previously, a study that investigated natural antioxidants as dietary supplements was shown to be effective in regressing cancer[3]. Over the years, microbes have risen on the stage of prominence as producers of useful drugs against debilitating diseases[4–10].

The bacteria genus of *Streptomyc*es is characterized as a gram-positive, filamentous, soil-dwelling and saprophytic in nature[11, 12]. The soil consists of a diverse microbial community which includes bacteria such as *Streptomyces*[13] and competition for nutrients can be extremely intense[14]. Nevertheless, *Streptomyces* adapted well to the surrounding environment and this may be justified by the fact that they carry a large genome size with high G-C content[15–21] that enable the production of vast array of enzymes, proteins and secondary metabolites to process variety of materials needed for growth and survival[22–26]. The genus of *Streptomyces* under the Actinobacteria phylum are by far the most recognized producers of current drugs and secondary metabolites with diverse biological activities[4,27–31]. There is renewed interest in the *Streptomyces* strains and species lurking in understudied ecological niches and awaiting discoveries which can aid development of new and needed drugs[32–34]. Additionally, there is growing evidence suggesting *Streptomyces* from the understudied mangrove habitat as potential sources of antioxidants in the pharmaceutical industries[35–50].

The mangrove forest is a unique ecosystem which consists of stretch of forests at the confluence of land and the marine ecosystems. Concentrated mostly in 15 countries with an estimated coverage of 75% of their coastline margin[51], mangrove forests are home to a rich microbial community[52] which remain relatively understudied for their biologically active properties[53–56]. Herein, we report a *Streptomyces* sp. strain MUSC 5 with antioxidant potential that was previously isolated from the soil in the mangrove forest of Malaysia. In addition, GC-MS analysis detected 11 known compounds present in the methanolic extract of *Streptomyces* sp. strain MUSC 5. Overall, this study supports the notion of mangrove forest in Malaysia harbouring promising *Streptomyces* that have yet to be investigated for important biologically active compounds.

Materials and Methods

Sampling and maintenance of *Streptomyces* sp. strain MUSC 5

The soil sample for the present study, was collected from mangrove forest in Tanjung Lumpur, Malaysia (MUSC-TLS4 3°48’21.3” N 103°20’3.3”E) in December 2012[43,57,58]. Soil sample collected consisted of portion of the soil layer just beneath 2–3 mm of the surface with a depth up to 30 cm and was achieved by a sterile trowel. Soil samples were aseptically packed into a plastic bag and delivered safely to be stored at - 20 ºC prior to air drying. Air-dried samples were then ground and processed by wet heat sterilization. Pre-treated sample was suspended in previously autoclaved water, diluted and plated uniformly across ISP 2 media supplemented with antifungal drugs which selectively promoted growth of *Streptomyces*. Growth was monitored by continuous sub-culture onto freshly made ISP 2 media until pure isolates was achieved. Pure isolates were then kept on ISP2 agar slant and 20 % glycerol at - 20 ºC as stocks for future work.

Phylogenetic analysis of *Streptomyces* sp. strain MUSC 5

The isolation of genomic DNA (gDNA) was for the purpose of amplifying the 16S rRNA region in the genome[59, 60]. The sequenced 16S rRNA gene of *Streptomyces* sp. strain MUSC 5 was then entered into GenBank/ EMBL/ DDBJ database to retrieve member type strains with the closest match. Multiple alignment for all the member type strains retrieved from GenBank/EMBL/DDBJ database was achieved using CLUSTAL-X software[61]. The stability of the generated phylogenetic tree was verified by following Felsenstein method[66].

Phenotypic characterization of *Streptomyces* sp. strain MUSC 5

Phenotypic characterization of *Streptomyces* sp. strain MUSC 5 consisted of growth characteristics, physiological tolerance levels and production of extracellular enzymes. Growth characteristics of *Streptomyce*s sp. strain MUSC 5 cultured at 28 °C for 7–14 days, was visually inspected on conventional nutrient media — International *Streptomyces* Project (ISP) 2, ISP3, ISP4, ISP5, ISP6, ISP7[67], *Streptomyces* agar (SA)[68], Nutrient agar (NA) [69], Actinomycete isolation agar (AIA)[70] and starch casein agar (SCA)[71]. Soluble pigment and colony colour on each growth media were monitored[72]. Physiological tolerance assessment of *Streptomyces* sp. strain MUSC 5 was evaluated by growing at temperature range of 4–50 °C, salinity levels of 0–10 % w/v and pH 2–10. Biochemical properties of *Streptomyces* sp. strain MUSC 5 evaluated the production of extracellular enzymes. Presence of catalase was investigated by observing bubble formation following the dropping of 3 % (v/v) hydrogen peroxide onto a culture of *Streptomyces* sp. MUSC 5[73]. Test to detect hemolysis activity was carried out on a 5-day old culture grown on blood agar media[74]. Production of extracellular enzymes were determined on ISP 2 media[75].

Fermentation process and extract preparation

A 10-day culture of *Streptomyces* sp. strain MUSC 5 in 10 mL of Tryptone Soya Broth (TSB) media and afterwards inoculated in 200 mL sterile Han’s Fermentation Media 1 (Biomerge, Malaysia) shaken in incubator at 28 °C, 220 rpm for 10 days. Secondary metabolites in supernatant was collected by initial centrifuging followed by filtration and freeze-drying[60]. Extraction of secondary metabolites from freeze-dried supernatant was performed using organic solvent methanol. Crude methanolic extract was collected after evaporation of methanol using of rotary evaporator and stored conveniently at – 20 °C for future use[43].

Antioxidant assays

Scavenging of ABTS radical

Scavenging of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical by the methanolic extract was examined according to Tan *et al.* (2017) [41]. In short, ABTS radical ($ABTS^{\bullet+}$) was obtained from the reaction of 7 mM of ABTS with 2.45 mM of potassium persulfate ($K_2S_2O_8$). The six concentrations (0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 4 mg/mL) of methanolic extract were prepared by 2-fold dilution in 96 - well plate. The ABTS radical was then introduced into the 96 well plate. The plate was then kept in dark for 20 minutes prior to reading of the UV absorbance at 734 nm. Gallic acid served as the standard for this experiment. The following formula was used to calculate the radical scavenging activity of methanolic extract in percentage (%):

$$\% \text{ ABTS scavenging activity} = \frac{\text{Absorbance of control - absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Scavenging of DPPH radical

Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical by the methanolic extract was performed according to Tan *et al.* (2017)[41]. The test was run in a 96-well microplate. A series of concentration of methanolic extract was prepared in the 96-well plate by 2-fold dilution ranging from 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 4 mg/mL. A solution of DPPH ethanol (0.016 % w/v) was then added into 96 well plates containing the methanolic extract and left standing in the dark for 20 minutes at room temperature. The UV absorbance of the mixture was read at a wavelength of 515 nm. Gallic acid was used as the control for this test. The following formula was used to calculate the radical scavenging activity of methanolic extract in percentage (%):

$$\% \text{ DPPH radical scavenging activity} = \frac{\text{Absorbance of control - absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Chelation of metal ions

The metal chelating assay was assessed according to Adjimani and Asare[76] and performed in a 96 well plate. Methanolic extract were prepared in the concentration of 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 4 mg/mL and placed in individual wells. A 2mM ferrous sulfate ($FeSO_4$) was afterwards added into the wells followed by the addition of 5 mM ferrozine. In this reaction mixture, both methanolic extract and ferrozine compete with each other for ferrous ion. Ethylenediaminetetraacetic acid (EDTA) was the standard used in this experiment. The following formula was used to calculate the metal chelating activity of methanolic extract in percentage (%):

$$\% \text{ Metal chelating activity} = \frac{\text{Absorbance of control - absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Ferric reduction antioxidant power (FRAP) assay

Reduction of ferric ion was evaluated according to Adjimani and Asare[76] with few alterations made. Series of concentration of methanolic extract ranging from 5 mg/mL, 10 mg/mL, 20mg/mL, 40 mg/mL and 80 mg/mL, were prepared in 25 µL in a 1.5 mL microcentrifuge tubes. A 25 µL from phosphate buffer (0.2 M) and 25 µL from potassium ferricyanide (1 %) were subsequently added into sterile 1.5 mL. The reaction mixtures were then heated to 50 °C and temperature kept constant for 20 minutes before cooling down to room temperature. A 25 µL of 10 % trichloroacetic acid (TCA) was afterwards added to cease the reaction. An 80 µL was transferred to wells in 96 well plate with a further addition of 20 µL of ferric chloride ($FeCl_3$). The UV absorbance reading was taken at wavelength of 700 nm and results expressed in equivalent dose of ascorbic acid.

Detection of compounds in methanolic extract with GC-MS

Profiling of individual constituents in the methanolic extract was achieved by using Agilent Technologies 6980N with a 5979 Mass Selective Detector [42]. A HP-5 MS (5 % phenyl methyl siloxane) capillary column was used as helium gas carrier at 1 mL every 1 minute. Heat was gradually applied until 40 °C was reached whilst keeping it constant for 10 minutes; then, increased by 3 °C every minute until peak temperature of 250 °C keeping it constant for 5 another minutes. MS was functioning at 70 eV. Individual compounds detected by GC-MS were matched with NIST 05 reference library.

Statistical analysis

Antioxidant tests were repeated three times and the result expressed in means ± standard deviation (SD). Statistical analysis of antioxidant result was computed using Statistical Package for the Social Sciences software (SPSS) and included using one-way analysis of variance (ANOVA) whilst Tukey's *post hoc* determined the statistical significance at p-value < 0.05. In order to correlate the antioxidant activity to phenolic content in methanolic extract, a Pearson's correlation in SPSS software was performed.

Results

Genomic and phylogenetic analysis of *Streptomyces* sp. strain MUSC 5

The 1489 bp 16 S rRNA sequence of *Streptomyces* sp. strain MUSC 5 (GenBank accession number KP998433) from its gDNA enabled retrieving representative of closely related taxa and manually aligned. The phylogenetic tree of *Streptomyces* sp. strain MUSC 5 is depicted in Figure 1. The phylogenetic tree constructed showed strain MUSC 5 forming sister clade with *Streptomyces drozdowiczii* NBRC 101007^T at bootstrap value of 63 %. Closest representation of taxa was established between *Streptomyces drozdowiczii* NBRC101007^T (99.52 %) followed by *Streptomyces laculatispora* BK166^T (99.37%) and *Streptomyces brevispora* BK160^T (99.30 %).

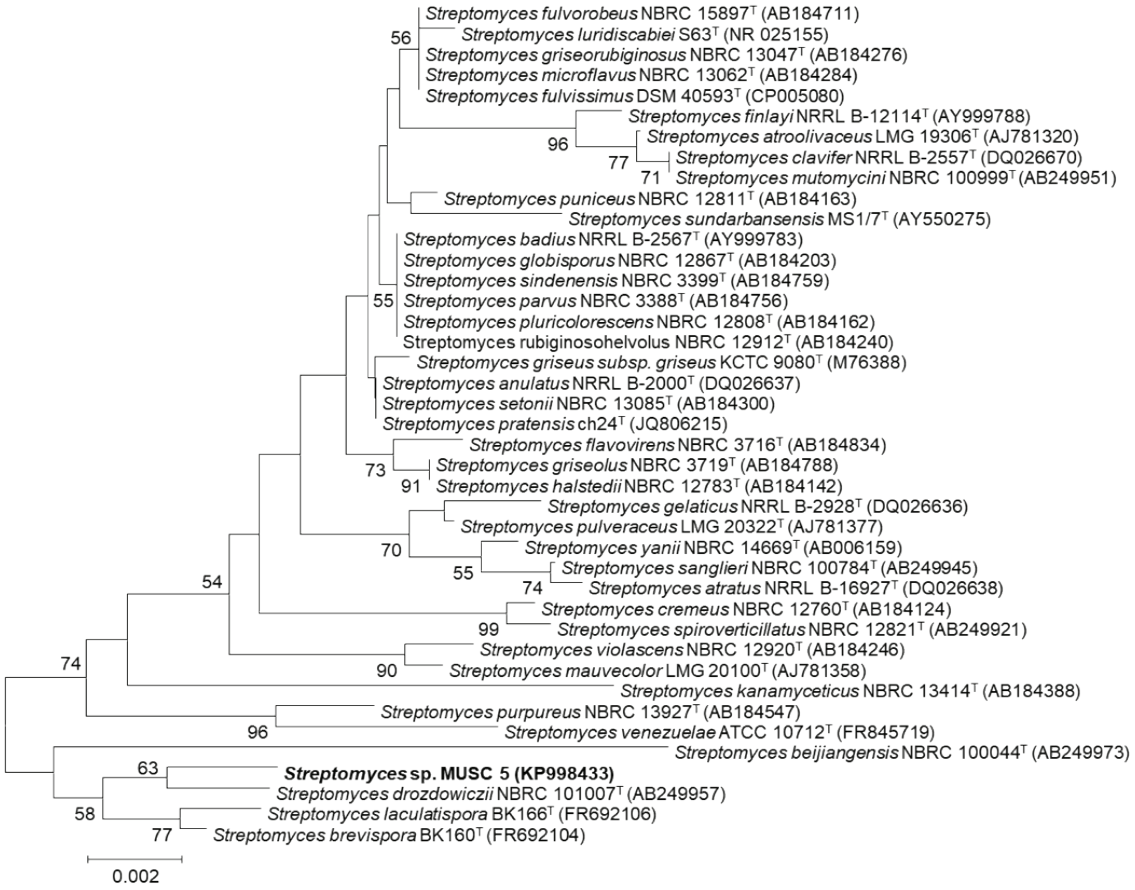

Figure 1. Neighbour-joining phylogenetic tree based on 1489 nucleotides of 16S rRNA gene sequence showing the relationship between strain MUSC 5 and closely related member strains. Numbers and nodes indicate percentages (> 50 %) of 1000 bootstrap re-sampling. Bar, 0.002 substitutions per site.

Cultural characterization, physiological tolerance and biochemical characterization

The growth of *Streptomyces* sp. strain MUSC 5 on various media is shown in Table 1. *Streptomyces* sp. strain MUSC 5 grew abundantly on ISP 2, ISP5, ISP6, ISP7 and SCA and SA after 7–14 days at 28 °C. This is in agreement by Gottlieb and Shirling who recommend ISP media for the growth of *Streptomyces*[67]. Colour of colony of mycelia were noted on all media grown, except ISP4. A light greyish olive and dark yellow pigments were visible on ISP 6 and NA, respectively (Table 1). Optimal conditions for growth were temperature of 26 °C, pH of 6–7 and salinity concentration of 2 % w/v. *Streptomyces* sp. strain MUSC 5 was tested positive for catalase. Results of the biochemical analysis suggested the production of catalase, xylanase and cellulase and amylase by *Streptomyces* sp. strain MUSC 5 (Table 2).

Table 1. Cultural characteristics of *Streptomyces* sp. strain MUSC 5.

Media	Growth	Colony colour		Soluble pigments
		Aerial mycelia	Substrate mycelia	
ISP 2	Well	Brilliant Greenish Yellow	Vivid Yellow	-
ISP 3	Poor	Light Olive Grey	Moderate Olive	-
ISP 4	-	-	-	-
ISP 5	Well	Dark Greyish Yellow	Light Yellowish Brown	-
ISP 6	Well	Pale Yellow	Moderate Yellow	Light greyish olive
ISP 7	Well	Light Olive Grey	Light Greyish Olive	-
AIA	Moderate	Pale Greenish Yellow	Pale Greenish Yellow	-
SCA	Well	Medium Grey	Light Greyish Olive	-
SA	Well	Light Orange Yellow	Brilliant yellow	-
NA	Moderate	Yellowish Grey	Light Greyish Yellow Brown	Dark yellow

- No growth on ISP 4 and no production of soluble pigment

Table 2. Biochemical and physiological characteristics of *Streptomyces* sp. strain MUSC 5.

Catalase	+
Haemolytic	-
Enzymatic test	
Chitinase activity (2.5 % chitin)	-
Xylanase activity (0.5 % xylan)	+
Amylolytic activity (0.2 % starch)	(+)
Protease activity (2 % casein)	-
Lipase activity (1 % tributyrin)	-
Cellulase activity (0.5 % CMC)	+
Temperature (°C)	
Growth	26-37
Optimum	26
NaCl (%) tolerance	
Growth	0-6
Optimum	2
pH tolerance	
Growth	6-8
Optimum	6-7

$^{-}$ No activity; $^{+}$ activity; $^{(+)}$ weak activity

ABTS radical scavenging assay

Scavenging of ABTS radical by the methanolic extract was assessed by reacting ABTS radical cation with methanolic extract and thereafter observing visible colour change from blue-green to colourless. The colour change is suggestive of ABTS scavenging activity. The UV absorbance of free ABTS radical was taken at 743 nm with result showing a concentration dependent scavenging of ABTS radical ($p < 0.05$) with 24.97 ± 0.99 % as the highest activity measured at 4 mg/mL (Table 3).

DPPH radical scavenging assay

Scavenging of DPPH radical by methanolic extract was assessed based on the visible colour change from purple (DPPH radical) to yellow (diphenylpicrylhydrazine) in the reaction mixture. Quantitative analysis of this antioxidant activity was based on the UV absorbance reading taken at 515 nm which is the wavelength that detects free DPPH radical. The result of this experiment demonstrated the DPPH radical scavenging potential of methanolic extract MUSC 5 with an activity ($p < 0.05$) of 22.95 ± 3.21 % at its highest concentration of 4 mg/mL (Table 3).

Metal chelating assay

In this experiment, the ferrozine reagent was used to assess the ferrous ion (Fe^{2+}) chelating ability of methanolic extract. The metal chelating potential of the methanolic extract was thereafter evaluated by taking the absorbance of complex of Fe^{2+}-ferrozine at 562 nm. A low absorbance reading normally suggests that most of the ferrous iron have been prevented to form complex with ferrozine by the metabolites within the methanolic extract. The result of this study indicated that methanolic extract had a metal chelating activity ($p < 0.05$) of 26.81 ± 1.05 % at 4 mg/mL (Table 3).

Table 3. Scavenging of free radicals and chelation of metal ion by methanolic extract.

Concentration (mg/mL)	Antioxidant activities (%)		
	ABTS radical scavenging activity	DPPH radical scavenging activity	Metal chelating activity
0.125	3.04 ± 0.54*	2.11 ± 4.92	3.05 ± 2.43
0.25	4.65 ± 0.95*	2.08 ± 4.73	4.13 ± 1.81*
0.5	4.61 ± 1.14*	4.35 ± 6.12	4.97 ± 0.65*
1	6.00 ± 1.15*	6.15 ± 6.73	8.60 ± 1.78*
2	14.55 ± 0.68*	16.39 ± 5.69*	12.94 ± 2.13*
4	24.97 ± 0.99*	22.95 ± 3.21*	26.81 ± 1.05*
Gallic acid[a]	42.50 ± 0.60*	-	-
Gallic acid[b]	-	53.99 ± 4.06*	-
EDTA[c]	-	-	68.49 ± 7.68*

*Statistically significant at $p < 0.05$; [a]Activity of Gallic acid at 12.5 µg/mL; [b]Activity of gallic acid at 10 µg/mL; [c]Activity of EDTA at 0.125 mg/mL

FRAP assay

Reduction potential of ferric ion to Fe^{2+} form by methanolic extract was assessed through FRAP assay. The amount of Fe^{2+} - Fe^{3+} complex formed was measured with UV wavelength light absorbance of 700 nm. Given there is activity, the colour of the reaction mixture changes to Prussian blue and indicates the methanolic extract has reducing power. The result showed that methanolic extract absorbance was 0.77–0.85, in the dose range of 1–2 mg (Figure 2) which was equivalent to 1.73–2.15 µg of ascorbic acid.

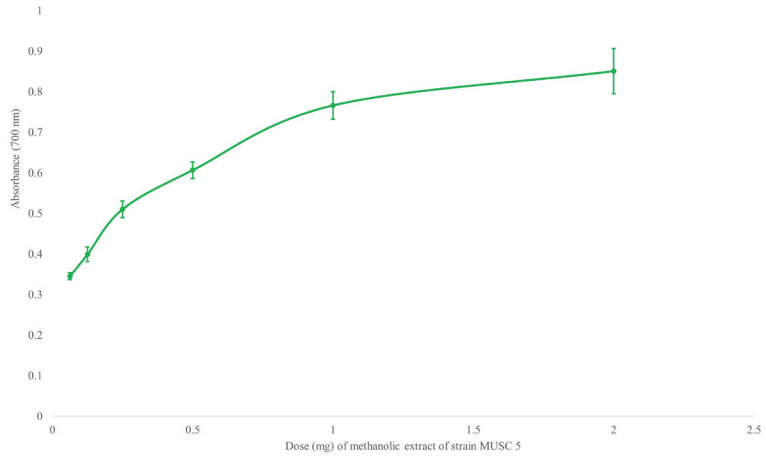

Figure 2. FRAP of the methanolic extract of Streptomyces sp. MUSC 5. The 6 doses (0.0625 mg, 0.125mg. 0.25 mg, 0.5 mg, 1 mg and 2 mg) represents the 6 test concentrations employed (2.5 mg/mL, 5 mg/mL, 10mg/mL, 20 mg/mL, 40 mg/mL and 80 mg/mL). Values are based on experiment run in triplicates ± standard deviation.

GC-MS Chemical profiling of methanolic extract of *Streptomyces* sp. MUSC 5

Chemical profiling of various constituents was achieved by the use of the GC- MS together with the mass spectral data provided by the NIST library. From this, 11 compounds identified belonged to the class of pyrrolopyrazine, esters, fatty acid esters, triterpene and an alkane. Further information regarding individual compounds are provided in Table 4 and Figure 3.

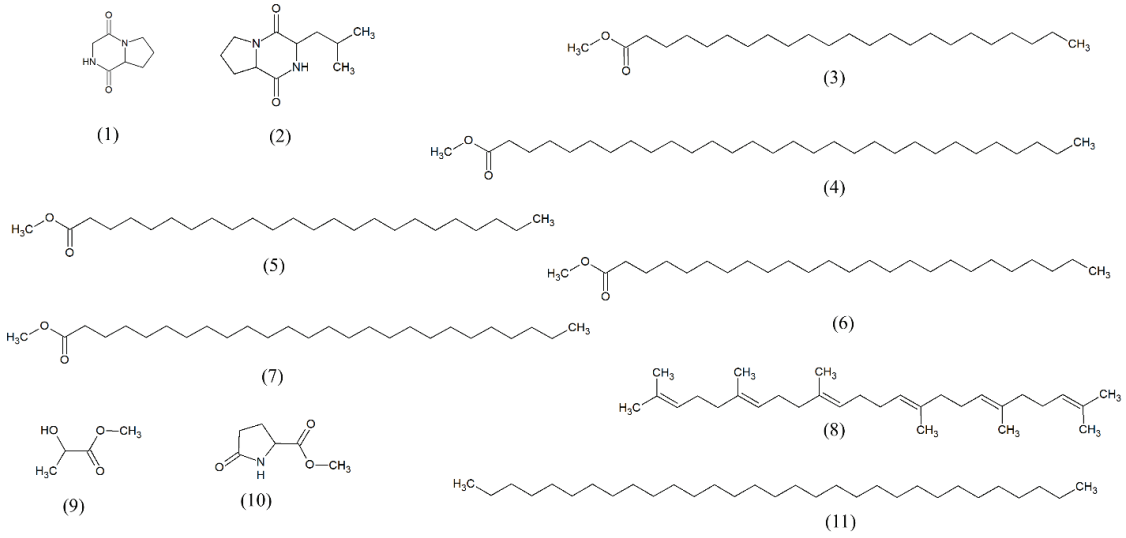

Figure 3. The molecular structures of the chemical compounds detected by GC-MS in the methanolic extract of Streptomyces sp. strain MUSC 5.

Table 4. Compounds detected by GC-MS.

No.	Constituents	Retention time (min)	Molecular Formula	Molecular weight	Similarity (%)
1	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	53.752	$C_7H_{10}N_2O_2$	154	94
2	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	55.048	$C_{11}H_{18}N_2O_2$	210	81
3	Tricosanoic acid, methyl ester	58.797	$C_{24}H_{48}O_2$	368	80
4	Triacontanoic acid, methyl ester	63.184	$C_{31}H_{62}O_2$	466	86
5	Tetracosanoic acid, methyl ester	65.669	$C_{25}H_{50}O_2$	382	93
6	Pentacosanoic acid, methyl ester	77.07	$C_{26}H_{52}O_2$	396	93
7	Hexacosanoic acid, methyl ester	81.538	$C_{27}H_{54}O_2$	410	93
8	Squalene	76.578	$C_{30}H_{50}$	410	95
9	Propanoic acid, 2-hydroxy-, methyl ester	3.852	$C_4H_8O_3$	104	99
10	DL-Proline, 5-oxo-, methyl ester	38.665	$C_6H_9NO_3$	143	96
11	Hentriacontane	79.802	$C_{31}H_{64}$	436	96

Discussion

The 16S rRNA is widely recognised as the golden standard for identification of bacteria at the genus level[77] and was therefore applied to validate that the strain belonged to the *Streptomyces* genus[12]. Having acquired the 16S rRNA gene sequence of *Streptomyces s*p. strain MUSC 5 with a 1489 bp, assisted with the construction of phylogenetic tree (Figure 1). Analysis of the tree placed the strain MUSC 5 within the *Streptomyces* genus. Apart from conferring the status of *Streptomyces* to the strain MUSC 5, close relations were also investigated revealing *Streptomyces drozdowiczii* NRBC 101007^T at bootstrap value of 63 %. Closest relation was established with *Streptomyces drozdowiczii* NBRC101007^T (99.52 %) followed by *Streptomyces laculatispora* BK166^T (99.37 %) and *Streptomyces brevispora* BK160^T (99.3 %). Further information regarding the physical, physiological and biochemical characteristics of *Streptomyces* sp. strain MUSC 5 was also conducted in the current study to provide phenotypic characterization and could be also useful for other research purposes. The strain was able to utilize a wide range of nutrients, produce soluble pigment and coloured colony, as shown by the result presented in Table 1. The strain showed potential in producing extracellular enzymes as well as tolerating different temperatures, salinity and pH (Table 1).

The methanolic extract was evaluated for antioxidant potential utilized a combination of radical scavenging assays and reduction power of antioxidants. The radical scavenging assays was chosen on the basis of its sensitivity and ease of performance and thus the use of ABTS and DPPH[78]. Herein, we report the radical scavenging activity of methanolic extract as having an ABTS and DPPH activity of 24.97 ± 0.99 % and 22.95 ± 3.21 %, respectively, at 4 mg/mL (Table 3).

The metal chelation antioxidant assay was carried out to examine the potential of the methanolic extract to interfere with the formation of coloured complex between ferrozine with ferrous ion[41]. In the biological system, Fe^{2+} is involved as a catalyst in the formation of hydroxyl radical ($OH^{\bullet -}$) through the Fenton reaction[79]. The $OH^{\bullet -}$ are the most destructive of all ROS, and removing it from the system is critical for maintaining homeostasis. The use of antioxidants that are capable to terminate hydroxyl radicals by chelating with ferrous ion would proof worthwhile. The result of the metal chelating test show that methanolic extract had a moderate activity ($p < 0.05$) of 26.81 ± 1.05 % at 4 mg/mL (Table 3).

FRAP is an antioxidant assay that measures a different aspect of ROS by process known as dismutation. In this experiment, both the oxidation and reduction happen concurrently involving an exchange of electrons between a reductant and an oxidant[80]. Here, the methanolic extract and ferrocyanide undergo dismutation. Given that methanolic extract possess antioxidant potential, it will exchange its electron with ferrocyanide and become oxidised. The ferricyanide is transformed to ferrocyanide and later reacted with ferric chloride forming Fe^{2+}-Fe^{3+} complex which has an absorb UV-Vis light at 700nm. In this experiment, the FRAP value was found within the dose range of 1–2 mg (Figure 2). This was equivalent of 1.73–2.15 µg of ascorbic acid.

Given that methanolic extract demonstrated antioxidant activity, these findings have prompted further investigation into identification of the antioxidative compounds. The GC-MS has become an common tool for chemical profiling of bioactive compounds after determining their biological activities[81]. For this reason, GC-MS was used in the present study to detect chemical constituents in the methanolic extract MUSC 5 (Table 4 and Figure 3). The GC-MS analysis led to the detection of 11 known compounds, including Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (**1**), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (**2**), Tricosanoic acid, methyl ester (**3**), Triacontanoic acid, methyl ester (**4**), Tetracosanoic acid, methyl ester (**5**), Pentacosanoic acid, methyl ester (**6**), Hexacosanoic acid, methyl ester (**7**), Squalene (**8**), Propanoic acid, 2-hydroxy-, methyl ester (**9**), DL-Proline, 5-oxo-, methyl ester (**10**) and Hentriacontane (**11**).

Pyrrolopyrazines exist as a class of heterocyclic compounds frequently recovered from microbial extracts. For example, compound (**1**) isolated from a *Bacillus* strain was identified as the active compound with strong DPPH radical scavenging activity as well as exhibiting activity against multi-drug resistant *Staphylococcus aureus*[82].Compound (2) among the 3 constituents present in ethyl acetate extract was identified in a marine *Streptomyces* sp. S2A as the major constituent responsible for the antioxidant activities (DPPH, ABTS, metal chelating and FRAP), antibacterial, enzyme inhibitory and cytotoxic effects[83].

In addition to the two pyrrolopyrazine compounds, several of the fatty acid esters were also detected by GC-MS in the current study. In the current study, GC-MS detected 5 fatty acid methyl esters (**3–7**) in the methanolic extract of *Streptomyces* sp. strain MUSC 5. Compound **(3)** has been previously detected by GC-MS in microbes[84]. Triacontanoic acid, methyl ester (**4**) present in a plant extract showed anticancer property[85]. Another plant extract displaying antidiabetic activity was found to contain tetracosanoic acid, methyl ester, **(5)**[86]. Both the ethanolic and water extract of propolis demonstrated antioxidant and antimicrobial activities and pentacosanoic acid, methyl ester (**6**) was confirmed in both extracts[87]. Hexacosanoic acid, methyl ester (**7**) was reported in a plant extract demonstrating anticancer properties[88].

Interestingly, GC-MS detected squalene (**8**), a biologically active triterpene, which was first reported from a shark liver oil by Tsujimoto (1916)[89]. To date, shark liver oil has been the major source of squalene, although this compound is also naturally produced in minute quantity by *Streptomyces*. For instance, *Streptomyces* sp. QC45B was shown to synthesize squalene via the methylerythritol phosphate pathway (MEP) [90].

Other miscellaneous compounds detected by GC-MS in the current study include propanoic acid, 2-hydroxy-, methyl ester **(9)** commonly known as methyl lactate. It is a volatile oil produced by plants and was detected by GC-MS in studies investigating the chemical composition of coffee

beans[91]. Compound (**10**) was detected in methanolic extract of *Bacillus* sp. strain SB1 and *Halobacillus* sp. strain SB2 having demonstrated antioxidant activity[92]. A long chain alkane hentriacontane (**11**) in a plant extract displayed biological activities including, antimicrobial and antioxidant activity[93].

Conclusion

The methanolic extract of *Streptomyces* sp. strain MUSC 5 has demonstrated antioxidant activity by radical scavenging and FRAP assays. Furthermore, GC-MS analysis detected the presence of 11 compounds in the methanolic extract having a variety of known biological activity. This study adds support to the notion that understudied mangrove forest in Malaysia hold promising *Streptomyces* with antioxidant metabolites that could be resourceful in the development of safer antioxidant agents in addressing oxidative stress which has been associated with several medical conditions.

Author Contributions

K-HM, LT-HT and B-HG executed the experiments, analysed data and participated in the writing of the manuscript. Additional technical supports and proofreading were provided by LT-HT, H-LS, JW-FL. B-HG, K-GC also provided funding of the project. B-HG developed the research project.

Conflict of Interest

The authors hereby declare no competing interest.

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Original Research Article

Progress in Microbes and Molecular Biology

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Genomic analysis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) strains isolated in Malaysia

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Abstract: Using seven complete genomes of human SARS-CoV-2 (retrieved from GISAID) isolated in Malaysia for phylogenetic tree construction, the current study showed that these strains formed four distinct clades when compared with other representative strains from Asia, Europe and US. In light of that, the genome sequences of these strains isolated in Malaysia suggested that there is currently more than one "type""of strain within the country. Complementing with epidemioogical and experimental studies, these findings allow better understanding the prevalence of certain types in Malaysia and permits further in-depth studies on the virulence and pathogenic mechanisms of these strains which is particularly critical to speed up the development of effective treatment regime.

Keywords: SARS-CoV-2; Malaysia; genome; phylogenetic analysis; SNVs; mutations

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Introduction

Taking a closer look at coronavirus, this notorious bug has caused several global outbreaks throughout human history, with notable instances such as 1918 (H1N1) influenza pandemic, and more recently Middle East Respiratory Syndrome (MERS) caused by MERS-CoV in 2012. Fast forward to the last month of 2019, the WHO China Country Office was informed of pneumonia cases with unknown etiology, which later on determined a new type of coronavirus known as SARS-CoV-2[1]. As of 30th April 2020, the World Health Organization reported that there are more than 3 million confirmed COVID-19 cases globally and has impacted more than 210,000 COVID-related deaths[2–6]. At the time of writing, Malaysia's government announced to ease the "partial lockdown" of more than six weeks, allowing almost all economic sectors to reopen, in parallel with the decreasing trend of COVID-19 confirmed cases during the end month of April 2020. In the South East Asia region, Malaysia was among the first few countries to implement the Movement Control Order (MCO) to curb the spread of the coronavirus on 18th March 2020 (Figure 1).

The first case of COVID-19 in Malaysia was detected on 24th January 2020[7]. The first case involved a Chinese national from Wuhan, who had travelled from Singapore to Johor Bahru in a group of eight for holiday on 22nd January 2020. They were quarantined at a hotel on the following days after coming into contact with an infected patient in Singapore. Since then, there were around 22 positive COVID-19 cases as the first wave of outbreak cases in the country, of which all the patients were discharged upon recovery[8]. However, a gradual increase in positive cases begun on 27th February before a sudden surge was observed on 15th March 2020 reaching as high as 428 cases after 11 days of zero reported case (i.e. from 16th to 26th February 2020). These cases were regarded as the second wave of outbreak and attributed to a religious gathering event which was attended by more than ten-thousands of people[8,9]. As a consequence, the shift and firm response from the government into the MCO implementation has been critical to limiting the spread of COVID-19.

To gain further understanding on the molecular epidemiology of the COVID-19 outbreak in Malaysia, we used seven publicly available whole genome sequences sampled up 14th April 2020 to analyze the phylogenetic evolution of the SARS-CoV-2 strains isolated from the patients in Malaysia with worldwide SARS-CoV-2 genome sequences. Comparisons of the single nucleotide variants (SNV) are often used for evolutionary studies as the virus genome is subjected to frequent mutations due to an error-prone RNA-dependent RNA polymerase responsible for the virus genome replication. These mutations might be associated with the changes in transmissibility and virulence of the virus. Thus, we also performed the SNV analysis to investigate the genotype changes during the transmission of SARS-CoV-2 in the country.

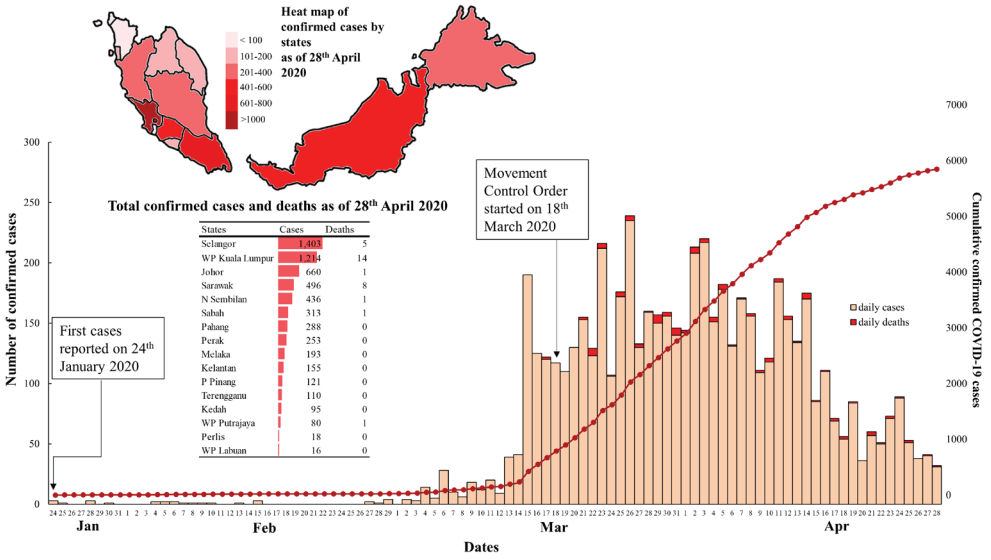

Figure 1. The daily confirmed COVID-19 cases and deaths reported in Malaysia up to 28th April 2020 based on statistics obtained from Ministry of Health Malaysia. The stacked bar graph shows the daily confirmed cases and deaths since the first case reported in Malaysia while the line graph shows the cumulative confirmed cases. A heat map shows the distribution of confirmed cases from different states in Malaysia.

Materials and methods

Phylogenetic and SNV analysis using complete genome of SARS-CoV-2

Complete genome sequences of SARS-CoV-2 were retrieved from GISAID (https://www.gisaid.org) and NCBI database[10,11]. NC_045512 genome sequence fetched from NCBI was used for reference and genomic coordinates in this study are based on this reference genome. The alignment of sequences were formed via ClustalX software, verified manually and adjusted prior to the reconstruction of phylogenetic trees[12]. Phylogenetic trees were constructed with the maximum-likelihood algorithm (Figure 2) using Molecular Evolutionary Genetics Analysis across Computing Platforms (MEGA) version 7.0[13–15]. Support for the tree topology was estimated with 1,000 bootstrap replicates. SNV composition of the selected strains based on the phylogenetic tree was analyzed and compared with the reference sequence NC_045512 using Bioedit and 2019 Novel Coronavirus Resource (2019nCoVR, https:// bigd.big.ac.cn/ncov)[16].

Data availability statement

All data are available in the main text and the supplementary materials. The genome sequences used in the current study is retrieved from GISAID (https://www.gisaid.org/) and NCBI databases (https://www.ncbi.nlm.nih.gov/nuccore/NC_045512).

Results

Phylogenetic analysis using maximum likelihood algorithm

In order to observe the genomic relationship between SARS-CoV-2 strains isolated in Malaysia, a dataset of 7 publicly available complete genomes of SARS-CoV-2 from different countries was retrieved from GISAID (https://www.gisaid.org, accessed on 14th April 2020; Supp Table 1)[10,11]. A phylogenetic tree which was constructed using the maximum-likelihood algorithm revealed that the seven strains isolated in Malaysia formed four major clades (Figure 2). Majority of the strains (n = 3) showed that they were closely related, forming Clade III with the closest related strain isolated from Singapore (GISAID accession ID: EPI_ISL_407987). On the other hand, another strain isolated from a 45-year old male patient in Clade I (GISAID accession ID: EPI_ISL_416886) displayed a close evolutionary relationship with strain from US (GISAID accession ID: EPI_ISL_404895) and China (Fujian, GISAID accession ID: EPI_ISL_411060). The fourth clade (i.e. Clade IV) which consist of strain isolated from a 67-year old female patient (GISAID accession ID: EPI_ISL_416907) was closely related to another strains from Asia region including China (GISAID accession ID: EPI_ISL_408486, EPI_ISL_403930), Japan (GISAID accession ID: EPI_ISL_410532, EPI_ISL_413459, EPI_ISL_412969) and Singapore (GISAID accession ID: EPI_ISL_406973). In addition, the other two strains EPI_ISL_417917 and EPI_ISL_417418 which were isolated later in late March formed a separate clade (Clade II).

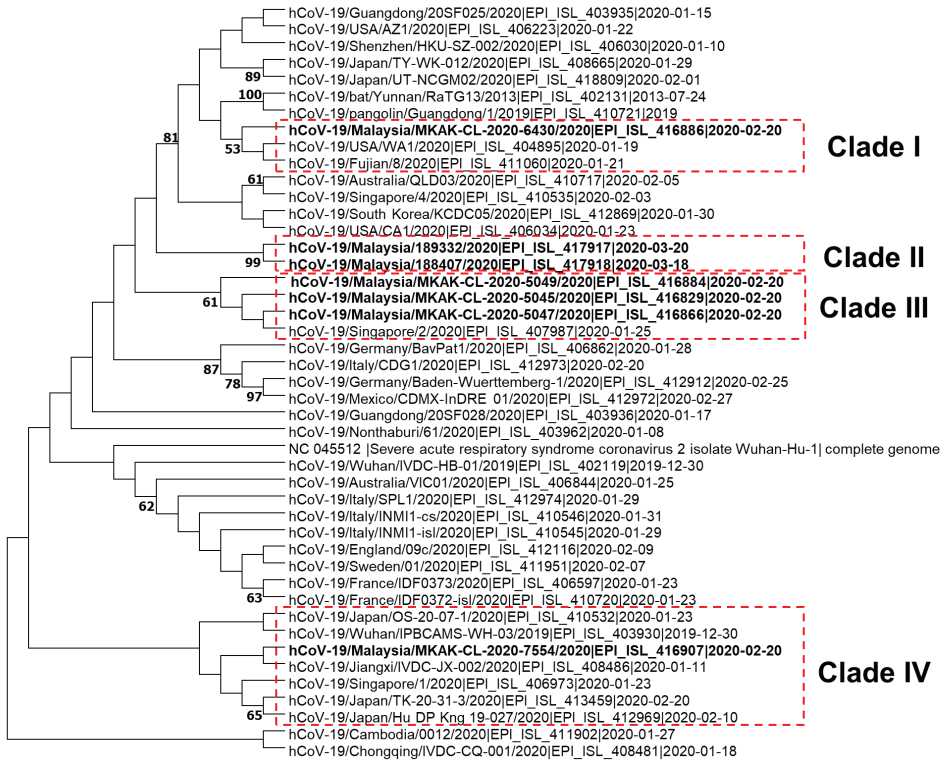

Figure 2. Phylogenetic analysis of forty-five SARS-CoV-2 complete genome sequences (-29880 nucleotides) showing relationship between seven Malaysian strains with representatives' complete sequences from different countries. It is a maximum-likelihood tree with bootstrap values (>50%) based on 1000 re-sampled datasets are shown at branch nodes.

Single nucleotide variants (SNVs) analysis

The genome-wide SNVs for selected strains are as reported in Table 1 using a strain isolated from China in December 2019 (NCBI accession number: NC_045512) as reference strain. A total of 30 SNVs was identified. EPI_ISL_417917 showed the highest number of SNVs, with 9 positions showing variance compared to the reference strain. Furthermore, one of the closely related strain isolated in Malaysia, EPI_ISL_417418 (based on phylogenetic analysis) revealed 8 SNVs; this strain shared 7 common SNVs with EPI_ISL_417917 with addition to a SNVs at 25473 nt (compared to NC_045512) where gene M is located. Additionally, all members of Clade III showed 27147G>C and one missense variation in gene M which encodes for matrix protein. Among the seven strains isolated in Malaysia, EPI_ISL_416866 displayed the lowest number of SNVs only one at 27147 nt (gene M). Similar pattern was observed with amino acid variations as shown in Table 2.

Discussion

The availability and data-sharing of SARS-CoV-2 whole genome sequences allow researchers to study the evolutionary relationships and patterns of molecular divergence between coronaviruses around the globe. In fact, nearly two-thirds of the viral genome falls within the first ORF (ORF1a/b) which translate to (non-structural) two polyproteins (pp1a and pp1ab), while the remaining genome encodes four essential structural proteins, including spike (S) glycoprotein, small envelope (E) protein, matrix (M) protein, and nucleocapsid (N) protein[17,18]. Along with these, there are a total of six accessory proteins coded by ORF3a, ORF6a, ORF7a, ORF7b, ORF8 and ORF10 genes located across the ~29kb genome of SARS-CoV-2. In the current analysis, a total of seven strains isolated in Malaysia was used to perform in the comparison along with representative strains from Asia, US and Europe (note: all sequences of this study were retrieved from NCBI or GISAID database). Most of these strains were closely related with strains isolated from Asia region based on Figure 2. However, some strains displayed more SNVs than the rest of the strains isolated in Malaysia (Figure 3). For instance, EPI_ISL_417917 and EPI_ISL_417918 formed Clade II; analyses on SNVs and amino acid variations reflected that these strains have more than 90% common nucleotide/amino acid in several genes. For these strains, most of the variations occurred within the ORF1ab genes including 6310C>A, 6312C>A, 11083G>T, 13730C>T, 19524C>T. These SNVs then corresponded for four missense variants and one synonymous variant for polyproteins 1a/1ab. In addition, there are two more variations occurring only in EPI_ISL_417917 (but not EPI_ISL_417918) at position 2737T>A (synonymous variant) and 13975G>A (missense variant). In addition to that, there is a SNV observed in gene N at 28311C>T in both strains which encodes for nucleocapsid protein (structural protein) that wraps the genomic RNA (gRNA) into a helical structure. One of the important points to note is that these two strains were obtained from patients in March 2020. As suggested by previous literature, RNA viruses like coronavirus can have high mutation rates and more often than not, these mutations are correlated with virulence modulation and

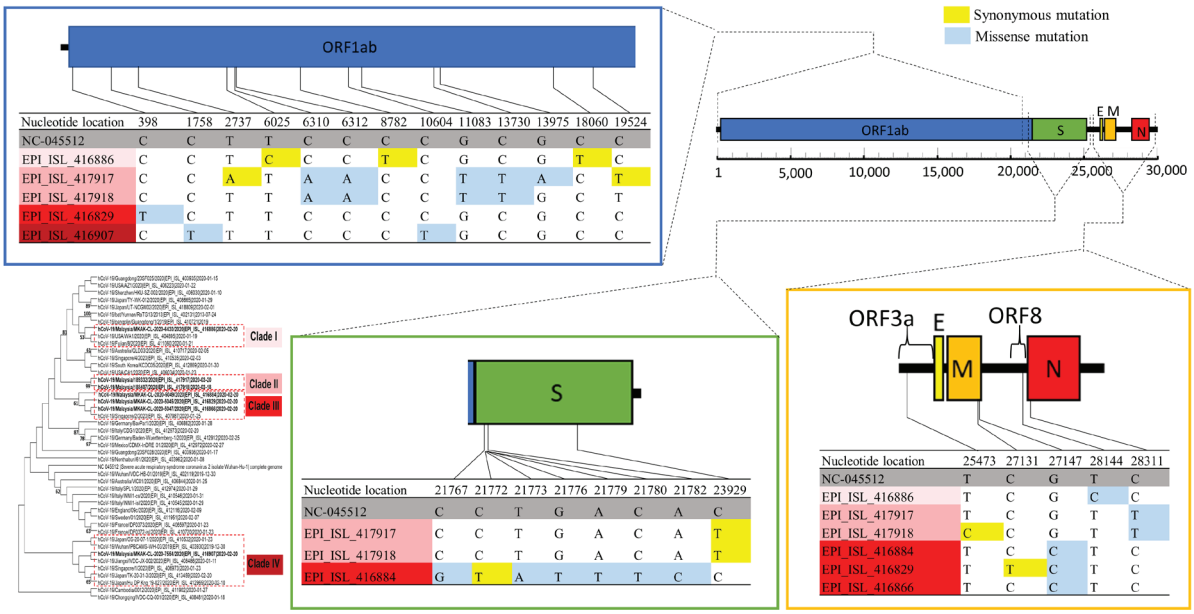

Figure 3. Single nucleotide variants detected from the seven Malaysia strains of SARS-CoV-2 in comparison to the reference strain (NC_045512).

evolvability, thereby improving their adaptation ability[19–21].

Five of the seven strains, EPI_ISL_416886, EPI_ISL_416866, EPI_ISL_416884, EPI_ISL_416829, EPI_ISL_416907 (Clade I, III and IV) were isolated in late February in Malaysia. Interestingly, EPI_ISL_416886 reflected several SNVs within two well-studied genomic regions including ORF1ab (6025T>C, 8782C>T and 18060C>T) and ORF8 (28144T>C). As a matter of fact, EPI_ISL_416886 is the only unique strain isolated from Malaysia that exhibited co-variations at these two locations (i.e. 8782C>T and 28144T>C). As it has been noted that most of 8782C>T and 28144T>C variant sub-strains are found outside of Wuhan, one of the two closely related strains of EPI_ISL_416886 was identified to be strain from Fujian (EPI_ISL_411060) which also carried SNVs at these two locations. A simple pairwise comparison showed that EPI_ISL_416886 exhibited ~99.7 % similarity to closely related strains EPI_ISL_411060 and EPI_ISL_404895 which isolated in US. There have been ongoing discussions on the topic of characterizing SARS-CoV-2 strains based on variation at 8782 (C or T) and 28144 (T or C), either (a) to trace the routes of infections by observing their network nodes, or (b) to study the virulence of strains (i.e. S or L subtypes)[22,23]. Having said that, the actual function of ORF8 in SARS-CoV-2 is yet to be discovered given that it lacks a known useful motif or region and seems to be highly divergent from ORF8b in SARS-CoV which induce intracellular stress pathways[17,24,25]. In addition to this, EPI_ISL_416886 carried a SNV at position 18060 nucleotide belonging to ORF1ab (which encodes for nsp14). Even though Pachetti *et al.*[20] reported that this SNV was mostly related to SARS-CoV-2 strain isolated from North America, further investigation into the travel history of the patient whom EPI_ISL_416886 was isolated from may be worthwhile to obtain a clearer understanding on how the strain acquired these SNVs, whether it's a spontaneous mutation and/or how fast before these SNVs emerged. Still and all, the changes at this position (i.e. 18060) from C>T resulted in a synonymous variant in amino acid sequence, thus the actual effect of this substitution is still pending to be discovered.

S glycoprotein encoded by gene S is one of the crucial factors in determining host range and pathogenicity of SARS-CoV-2. A study in early February by Zhou *et al.*[26] has confirmed that SARS-CoV-2 shares similarity in the target receptor for cellular entry, recognized as angiotensin converting enzyme II (ACE2) receptors. In other words, the S glycoprotein of SARS-CoV-2 can attach to ACE2 receptors available on several types of human cells before hijacking the host machinery[26–30]. From Table 1, a total of 9 SNVs was found to be associated with S glycoprotein (or gene S). Despite of that, three strains (EPI_ISL_416884, EPI_ISL_417917 and EPI_ISL_417918) isolated in Malaysia carried one or more SNVs within this gene. EPI_ISL_416884 (Clade III) displayed highest number of SNVs within gene S with 7 SNVs, and subsequently resulted in 1 synonymous and 5 missense (amino acid) variants in the S glycoprotein, while EPI_ISL_417917 and EPI_ISL_417918 only reflected one SNV (i.e. 23929C>T) without changes in the amino acid composition (i.e. synonymous variant). At the time of writing, these 7 SNVs found in EPI_ISL_416884 (21767C>G, 21772C>T, 21773T>A, 21776G>T, 21779A>T, 21780C>T, 21782A>C) seems to be novel" and the true effect of these changes are still unknown. Taking note that these SNVs are mostly located near the N-terminal domain of the S1 subunit of S protein which is not directly involved with binding to ACE2, hence the variations may not be influencing/altering the binding ability of the virus[31]. On the flip side, it is quite conservative or safe to mention that the actual functional impact of

Nt	Gene	Ref	Clade I			Clade II		Clade III				Clade IV							Proportion of strains with SNVs
		NC_045512	EPI_ ISL_416886	EPI_ ISL_404895	EPI_ ISL_411060	EPI_ ISL_417917	EPI_ ISL_417918	EPI_ ISL_416884	EPI_ ISL_416829	EPI_ ISL_416866	EPI_ ISL_407987	EPI_ ISL_410532	EPI_ ISL_403930	EPI_ ISL_416907	EPI_ ISL_408486	EPI_ ISL_406973	EPI_ ISL_413459	EPI_ ISL_412969	
398	OR-F1ab	C	C	C	C	C	C	C	T	C	C	C	C	C	C	C	C	C	1/16
1758		C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	C	C	1/16
2737		T	T	T	T	A	T	T	T	T	T	T	T	T	T	T	T	T	1/16
6025		T	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	1/16
6310		C	C	C	C	A	A	C	C	C	C	C	C	C	C	C	C	C	2/16
6312		C	C	C	C	A	A	C	C	C	C	C	C	C	C	C	C	C	2/16
6996		T	T	T	T	T	T	T	T	T	T	T	C	T	T	T	T	T	1/16
8274		G	G	G	G	G	G	G	G	G	G	G	G	G	A	G	G	G	1/16
8782		C	T	T	T	C	C	C	C	C	C	C	C	C	C	C	C	C	3/16
10604		C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	C	C	1/16
11083		G	G	G	G	T	T	G	G	G	G	G	G	G	G	G	T	T	4/16
13730		C	C	C	C	T	T	C	C	C	C	C	C	C	C	C	C	C	2/16
13975		G	G	G	G	A	G	G	G	G	G	G	G	G	G	G	G	G	1/16
18060		C	T	T	T	C	C	C	C	C	C	C	C	C	C	C	C	C	3/16
19524		C	C	C	C	T	T	C	C	C	C	C	C	C	C	C	C	C	2/16
21767	S	C	C	C	C	C	C	G	C	C	C	C	C	C	C	C	C	C	1/16
21772		C	C	C	C	C	C	T	C	C	C	C	C	C	C	C	C	C	1/16
21773		T	T	T	T	T	T	A	T	T	T	T	T	T	T	T	T	T	1/16
21776		G	G	G	G	G	G	T	G	G	G	G	G	G	G	G	G	G	1/16
21779		A	A	A	A	A	A	T	A	A	A	A	A	A	A	A	A	A	1/16
21780		C	C	C	C	C	C	T	C	C	C	C	C	C	C	C	C	C	1/16
21782		A	A	A	A	A	A	C	A	A	A	A	A	A	A	A	A	A	1/16
23929		C	C	C	C	T	T	C	C	C	C	C	C	C	C	C	C	C	2/16
25060		A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	1/16
25473	ORF3a	T	T	T	T	T	C	T	T	T	T	T	T	T	T	T	T	T	1/16
27131	M	C	C	C	C	C	C	C	T	C	C	C	C	C	C	C	C	C	1/16
27147		G	G	G	G	G	G	C	C	C	C	G	G	G	G	G	G	G	4/16
28144	ORF8	T	C	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	3/16
28311	N	C	C	C	C	T	T	C	C	C	C	C	C	C	C	C	C	C	2/16
29635	ORF10	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	T	2/16
Proportion of SNVs		-	4/30	3/30	3/30	9/30	8/30	8/30	3/30	1/30	1/30	0/30	1/30	2/30	1/30	1/30	2/30	2/30	

Table 1: Single nucleotide variations (SNVs) deduced by comparison of complete whole genome sequences of SARS-CoV-2 isolated in Malaysia and selected closely-related sequences (n = 16 sequences) using NC_045512 as reference genome (Blue: variant).

Aa	Gene	Ref	Clade I			Clade II		Clade III				Clade IV							Proportion of strains with variants
		NC_044512	EPI_ ISL_416886	EPI_ ISL_404895	EPI_ ISL_411060	EPI_ ISL_417917	EPI_ ISL_417918	EPI_ ISL_416884	EPI_ ISL_416829	EPI_ ISL_416866	EPI_ ISL_407987	EPI_ ISL_410532	EPI_ ISL_403930	EPI_ ISL_416907	EPI_ ISL_408486	EPI_ ISL_406973	EPI_ ISL_413459	EPI_ ISL_412969	
45	OR-F1ab	H	H	H	H	H	H	H	Y	H	H	H	H	H	H	H	H	H	1/16
498		A	A	A	A	A	A	A	A	A	A	A	A	V	A	A	A	A	1/16
824		T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	1/16
1920		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	1/16
2015		S	S	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S	2/16
2016		T	T	T	T	K	K	T	T	T	T	T	T	T	T	T	T	T	2/16
2244		I	I	I	I	I	I	I	I	I	I	I	T	I	I	I	I	I	1/16
2670		C	C	C	C	C	C	C	C	C	C	C	C	C	Y	C	C	C	1/16
2839		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	3/16
3447		P	P	P	P	P	P	P	P	P	P	P	P	S	P	P	P	P	1/16
3606		L	L	L	L	F	F	L	L	L	L	L	L	L	L	L	F	F	4/16
4489		A	A	A	A	V	V	A	A	A	A	A	A	A	A	A	A	A	2/16
4571		G	G	G	G	S	G	G	G	G	G	G	G	G	G	G	G	G	1/16
5932		L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	3/16
6420		L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	2/16
69	S	H	H	H	H	H	H	D	H	H	H	H	H	H	H	H	H	H	1/16
70		V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	1/16
71		S	S	S	S	S	S	T	S	S	S	S	S	S	S	S	S	S	1/16
72		G	G	G	G	G	G	W	G	G	G	G	G	G	G	G	G	G	1/16
73		T	T	T	T	T	T	S/I	T	T	T	T	T	T	T	T	T	T	1/16
74		N	N	N	N	N	N	H	N	N	N	N	N	N	N	N	N	N	1/16
789		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	2/16
1166		L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	1/16
27	ORF3a	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	1/16
203	M	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	1/16
209		D	D	D	D	D	D	H	H	H	H	D	D	D	D	D	D	D	4/16
84	ORF8	L	S	S	S	L	L	L	L	L	L	L	L	L	L	L	L	L	3/16
13	N	P	P	P	P	L	L	P	P	P	P	P	P	P	P	P	P	P	2/16
26	ORF10	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	2/16
Proportion of variants		0/29	4/29	3/29	3/29	9/29	8/29	7/29	3/29	1/29	3/29	0/29	1/29	2/29	1/29	1/29	2/29	2/29	

Table 2: Amino acid variations deduced by comparison of complete whole genome sequences of SARS-CoV-2 isolated in Malaysia and selected closely-related sequences (total n = 16 sequences) using NC_045512 as reference strain (Blue: missense; yellow: synonymous).

these variants remain unclear and further investigations are neccessary to assess its importance, particularly for the development of vaccines[25,32].

Above all, these findings of this study were consistent with reports from other research teams, with most of the strains showing high genomic similarities between strains derived from the Asia region. In the current study, regardless of sampling time (February or March), all the strains isolated in Malaysia reflected highly similar "pattern" of SNVs when compared to the other strains derived from Asia including China (EPI_ISL_411060, EPI_ISL_403930 and EPI_ISL_408486), Singapore (EPI_ISL_407987 and EPI_ISL_406973), and Japan (EPI_ISL_410532, EPI_ISL_413459 and EPI_ISL_412969). When compared within the seven strains isolated in Malaysia, members of Clade II EPI_ISL_417917 and EPI_ISL_417918 were shown to be more closely related to each other (<0.15% difference by pairwise comparison) compared to the other five strains (Supp Table 2). A recently published study by Forster *et al.*[22] reconstructing evolutionary paths of SARS-CoV-2 using 160 complete human SARS-CoV-2 and found three central variants distinguished by amino acid changes which was named A, B, and C, with A being the ancestral type according to the bat outgroup coronavirus. Based on this finding, two members of Clade I (EPI_ISL_404895 and EPI_ISL_411060) in this study were determined to be A type, while most of the members of Clade III and IV were determined as derived-B type which as suggested by its name derived from A type. Forster *et al.*[22] mentioned that SARS-CoV-2 would to first mutate into derived B type (from A type), before finally turning into B type which is the most common type in East Asia. Altogether, these findings may indicate that there are currently more than one subtype present in Malaysia. Nonetheless, there is still much more to be explored, particularly on the transmittance of SARS-CoV-2: how these variations/mutations emerged over time in Malaysia, and then how these changes caused to the behavior of the virus (e.g. on their pathogenicity and virulence). Given the recent emergence of SARS-CoV-2 around the world, researchers have witnessed the growing number of genome sequences deposited in publicly available database like GISAID and NCBI. The policy on data sharing can potentially hasten the identification of COVID-19 infection sources while expediting the drug designing process and vaccine(s) development based on the availability of these complete viral genomes derived from different parts of the world[33]. All and all, by garnering a more thorough perspective on COVID-19 infection sources, the current study could serve as the launching pad to inspect and understand the dynamics of the local transmission of SARS-CoV-2 in Malaysia. Complementing with epidemiological studies, these findings could essentially gather valuable information on the prevalence of certain strains in Malaysia, whereas in-depth experimental research would then shed some light on the virulence and pathogenic mechanisms of these strains.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

The experiment and data analysis were performed by H-LS, LT-HT, L-HL, the manuscript was written and proof by H-LS, LT-HT, JW-FL, VL, N-SAM and L-HL. The project was founded by H-LS and L-HL.

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Original Article

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Risk factors affecting COVID-19 case fatality rate: A quantitative analysis of top 50 affected countries

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Abstract:

Background: Latest clinical data on treatment on coronavirus disease 2019 (COVID-19) indicated that older patients and those with underlying history of smoking, hypertension or diabetes mellitus might have poorer prognosis of recovery from COVID-19. We aimed to examine the relationship of various prevailing population-based risk factors in comparison with mortality rate and case fatality rate (CFR) of COVID-19.

Methods: Demography and epidemiology data were used, which have been identified as verified or postulated risk factors for mortality of adult inpatients with COVID-19. The number of confirmed cases and the number of deaths until April 16, 2020 for all affected countries were extracted from Johns Hopkins University COVID-19 websites. Datasets for indicators that are prevailing or postulated factors of COVID-19 mortality were extracted from the World Bank database. Out of 185 affected countries, the top 50 countries were selected for analysis in this study. The following seven variables were included in the analysis, based on data availability and completeness: 1) proportion of people aged 65 above, 2) proportion of male in the population, 3) smoking prevalence, and 4) number of hospital beds. Linear regression analysis was carried out to determine the relationship between CFR and the aforementioned risk factors.

Results: United States shows approximately 0.20% of confirmed cases and it has about 4.85% of CFR. Luxembourg shows the highest percentage of confirmed cases of 0.55% but a low 2.05% of CFR, showing that a high percentage of confirmed cases does not necessarily lead to high CFR. There is a significant association between CFR, people aged 65 and above (β=4.70; p = 0.035).

Conclusion: Countries with high proportion of older people above 65 years old have a significant risk of having high CFR from COVID-19. Nevertheless, gender differences and smoking prevalence failed to prove a significant relationship with COVID-19 mortality rate and CFR.

Keywords: COVID-19, risk, epidemiology, demography, fatality, age, diabetes

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Introduction

On February 11, 2020, the World Health Organisation (WHO) renamed the highly contagious respiratory disease, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), as coronavirus disease-19 (COVID-19) [1]. The unprecedented increase in COVID-19 cases has led WHO to call it as a pandemic on March 11, 2020 [2,3].

As this pandemic continues to evolve, researchers are learning more about SARS-CoV-2 every day, including the fact that it can be transmitted from symptomatic, pre-symptomatic and asymptomatic people infected with COVID-19 [4]. Studies have shown that COVID-19 is primarily transmitted from symptomatic people to people who are in close contact through respiratory droplets, by

direct contact with infected persons or by contact with contaminated objects and surfaces [5]. Fever, tiredness and dry cough are the most common symptoms in symptomatic COVID-19 patients [6,7].

Current evidence indicated that certain group of people are at a higher risk of suffering from severe illness from COVID-19 [8,9]. Such risk factors include gender, Bacilllus Calmette-Guerin (BCG) vaccination, smoking and malaria prevalence [10,11]. Older people have a higher risk due to the decreasing function of the age-dependent lymphocytes, which resulting in their increased susceptibility to COVID-19 disease [7]. In addition, a study shows that there is a higher percentage of death among patients aged over 65 years (62%) than patients aged below 65 years old (37%) [12]. Furthermore, male gender is commonly observed in COVID-19 patients (73%) according to a retrospective study done on 113 deceased patients [13].

Another risk factor for COVID-19 mortality is existing comorbidities. A study by Guan et al. shows that COVID-19 are more commonly seen in patients with hypertension, diabetes, cardiovascular disease and a history of smoking [14]. Not only were these patients susceptible to the disease, they also had a higher chance of obtaining poor health outcomes after Immediate Care Unit (ICU) admission and may lead to death [12]. Moreover, a study on the correlation between COVID-19 mortality and BCG vaccination suggested that early BCG vaccination could help to decrease the mortality rate [10]. Other than that, malaria prevalence is also another risk factor of COVID-19 mortality. A higher number of COVID-19 cases were reported in countries with low malaria prevalence than countries that had higher malaria prevalence [15,16]. Apart from addressing risk factors, there are also parameters that may affect the COVID-19 mortality rate such as shortage of staff, lack of medical supply or equipment, insufficient hospital beds and the country's health expenditure.

As of late April 2020, SARS-CoV-2 virus has resulted in more than 3.1 million infections and over 217,000 deaths globally [1]. As COVID-19 has become a global pandemic issue, implementation of suitable interventions will be needed for the public, healthcare professionals and patients and also to ensure all sectors to work together cohesively and efficiently [17]. Even though COVID-19 originate from the same family as other known coronaviruses, SARS-CoV-2 has very different severity and contagion characteristics and much still needs to be learned about it. Thus, it is imperative to evaluate the relationship of postulated or verified risk factors with COVID-19 mortality, as presented in a recent analysis based on United Kingdom [18] and Spain [19]. It is absolute crucial to evaluate the risk factors of mortality among patients infected with COVID-19 at population level. By validating the relationship, patients with COVID-19 can be treated more aggressively than those without the risk factor [20]. The findings of the current study provide a picture of COVID-19 case fatality rate (CFR) in top 50 affected countries.

We aimed to determine the association between specific risk factors and COVID-19 CFR. These findings consolidate the evidence of crucial risk factors that front liners need to prioritise to decrease the COVID-19 mortality globally.

This is an ecological study that examines indicators or variables that could be associated with COVID-19 mortality. The unit of observation in an ecological study is the population of the particular area or specific country in which the disease rates were measured. One of the advantages of an ecological study is it provides a snapshot of a transforming event, in additional to the fact that the disease rate and indicator statistics could be mined from existing databases thus saving time [21]. The compared populations and disease of interest are normally defined based on temporal and spatial variation. It is apparent that the reported cases of COVID-19 tend to fluctuate and the sudden spike could be linked to so local transmission cluster. In term of geographical comparisons, epidemiologists are also interested to determine the geographical associations between disease incidence or mortality and the prevalence of risk factors, as exemplified by recent COVID-19 study by Whittle and Diaz-Artiles [22].

Methods

Data extraction

Demography and epidemiology data which have been identified as verified or postulated risk factors for mortality among adult inpatients with COVID-19 were used. The data were collected from World Bank (https://data.worldbank.org/) and Johns Hopkins University COVID-19 (https://coronavirus.jhu.edu/map.html) websites. The number of confirmed cases and the number of deaths for all affected countries were extracted from the latter [23], while datasets for indicators that are prevailing or postulated as the risk factors of COVID-19 mortality were extracted from the World Bank database [24]. Data extracted for this study was up until April 16, 2020. All data acquired were exported in excel format and arranged according to country rankings with the top having the highest number of confirmed cases as of April 16, 2020 and the bottom having the least. To facilitate comparison, out of about 185 affected countries, only top 50 countries were selected to be analyzed in this study.

The following seven variables were included in the analysis, based on data availability and completeness: 1) proportion of people aged 65 above, 2) proportion of male in the population, 3) smoking prevalence, and 4) number of hospital beds.

Data analysis

For each country, the percentage of confirmed COVID-19 case per country was calculated by dividing the number of confirmed COVID-19 cases by the total population for each country. Also, CFR was calculated by dividing the number of deaths related to COVID-19 by the confirmed COVID-19 cases.

Linear regression analysis was conducted to determine the risk factors of CFR for COVID-19. This method was

chosen so that the degree of association can be quantified and that this estimate can be adjusted with other potential risk factors. For this analysis, two variables (CFR and number of hospital beds) were standardized due to differences in scale and very large range. Standardization was done by subtracting each value by the mean and then dividing it with the standard deviation. However, data for four variables (diabetes prevalence, current health expenditure, and number of nurses and midwives) were not normally distributed. Transforming these variables to normality were performed but model over-fitting occurred, therefore further analysis was not pursued. All analyses were conducted using Microsoft Excel and R (ver. 3.6.0). A p-value < 0.05 was considered as statistically significant.

Results

Information of 2,017,444 confirmed COVID-19 cases and 137,166 deaths from each of the 50 top countries (Supplementary Information) were extracted. This constitutes about 93.3% and 92.8% of the global confirmed cases and deaths on the data collection date (April 16, 2020).

Case Fatality Rate (CFR) and mortality rate of COVID-19 in the top 50 countries

From data extracted up until April 16, 2020, the United States (US) reported to have the highest number of total confirmed cases and the highest total number of deaths of 639,733 cases and 31,002 cases respectively. Despite that, the US accounted about 0.20% (Table 1) of confirmed cases and it had about 4.85% of CFR (Table 1), indicating a moderate mortality rate of COVID-19 in comparison to other countries. Luxembourg, ranked 47th in the list of top 50 COVID-19 countries, had the highest percentage of confirmed cases of 0.55% but a low 2.05% of CFR (Table 1), indicating that a high percentage of confirmed cases does not necessarily lead to a high CFR. This is due to variations in number, transmission rate and severity of the disease regardless of the rankings [25]. Hence, it is important to evaluate the possible factors that can affect the increase of COVID-19 mortality rate globally.

Table 1: Percentage of confirmed CFR and COVID-19 cases (In sequence of the highest to lowest CFR%)

No.	Countries	Confirmed COVID-19 cases (%)	Case Fatality Rate (%)
1	Belgium	0.3	13.95
2	United Kingdom	0.15	13.82
3	Italy	0.27	13.11
4	France	0.2	12.77
5	Netherlands	0.17	11.32
6	Sweden	0.12	10.63
7	Spain	0.39	10.46
8	Indonesia	0.002	8.99
9	Mexico	0.005	7.68
10	Philippines	0.01	6.4
11	Iran	0.1	6.24
12	Brazil	0.01	6.07
13	Dominican Republic	0.03	5.23
14	Romania	0.04	5.09
15	Ecuador	0.05	4.94
16	United States	0.2	4.85
17	Switzerland	0.31	4.8
18	Denmark	0.12	4.54
19	Colombia	0.01	4.22
20	China	0.01	4.01
21	Poland	0.02	3.76
22	Canada	0.08	3.56
23	Ireland	0.26	3.54
24	India	0.001	3.4
25	Portugal	0.18	3.33
26	Germany	0.1	2.86
27	Ukraine	0.01	2.79

Table 1: Percentage of confirmed CFR and COVID-19 cases (In sequence of the highest to lowest CFR%) (Continued)

28	Panama	0.09	2.75
29	Austria	0.16	2.73
30	Czech Republic	0.06	2.63
31	Finland	0.06	2.23
32	Norway	0.13	2.21
33	Peru	0.04	2.21
34	Turkey	0.08	2.19
35	Korea, Rep	0.02	2.16
36	Japan	0.01	2.06
37	Luxembourg	0.56	2.05
38	Serbia	0.07	2.03
39	Pakistan	0.003	1.85
40	Thailand	0.004	1.72
41	Malaysia	0.02	1.62
42	Saudi Arabia	0.02	1.3
43	Chile	0.04	1.14
44	Israel	0.14	1.11
45	Australia	0.03	0.97
46	Belarus	0.04	0.95
47	Russian Federation	0.02	0.83
48	United Arab Emirates	0.06	0.62
49	Singapore	0.07	0.27
50	Qatar	0.15	0.17

Relationship between the different risk factors and COVID-19 CFR

The proportion of people aged 65 and above had a significant association with CFR (4.70 [95% CI: 0.34, 9.06], $p = 0.04$, Table 2). For every 1-unit increase in the proportion of people aged 65 years, the CFR increased by 4.7 units. This relationship is illustrated in Figure 1, where CFR sharply increases when the proportion of people aged 65 years is 0.18 and above.

Table 2: Linear regression analysis for different risk factors (independent variable) and COVID-19 CFR (dependent variable)

Independent variable	β estimate (95% Cl)	**Standard Errors**	***p*-value**
Age (above 65 years)	4.70 (0.34, 9.06)	2.17	**0.035**
Gender - Male	-0.33 (-0.92, 0.26)	0.29	0.26
Smoking Prevalence (total % of people aged 15 and above)	0.009(-0.03, 0.04)	0.02	0.60
Hospital Beds (per 1,000 people)	-0.097 (-0.39, 0.19)	0.14	0.50

Discussion

There are still a lot of unknown regarding COVID-19 disease. However, good clinical findings are made available now to understand the risk factors that could affect its treatment outcomes.

Studies have shown that age is a clear risk factor for severe COVID-19 disease. This has been confirmed by our study where the proportion of people aged 65 and above has shown a significant association with CFR. This indicates that countries with a higher proportion of people aged 65 and above may result in higher COVID-19 mortality

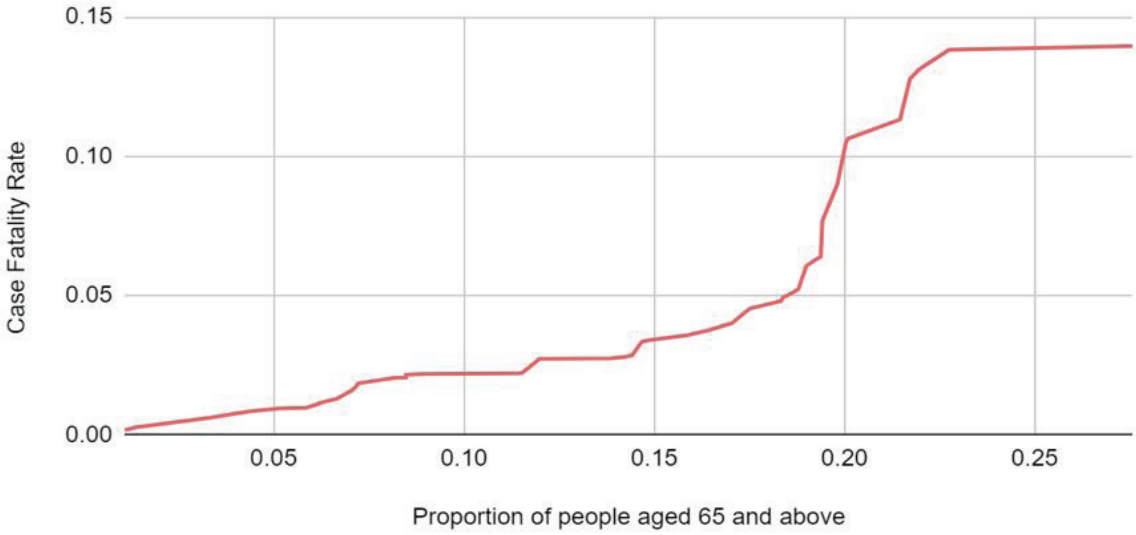

Figure 1: Association between case fatality rate and proportion of people aged 65 and above.

rate (Figure 1). Bhatraju et al. (2020) has shown that in Seattle, the US reported more than 60% of COVID-19 deaths in patients aged 65 years and above than those who are younger than 65 years old [12]. Verity et al. (2020) has shown that the a stark difference in the CFR between those aged below and above 60 years (1.4% versus 4.5%, respectively) [9]. This suggests that the older the country population, the higher the CFR. For those 80 years old and above, COVID-19 appears to have a 13.4% fatality rate [9]. Notably, the average age of deceased and recovered patients were found to be 68 and 51 years, respectively [13]. These studies show that COVID-19 disproportionately impacts certain groups, and that older people [26] and pregnant women [27] are among the vulnerable groups. This could be due to the weakening effects of ageing on the immune system. As age increases, there is an increase of deficiency in T-cell and B-cell function and overproduction of type 2 cytokines [7]. This may promote viral replication and extend the duration of pro-inflammatory responses, leading to poor prognosis [7]. Furthermore, older people tend to have more underlying conditions that may also be risk factors for severe COVID-19 [9,14].

Studies have shown that many of the severe COVID-19 patients also have underlying medical conditions, such as diabetes and cardiovascular diseases [13,28]. Patients with existing comorbidities, including hypertension, diabetes, cardiovascular disease and history of smoking, seems to be associated with COVID-19 more severely [14]. With reference to a retrospective study of 113 deceased patients from COVID-19, 48% of the patients had chronic hypertension and 14% of them had cardiovascular diseases [13]. In addition to that, COVID-19 patients who have hypertension were closely associated with poor health outcomes after hospital admission. This may be due to factors such as vascular aging, reduced renal function and medication interactions [29]. Although smoking prevalence has shown no significant association with COVID-19 (p=0.60), it cannot be assumed that there is no association between other co-morbidities and COVID-19 CFR since not all factors were considered in this study, such as hypertension and cardiovascular diseases [6].

COVID-19 is a rapid spreading communicable disease and general public are responsible in controlling this COVID-19 pandemic. Various levels of well-preparedness plans are needed to tackle this pandemic situation [30]. These include availability of medicines or medical supplies to treat COVID-19 patients, availability of suitable places to quarantine or self-isolate these patients, number of healthcare professionals and a strategized interventions, such as social distancing, quarantine, isolation actions and proper management, for the patients and public to flatten the curve and to reduce healthcare burden. It is necessary for some countries which may need more supplies than others to cater all sick patients, and thus increasing the health expenditure of the country [2]. Some of the medical supplies include personal protective equipment, mechanical ventilators, COVID-19 testing kits and extracorporeal membrane oxygenation. Due to surge of demand on healthcare system, countries with low income and poor healthcare infrastructure suffer the most [14]. However, in our study, current health expenditure of the top 50 countries was not tested due to aforementioned reason.

Sufficient hospital capacity in term of hospital bed is necessary to accommodate unforeseen pandemic situations [31]. Despite the insignificant association to COVID-19 CFR (p=0.50), accessibility to adequate hospital beds for COVID-19 patients can potentially affect the CFR as the number of beds required depends on the number of confirmed cases in each country. Apart from hospital beds, other medical supplies such as medical grade face mask and mechanical ventilators must be sufficient as they are the key equipment for frontline healthcare workers [32–34].

Evidence of high numbers of infections and deaths among healthcare workers due to lack of face mask and medical gowns were reported in Italy [32]. In the US, recent estimates have suggested that the estimated ventilators needed is ranging from several hundred thousand to a million, far more than what are currently available [32]. It is difficult to estimate the exact number of ventilators needed as it depends on the number, transmission rate and severity of the disease in each country.

There are a number of limitations in this study. The nature of the study design (ecological study) and type of analysis used (linear regression analysis) allowed us to use country-level aggregated data to determine the relationship between CFR and specific risk factors. Since aggregated data were used, this means that the results are only applicable at a country-level, instead of individual-level. Some factors had to be excluded due to incomplete data such as malaria prevalence and BCG vaccination. Even if the data were available, the data may come from different years. The years from which the data were retrieved were not consistent for all indicators. The data collected were also limited by unavailability of certain data to sufficiently make an overall conclusion for several factors, including comorbidities. There were four other proposed comorbidities to be analyzed but only two indicators' datasets were available in World Bank Data, which are diabetes and smoking prevalence. Of note, our study did not able to test the association between diabetes prevalence and CFR. Therefore, more research should be conducted to further understand the relationship between comorbidities and CFR. This would help to identify and to better understand other possible factors that may also affect CFR. However, even with these limitations, it is important to note that the aim of our report is to determine the association between specific risk factors and COVID-19 case fatality rate globally. Also, our analysis is limited by data derived from confirmed COVID-19 cases as of April 16, 2020, yet the number of global cases continues to increase. It is also important to note that transmissibility rate and various periods of the pandemic were not taken into account, as it may differ at different countries or even area within the same country [6,35,36].

Conclusion

As COVID-19 is such a new disease, much still needs to be learned about it. Age is a clear risk factor for severe COVID-19 and death. COVID-19 is an illness that disproportionately impacts older people. However, other risk factor such as smoking should not be neglected. Prediction alone is not efficient, but well-planned and suitable interventions should also be carried out. In addition to that, potential risk factors need a lot more research in order to understand the risks for the worst forms of COVID-19 and what we ought to learn to best protect the people with higher risk. Participation and involvement of every individual, including patients, public and healthcare professionals, is necessary and everyone should work together towards combating COVID-19 disease.

Conflict of interest

None declared.

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None.

Author contributions

LCM conceived the project. HPG, WIM, NIA, LLC, LCM analyzed results and interpreted the data and wrote the manuscript draft. NK, BHG, KWG, SFY revised the manuscript. All authors read and approved the final manuscript.

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Review Article

Progress in Microbes and Molecular Biology

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Critical review of fermentation and extraction of anti-*Vibrio* compounds from *Streptomyces*

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Abstract: A single *Streptomyces* strain often have the potential to produce more than one bioactive compound. Fermentation parameters include media compositions, temperature and pH, have great impact on the secondary metabolism of *Streptomyces* and subsequently on production of different microbial products. This review aims to consolidate the studies on the cultivation parameters used to enhance the production of secondary metabolite with anti-*Vibrio* activity from a single *Streptomyces* strain. In turn, this review sheds light on the possible alterations of the cultivation parameters to obtain desired anti-*Vibrio* compounds from *Streptomyces* sp. Furthermore, the bioactive compounds with anti-*Vibrio* activity identified from *Streptomyces* sp. were demonstrated to exhibit immense values for future antibacterial agent developments.

Keywords: *Streptomyces*; *Vibrio*; fermentation; extraction; secondary metabolites

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INTRODUCTION

Fermentation is an important process for the production of various structurally-diverse bioactive substances from microorganisms, including antibiotics, anticancer, antiviral and immunosuppressants[1,2]. Given that the limited quantity of bioactive substances is usually produced by these microorganisms, fermentation is one of the feasible processes to continuously supply majority of these clinically useful drugs in the market currently. This is because the total chemical synthesis is way too complicated and costly than fermentation. For instance, antimicrobial peptides such as a novel class of antibiotics, which recently have received much attention, is not economically feasible to be synthesized chemically if involve larger or more complex peptides[3]. Furthermore, medium optimization remains one of most critical steps in fermentation technology to enhance the production of valuable bioactive compounds. To achieve maximum production of desirable compounds, the production medium containing appropriate components (e.g., carbon, nitrogen, NaCl, *etc*.) coupled with optimal fermentation conditions are required to be identified and optimized accordingly[1].

Actinobacteria have been regarded as the most prolific producers in the microbial world[4–8], especially from the genus *Streptomyces*[9,10]. The genus *Streptomyces* has responsible for the production of more than 70% of commercially important antibiotics[2,11], as well as many bioactive compounds of pharmacological and agricultural interest[12–22]. The discovery of antibiotic from *Actinobacteria* is highly dependent on the effect of growth conditions on the production of secondary metabolites[23–28]. These soil bacteria are known to have complex life cycle which is composing of different stages. Secondary metabolites are usually produced by *Streptomyces* sp. at the end of the active vegetative growth and during the dormant or reproduction stage[29]. The secondary metabolism of *Streptomyces* is based on its unique genetic make-up but the expression can be

influenced by the surrounding manipulations[30]. Therefore, the productions of secondary metabolites are often associated with the limitation of nutrients, presence of inducer or reduction of growth rate in *Streptomyces*[23]. It is well known that secondary metabolite production can be repressed by readily available carbon source, high levels of nitrogen and phosphorus, all of which keeping the bacteria at active proliferative stage. This indicated that the production of secondary metabolites can be influenced significantly by various fermentation parameters including the nutrient availability, pH, temperature, mineral salts, inducers and inhibitors[31]. Small modifications in the composition of growth media can result variation of the quantity of specific compounds, also these modifications could result in the production of a completely distinct pattern of molecules[32].

Vibrio spp. is autochthonous to various aquatic environments, including estuarine, coastal waters and sediments[33–36]. *Vibrio* spp. was known to be susceptible virtually to most of the antimicrobial agents[37,38]. However, antimicrobial resistance has emerged and evolved in many bacterial genera[39–41], including *Vibrio* spp. as a result of excessive use of antimicrobial agents in various settings[42]. For instance, applications of antibiotics in aquaculture water as prophylactics to control infectious diseases in fish and aquatic organisms. Furthermore, certain *Vibrio* species, in particular *V. parahaemolyticus* and *V. vulnificus* are significant foodborne human pathogens[43–47]. Hence, the increase in emergence of antibiotic-resistant bacterial pathogens, including *Vibrio* spp. is a major public health concern[39,40,42]. This issue not only has immense impact on human health, it is also a concern on the future ability to treat the diseases as antibiotic resistance has developed over time, from single classes of antibiotics to multidrug resistance and eventually emergence of superbug with extreme drug resistance[48,49]. Therefore, it has increased the interest of research on the search for more effective alternatives to cope with the issue of antibiotic resistant bacteria, including *Vibrio* pathogens[50,51]. The exploration of bioactive compounds sourcing from natural resources, including plant[52–56], animal[57] or microbial origins[58–64] constitute an attractive bioprospection strategy among the drug discovery scientists. In fact, numerous efforts have demonstrated that the genus *Strepto-myces* capable to synthesize various bioactive compounds against *Vibrio* pathogens, representing a valuable source for antibacterial agents with anti-*Vibrio* activities[65–67].

In the light of the promising potential of *Streptomyces* as the bioresource of anti-*Vibrio* compounds, this review provides the rationale for the designing and optimizing of fermentation medium to facilitate the process of anti-*Vibrio* metabolite production in *Streptomyces* sp. Furthermore, the importance of extraction techniques for optimum yield of the desired bioactive compounds is discussed in this review (Figure 1).

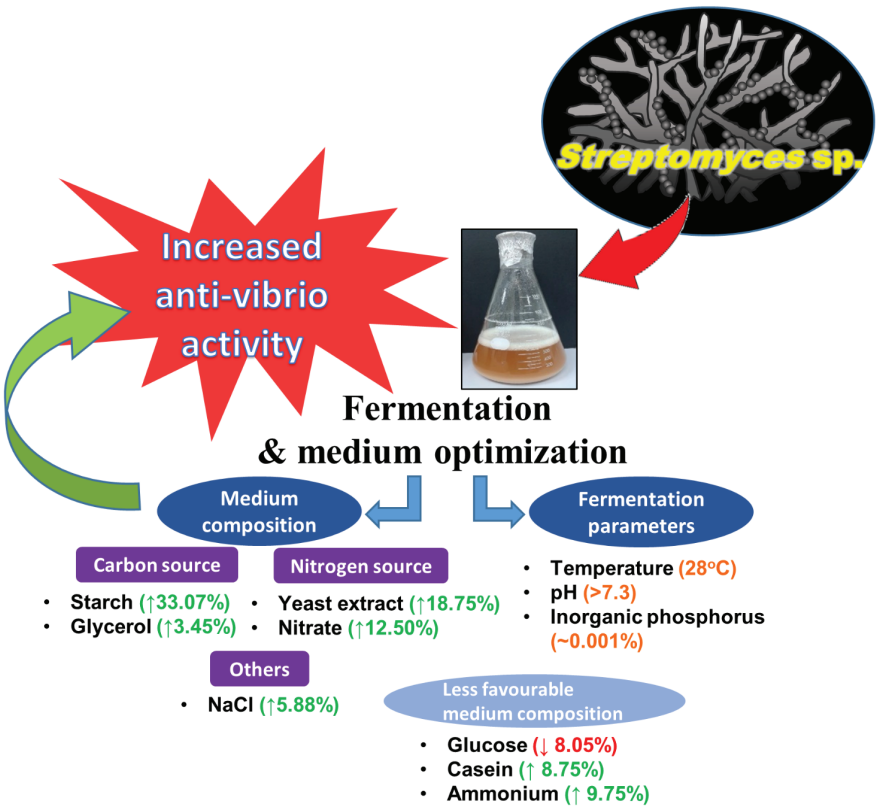

Figure 1. Fermentation and medium optimization for the production of anti-*Vibrio* compounds by *Streptomyces* sp. Fermentation is conducted to induce the production of anti-*Vibrio* active metabolites by *Streptomyces*. Starch and glycerol are both good carbon sources for the production of metabolites with better anti-*Vibrio* activity (percentage shows the changes in the anti-*Vibrio* activity when added with the indicated component in the fermentation medium). Likewise, yeast extract and nitrate are the preferable nitrogen source as compared to casein and ammonium for the production of metabolites with better anti-*Vibrio* activity by the *Streptomyces*. The fermentation parameters presented are the optimum conditions for the production of anti-*Vibrio* active metabolites.

FERMENTATION PROCESS FOR PRODUCTION OF ANTI-*VIBRIO* COMPOUNDS BY *STREPTOMYCES* SP.

Within the 64 studies analyzed in this review, a total of 38 studies conducted secondary screening of the metabolites produced by the anti-*Vibrio Streptomyces* via submerged fermentation process. This implies that 59.4% of the studies showed the anti-*Vibrio Streptomyces* strains displayed the antagonistic activities against different *Vibrio* sp. through the production of bioactive secondary metabolites. Thus, more study should perform fermentation in order to fully unravel the potential of the anti-*Vibrio Streptomyces* strains in the production of bioactive compounds against *Vibrio* sp. Solid state fermentation was reported as an alternative fermentation process to facilitate the secondary metabolites production from the anti-*Vibrio Streptomyces*[65]. The solid state fermentation involves the use of solid particles free of water or with little moisture for microbial growth and secondary metabolites production[68]. Mohana and Radhakrishnan (2014)[65] indicated that solid state fermentation process was more suitable for *Streptomyces* MA7, a strain derived from mangrove rhizosphere sediment in producing anti-*Vibrio* bioactive metabolites against *Vibrio* pathogens such as *V. cholerae* O1, *V. cholerae* O139, *V. parahaemolyticus* and *V. mimicus*. However, there is limited information on studies comparing the two different fermentation techniques in the production of secondary metabolites with anti-*Vibrio* activities. More study could be performed to investigate the optimal fermentation techniques for the production of anti-*Vibrio* compounds from *Streptomyces* at a higher yield. Nevertheless, there was study suggested that solid-state fermentation is better for antibiotic production by *Streptomyces* in the aspects of its stability and quantity[69]. For instance, solid-state fermentation of *Streptomyces* species resulted in higher yield and stability of well-known antibiotics including tetracycline[70], neomycin[71], cephamycin C[72] and oxytetracycline[73].

FERMENTATION PARAMETERS AFFECTING ANTI-*VIBRIO* COMPOUNDS PRODUCTION

Media composition

Media composition plays an important role in determining the microbial secondary metabolites as it comprises of components that may act as activators of certain signaling pathway in the production of secondary metabolites[31]. Thus, a single strain, grown under different condition may result in production of substantially different compounds. A study reported that by using a defined medium resulted in production of new metabolites which were not found in other media used to cultivate *Streptomyces* sp. C34, and exhibited antibacterial activity towards *V. parahaemolyticus*[74]. The defined medium (Table 1) containing 2 mM fluoride employed by the study was previously developed for the production of fluorinated secondary metabolites by *Streptomyces*[75]. The mechanism for the production of the novel metabolites by *Streptomyces* C34 has yet to be elucidated. Nevertheless, it was suggested that the addition of fluoride salts could have activated the unique biosynthetic genes which responsible for the production of those new compounds[75]. Therefore, other than depending on the biosynthetic potential of the microbes which determines the types of bioactive compounds, the composition of the media also plays a substantial role on the success of screening programs based on culture-dependent bioprospecting strategy. According to the 38 studies that performed submerged fermentation, different types of fermentation broths were used, including starch casein broth, soybean meal broth, potato dextrose broth, arginine glycerol broth, actinomycetes isolation broth and glycerol asparagine. Besides that, examples of fermentation broth with defined compositions used for the production of secondary metabolites from the anti-*Vibrio Streptomyces* can refer to Table 1.

Table 1. The composition of selected production media and fermentation conditions used for secondary metabolites production in the *Streptomyces* sp. displaying anti-*Vibrio* activity.

Parameters	Studies that utilized mixture of complex and simple carbon and nitrogen sources[#]													
Composition (% w/v)[*]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Glucose					1			0.4	2			0.5		0.2
Soluble starch	2	0.5	0.5	1	1	1			0.5	1	0.1	2		
Glycerol				1	1		1						1	
Myo-inositol							0.04							
Malt extract								0.4						
Soybean	2		0.5						0.5		0.1	1.5		
Casein		0.03		0.03						0.03				
Cornsteep powder					1									
Polypeptone					0.5									
Peptone						0.2			0.2					
Yeast extract					0.2	0.4		0.4	0.2			0.25		0.3
L-tyrosine													0.05	
L-asparagine													0.1	
MSG							0.5							
$CaCO_3$		0.002		0.002	0.32		0.025			0.002		0.1		0.004

NaCl		0.2	0.05	0.2	0.1		0.05			0.2			0.05	0.08
NaF							0.0084							
NH_4Cl							0.15							0.1
KBr						0.01								
KCl														0.01
K_2HPO_4			0.05	0.2			0.2		0.05	0.2			0.05	0.001
KNO_3	0.1	0.2		0.2						0.2	0.005			
$FeSO_4$		0.001		0.001		0.004	0.0025			0.001				
$MgSO_4$		0.005	0.05	0.005			0.05		0.05	0.005			0.05	0.02
$CoCl_2$							0.001							
$ZnSO_4$							0.001							
Seawater	+	+	-	-	-	-	-	+	+	+	+	+	+	-
pH	7.5	ns	ns	7.2	7.4	8	7	7.2	7.4	7.0±0.2	ns	7.5	7	7
Temperature (°C)	27	28	28	29	30	ns	28	28	28	28	27	28	28	28

* The percentage of each composition was calculated using: w/v% = (weight of solute (g)/volume of media(mL)) × 100

1 - Soybean medium[65], 2 - Starch casein broth[76], 3 - GsB broth medium[77], 4 - Casein glycerol/ starch medium[78], 5 - Production broth[79], 6 - A1BFe media[80], 7 - Defined medium[74], 8 - Fermentation broth[81], 9 - R2A medium[82], 10 - Starch casein broth[83], 11 - Soybean meal broth[84] 12 - Fermentation broth[85] 13 - Melanin production medium[86, 87],14 - Fermentation broth[88–92]

The influence of complex and simple carbon source on anti-Vibrio activity

The carbon source has significant effect on the production of antibiotic and the morphological development of *Streptomyces* sp. Several mechanisms have been described in the genus *Streptomyces* to illustrate the carbon catabolite repression effects on secondary metabolites production[93,94]. As for aim of this review, it is to consolidate and rationalize the information available on the effect of different media composition on *Streptomyces* toward the production of metabolites against *Vibrio* sp. Furthermore, major emphasis will be given towards the efficacy of the anti-*Vibrio* metabolites produced by *Streptomyces* in response to the presence of specific carbon source in the fermentation media. Based on the data of media composition presented in the reviewed studies, carbon sources such as starch, glycerol and glucose are commonly used as growth substrate in the fermentation media used to produce secondary metabolites. Majority of the studies incorporated starch (45.2%), a complex carbohydrate in the fermentation medium for the production of secondary metabolites with anti-*Vibrio* activity (Table 2).

Literatures demonstrated that the optimal production of secondary metabolites is generally achieved by culturing the microorganisms in media containing slowly assimilated nutrient sources while the readily utilized carbon source is often known to repress antibiotic production. For instance, the use of glucose as a carbon source had a negative influence on the production of nystatin as well as their morphology to a certain extent that resulted in termination of cell growth and nystatin production[95]. This is also commonly seen in other *Streptomyces* sp., such as in the production of streptomycin, chloramphenicol and cephamycin by *S. griseus*[96], *S. venezuelae*[97] and *S. clavuligerus*[97] respectively. However, previous study indicated novobiocin production by *S. niveus* is subjected to catabolite repression by citrate assimilation and not caused by glucose assimilation[98]. *Streptomyces avermitilis* was shown to assimilate glucose slowly and become the best carbon source in determining the production rate of avermec-tin[99]. Ikeda *et al.* (1988)[99] suggested that the activity of 6-phosphogluconate dehydrogenase of the pentose phosphate pathway is associated with avermectin production, in which the NADPH generated by the enzyme could be used as the intermediate for the biosynthesis of avermectin. Previous study also indicated that glucose is important for the biosynthesis of ε-rhodomycinone, an important aglycone precursor to anthracycline antibiotic in *Streptomyces*[100].

To identify the best carbon source for the production of anti-*Vibrio* metabolites by *Streptomyces*, the anti-*Vibrio* activities of the *Streptomyces* strains with or without the specific carbon source were compared based on the inhibition zones (Table 3). In Table 3, the anti-*Vibrio* activity of metabolites produced by *Streptomyces* strains increased by 33.1% in the presence of starch as carbon source. Furthermore, a ten folds increment of anti-*Vibrio* activity is demonstrated by *Streptomyces* metabolites produced in the presence starch when compared to the use of glycerol as carbon source. In contrast, the use of glucose as carbon source is shown to repress the anti-*Vibrio* activity of the *Streptomyces* metabolites by 8.1% based on the median inhibition zones. This information is in line with other studies, indicating starch is a good carbon source for anti-*Vibrio* metabolite production. The starch-based A1BFe medium (Table 1) resulted in production of twice the amount of anti-*Vibrio* compounds by *Streptomyces atrovirens* PK288-21 compare to culture in glucose-based TCG medium[80]. The study suggested that *Streptomyces atrovirens* PK288-21 utilized starch as the main carbon source that could increase the production of antibacterial compounds[80]. The continuous and gradual hydrolysis of starch could

avoid the carbon catabolite repression mechanisms that usually triggered by carbon sources that are more easily metabolized by the microorganism such as glucose[101]. In addition, the antibacterial compounds present were consisted of two benzaldehydes compounds identified from the fermented broth of *S. atrovirens* PK288-21. Both of the benzaldehyde derivatives demonstrated antibacterial activity against both *V. anguillarum* and *V. harveyi*, particularly against *V. harveyi* with lower MIC values reported as compared to ciprofloxacin (58 µg/mL). The work showed that the compound, 2-hydroxy-5-(3-methylbut-2-enyl)benzaldehyde **(9)** was as a new derivative while 2-hepta-1,5-dienyl-3,6-dihydroxy-5-(3-methylbut-2-enyl)benzaldehyde **(10)** was previously reported from fungus *Eurothium rubrum*. Similarly, another 4 studies (Table 2) also demonstrated the use of starch with concentrations ranging from 0.1 to 1% (w/v), as the sole carbon source in the fermentation medium for the production of secondary metabolites by *Streptomyces* strains, and exhibited diverse strength of antibacterial activity against *Vibrio* sp.[76,77,83,84]. Overall, starch is recommended to be a good carbon source for the production of anti-*Vibrio* metabolites from *Streptomyces*.

Table 2. The compositions of fermentation medium and the fermentation conditions extracted from the reviewed studies on *Streptomyces* with anti-*Vibrio* activity.

Parameters	Compositions	Concentration % (w/v)/ Units	Number of studies performed fermentation (n = 31)	Percentage (%)
Carbon sources	**Complex carbon source only**			
	Starch	0.1	1	
		0.5	2	
		1	2	
	Sugarcane	1	1	
	• Yeast extract	5	1	
			Total = 7	22.6
	• Glucose	0.2	5	
	• Glycerol	1	2	
	• Glycerol, myoinositol	1, 0.04	1	
			Total = 8	25.8
	Mixture of both complex and readily utilizable			
	• Starch, glucose	2, 1	1	
	• Starch & glycerol	1	1	
	• Glycerol, starch, glucose	1,1,1	2	
	• Malt extract, glucose	0.4, 0.4	1	
			Total = 5	16.1
	Media used by studies w/o specify the composition			
	• Starch casein broth	-	5	
	• Potato dextrose broth		1	
	• Arginine glycerol broth		1	
	• Glycerol asparagine broth		1	
	• ISP2		1	
	• Soybean meal medium		1	
	• Actinomycetes isolation medium		1	
			Total = 11	35.5
Nitrogen sources	**Complex nitrogen source only**			
	• Soybean	0.2	1	
		0.5	1	
	• Tryptone, yeast extract	1, 5	1	
	• Peptone, yeast extract	0.2, 0.4	1	
	• Soybean, yeast extract	1.5, 0.25	1	
	• Polypepton, yeast extract, corn steep liqour	0.5, 0.2, 0.1	1	
	• Soybean meal, peptone, yeast extract	0.5, 0.2, 0.2	1	
	• Malt extract, yeast extract	0.4, 0.4	1	

	• L-tyrosine, L-asparagine	0.05, 0.1	2	
			Total = 10	32.3
	Mixture of both complex and readily utilizable			
	• Soybean, KNO_3	0.1, 0.005	1	
	• Casein, KNO_3	0.03, 0.2	3	
	• Yeast extract, NH_4Cl	0.3, 0.1	5	
	• MSG, NH_4Cl	0.5, 0.15	1	
			Total = 10	32.3
	Media used by studies w/o specify the composition			
	• Starch casein broth	-	5	
	• Potato dextrose broth		1	
	• Arginine glycerol broth		1	
	• Glycerol asparagine broth		1	
	• ISP2		1	
	• Soybean meal medium		1	
	• Actinomycetes isolation medium		1	
			Total = 11	35.5
Phosphate	K_2HPO_4	0.001	5	
		0.05	4	
		0.2	3	
			Total = 12	38.7
Salt	NaCl	0.05	3	
		0.08	5	
		0.1	1	
		0.2	3	
		1	1	
			Total = 13	41.9
pH		7	9	
		7.2	1	
		7.4	1	
		7.5	2	
		8	1	
		Not specified	17	
			Total = 31	
Temperature (ºC)		23	1	
		25	1	
		27	2	
		28	13	
		29	1	
		30	5	
		32	1	
		35	1	
		26-30	1	
		28-32	1	
		Not specified	4	
			Total = 31	

Table 3. The effect of carbon, nitrogen and NaCl on the anti-*Vibrio* activity of *Streptomyces* metabolites.

Media composition (concentration range, w/v %)	Median of Inhibition zone (mm)		Percentage of changes in anti-*vibrio* activity (%)
	Absence	Presence	
Carbon sources			
Starch (0.32 – 2)	15.03 (n = 16)	20 (n = 10)	Increased by 33.07
Glucose (0.2 – 2)	17.40 (n = 18)	16 (n = 9)	Decreased by 8.05
Glycerol (0.12 – 1)	17.40 (n = 20)	18 (n = 8)	Increased by 3.45
Nitrogen sources			
Yeast extract (0.3 - 1)	16 (n = 16)	19 (n = 8)	Increased by 18.75
Casein (0.03 - 1)	16 (n = 15)	17.4 (n = 10)	Increased by 8.75
Ammonium salts, NH_4^+ (0.0001 - 0.12)	16.4 (n = 18)	18 (n = 5)	Increased by 9.75
Nitrate salts, NO_3^- (0.2)	16 (n = 16)	18 (n = 7)	Increased by 12.50
Others			
NaCl (0.05 - 1.2)	17 (n = 8)	18 (n = 16)	Increased by 5.88

Influence of organic and inorganic nitrogen source on anti-Vibrio activity

Nitrogen sources such as nitrate and ammonium salts which favorable for growth were shown to affect negatively on the production of secondary metabolites in *Streptomyces*. The readily utilized nitrogen sources were demonstrated to cause repression of enzymes responsible for tylosin in *Streptomyces fradiae*[102]. Complex protein source such as soybean meal and the slowly assimilated amino acid such as proline are good nitrogen source to promote high secondary metabolites production. Therefore, slow-metabolizing nitrogen sources are preferable to supply the essential nutrients to the antibiotic-producing strains. Yeast extract, corn steep liquor and soybean flour are commonly used complex organic nitrogen sources[31]. Based on the reviewed studies, soybean meal (0.2 and 0.5% w/v) was evidenced in studies[77,103] as a sole nitrogen source for the production of metabolites that exhibited anti-*Vibrio* activities by the *Streptomyces* strains (Table 2). Furthermore, the anti-*Vibrio* activity of the *Streptomyces* strains cultivated in different nitrogen sources were compared based on the median inhibition zone (Table 3). The usage of yeast extract as a complex organic nitrogen source is found to enhance the anti-*Vibrio* activity of the *Streptomyces* metabolites by 18.75%, when compared to the only 8.75% increment in the presence of casein as an organic nitrogen source. Besides that, nitrate is a more favorable inorganic nitrogen source when compared to the use of NH_4^+ in the fermentation media of the anti-*Vibrio Streptomyces*. None of the studies utilized ammonium or nitrate salts as the sole nitrogen source for the fermentation process. A total of 19 studies demonstrated the use of a mixture of readily and slowly utilizable nitrogen sources in the optimization of medium composition for the improvement of the yield of secondary metabolites (Table 1). As the readily utilizable sources such as ammonium salts and nitrate salts serve to support the exponential growth of the bacteria while the slowly used sources such as yeast extract and casein serve to sustain the production of metabolites during the stationary phase, as the rapidly assimilated sources are depleted[31]. Thus, the combination of yeast extract and nitrate salts could be used to serve as a good nitrogen sources in the production of anti-*Vibrio* metabolites in the genus *Streptomyces*.

Inorganic phosphate

Inorganic phosphorus is the common major growth-limiting nutrient in natural environments[31]. Literatures showed that high concentration of inorganic phosphate in culture media causes negative regulation on the synthesis of secondary metabolites in different *Streptomyces* sp.[104,105]. A total of 12 studies (38.7%) indicated the supplementation of dipotassium phosphate as a source of inorganic phosphate, with wide range of concentrations from 0.001 to 0.2% (w/v) (~ 0.5–115mM) in the fermentation medium for the production of anti-*Vibrio* secondary metabolites by *Streptomyces* (Table 2). None of the studies indicated the potential of inorganic phosphate that could resulted in lower production of anti-*Vibrio* compounds. Although some literatures demonstrated the supply of inorganic phosphate more than 3–5 mM are frequently inhibitory to antibiotic biosynthe-sis[105,106]. Liras *et al.* (1990)[106] indicated phosphate stimulates the expression of genes involved in the biosynthesis of macromolecules and house-keeping genes essential for growth whereas it often inhibits expression of genes encoding for biosynthesis of secondary metabolites. The *p*-ami-nobenzoic acid synthase (PABA synthase), that catalyzes the conversion of chorismic acid to *p*-aminobenzoic acid which is a precursor for candicidin (macrolide antibiotic) was found to be inhibited by potassium phosphate at 5 to 10 mM resulting in repression of candicidin biosynthesis in *Streptomyces griseus*[107]. Studies showed that the biosynthesis of several groups of antibiotic are

particularly sensitive to phosphate repression such as aminoglycosides[108], tetracyclines[109], macrolides[110] and polyenes[104]. Meanwhile, the biosynthesis of beta-lactam antibiotic and peptide secondary metabolites were poorly sensitive to high concentration of inorganic phosphate. For example, the production of cephalosporin is optimal at 25 mM phosphate but higher concentrations of phosphate resulted in 85% reduction of cephalosporin production in *S. clavuligerus*[111]. These evidences suggested that the genes encoding the enzyme for the secondary metabolites produced by the anti-*Vibrio Streptomyces* may have lower sensitivity toward phosphate repression. However, the concentration of inorganic phosphate to be used in fermentation media should be optimized to ensure maximum production of anti-*Vibrio* metabolites by the *Strep-tomyces*. By comparing the anti-*Vibrio* activity of the *Streptomyces* metabolites under different concentration of K_2HPO_4 (Table 4), based on the median of inhibition zone, it is observed that the anti-*Vibrio* activity reduced by 33.3% when the concentration of K_2HPO_4 used is increased from 0.001% to 0.2% (w/v). These data suggest that inorganic phosphate is recommended to be maintained at lower concentration such as at 0.001% (w/v) as a source of phosphorus in the fermentation media for optimal production of anti-*Vibrio* metabolites from *Streptomyces*.

Table 4. The effect of different compositions and the fermentation conditions on the anti-*Vibrio* activity of *Streptomyces* metabolite

Parameters	Concentration (w/v %) / Range	Median of inhibition zone (mm)
NaCl	0.05	15.02 (n = 2)
	0.08	30.00 (n =3)
	0.20	19.00 (n = 8)
	> 0.20	15.00 (n = 3)
K_2HPO_4	0.001	30.00 (n = 3)
	0.01 - 0.05	16.52 (n = 4)
	0.2	20.00 (n = 1)
pH	7	15.03 (n = 7)
	7.1 – 7.3	18 (n = 5)
	> 7.3	22.5 (n = 2)
Temperature (°C)	< 28	16.4 (n = 2)
	28	20 (n = 12)
	30	15 (n = 7)

Sodium chloride

The supplementation of sodium chloride in the fermentation medium is one of the non-nutritional stress factors influencing the secondary metabolites production[112,113]. Based on the reviewed studies, a total of 13 studies supplemented sodium chloride in the fermentation medium for the production of anti-*Vibrio* secondary metabolites from *Streptomyces* (Table 2). The concentration of sodium chloride used was ranging from 0.05 to 1% (w/v), showing production of anti-*Vibrio* metabolites from *Streptomyces*. In line with the literatures, the anti-*Vibrio* activity of *Streptomyces* metabolites is enhanced by 5.88% when cultivated in the presence of sodium chloride as compared to the metabolites produced in the absence of sodium chloride (Table 3). Barakat and Beltagy (2015)[114] indicated the *Streptomyces ruber* ERH2 supplemented with 1% sodium chloride (w/v) produced metabolites against *V. ordalii* fish pathogen, with high inhibition zone measured at 15mm. As indicated in Table 4, a small increase of sodium chloride concentration, such as from 0.05 to 0.08% (w/v) resulted in 99.7% increment in the anti-*Vibrio* activity, thus indicated the optimum concentration of sodium chloride for the production of anti-*Vib-rio* metabolites is at 0.08% (w/v) for *Streptomyces*. At the meantime, the further increase of sodium chloride in the fermentation media from 0.08% (w/v) to more than 0.2% (w/v) may reduce the anti-*Vibrio* activity from *Streptomyces* metabolites by 50%. Similarly, Syvitski *et al.* (2006)[115] demonstrated that the presence of salt in the growth medium could result in differential production of antibiotic by *Streptomyces*. Furthermore, this study indicated the addition of 2.5% of sodium chloride inhibited the production of actinorhodin, but activated the production of undecylprodigiosin[115]. The study also reported high salt conditions that resulted in differential expression of these genes, *act*II-ORF4 and *redD* encoding corresponding pathway specific transcriptional regulators for both actinorhodin and undecylprodigiosin biosynthesis in *Streptomyces coelicolor* A3(2)[115].

Temperature

An optimal temperature is often required for the production of secondary metabolites. Based on the reviewed studies, 28°C (41.9%) is the most common incubation temperature used for the secondary metabolite production. Slightly higher incubation temperature at 30°C is also reported in several studies (16.1%) (Table 2). There are also studies employed a lower incubation temperature ranging from 23-25°C[116,117]. The studies indicated that the optimal temperature for production of secondary metabolites can be varying considerably between the similar genera of *Actinobacteria*. Furthermore, some studies indicated that optimal temperature for production of secondary metabolites is generally lower, when compared to growth of *Streptomyces* sp. Thakur *et al.* (2009)[118] reported *Streptomyces* sp. 201 showed narrow range of incubation temperature for growth and antibiotic production, maximum mycelial growth was measured at 35°C while highest antibacterial activity was observed at 30°C. Thirumurugan and Vijayakumar (2015)[119] also reported a strain, *Streptomyces* ECR77 that produced anti-*Vibrio* secondary metabolites after cultivated at 28-30°C although this strain showed optimal growth at 35°C. Costa and Badino (2012)[120] also recommended that the reduction of temperature could be useful in increasing the production of clavulanic acid by *Streptomyces clavuligerus*. According to Table 4, repression effect could occur via increase of fermentation temperature from the optimum 28°C to 30°C, resulting in 25% reduction of anti-*Vibrio* activity (based on the median of inhibition zone) from the *Streptomyces* metabolites. Hence, these data suggest that lower incubation temperature results in lower cellular growth and substrate consumption which could minimize

the metabolite repression effects and also reduces end-product degradation, eventually increasing the yield of secondary metabolites production[120].

pH of fermentation media

The pH of the cultivation media has substantial effect on the growth of *Streptomyces* sp. and their antibiotic production ability[121,122]. Based on the reviewed studies, a narrow range of initial pH (7–8) of the fermentation media were used in cultivation of *Streptomyces* sp. for secondary metabolites production (Table 2). Kontro *et al.* (2005)[122] reported that the pH ranges for the optimal growth of *Streptomyces* sp. were species specific and strongly influenced by the nutrient compositions of the media. The use of neutral to slightly alkaline pH as described by majority studies suggested that these pH range are more preferable for developing a fermentation medium for antibiotic producing-*Streptomyces*. In agreement with others, anti-*Vibrio* activity from *Streptomyces* metabolites could be enhanced by performing the fermentation under slightly alkaline pH. As depicted in Table 4, the anti-*Vibrio* activity could be increased by 49.7% with a small increase of the fermentation media from pH 7 to 7.3. According to Guimaraes *et al.* (2004)[121] findings, the low pH level of the cultivation media (at the end of the shake flask fermentation) resulted in no detection of retamycin although the final cell concentrations of *S. olindensis* ICB20 reached 4 g/liter, indicating that the pH negatively affected the activity of the biosyn-thetic enzymes that involved in the secondary metabolism. Meanwhile, at higher pH of 8.0, it was reported to have negative effect on the excretion of the antibiotic, demonstrated by the higher intracellular content of retamycin was produced, rather than the yield of extracellular retamycin[121].

EXTRACTION OF SECONDARY METABOLITES

The extraction is a critical step to isolate the desirable secondary metabolites from the complex fermented products[123]. Solvent extraction is one of the most common extraction methods due to the high selectivity and solubility of target compositions. It has been widely utilized to extract fermentation-derived microbial products prior to the final purification of bioactive compounds by chromatography[74,123,124]. There are a wide range of approaches available for the recovery of microbial metabolites. Primarily, the types of extraction method employed is chosen depending on the compounds of interest residing whether it is excreted into the medium or produced intracellularly. Generally, direct solvent extraction is conducted if the desired product is present in the cell and the medium. However, the common practice in extraction of microbial product from the cultivation media involves the separation of the microorganism biomass by centrifugation or filtration prior to solvent extraction of the cell free medium[125,126]. Among the 31 studies that performed fermentation, 18 studies (58.1%) conducted solvent extraction method to extract and determine the antibacterial activity of the bioactive compounds present in the fermented product.

The selection of most appropriate solvent is critical in determining the successfulness of yielding the desired product. Nonpolar solvents (petroleum ether, chloroform and hexane) are useful in extracting lipophilic compounds such as alkanes, sterols, alkaloids, fatty acids, coumarins and some terpenoids. Some alkaloids and flavonoids are compounds with medium polarity can be extracted with medium polarity solvents such as ethyl acetate. Meanwhile the more polar compounds such as flavonoid glycosides, tannins and some alkaloids are extracted with the carbon-bonded oxygen-bearing extractants include alcohols, esters and ketones [123]. Table 5 shows the examples of bioactive compounds isolated from anti-*Vibrio Streptomyces* using different organic solvents.

Table 5. The bioactive compounds identified from the *Streptomyces* sp. displaying anti-*Vibrio* activities.

Source	Compounds	Antibacterial activity	References
Ethyl acetate of *Streptomyces rosa* var. *notoensis*	Nanaomycin A **(1)**	MIC: 6.3 µg/mL against *V. alginolyticus* 138-2 MIC: 3.1 µg/mL against *V. parahaemolyticus* K-1	[127]
	Nanaomycin D **(2)**	MIC: <0.05 µg/mL against *V. alginolyticus* 138-2 MIC: <0.05 µg/mL against *V. parahaemolyticus* K-1	
Methylene chloride extract of endophytic *Streptomyces* sp. NRRL30562 derived from plant, *Kennedia nigriscans*	Munumbicin B **(3)**	16 mm against *V. fischeri* PIC345	[116]
	Munumbicin C **(4)**	9 mm against *V. fischeri* PIC345	
	Munumbicin D **(5)**	12 mm against *V. fischeri* PIC345	
Methanol extract of desert soil-derived *Streptomyces* sp. C34	Chaxalactin A **(6)**	MIC: 12.5 µg/mL against *V. parahaemolyticus*	[74]
	Chaxalactin B **(7)**	MIC: 20 µg/mL against *V. parahaemolyticus*	
	Chaxalactin C **(8)**	MIC: 12.5 µg/mL against *V. parahaemolyticus*	
Acetone extract of *Streptomyces atrovirens* PK288-21 derived from marine seaweeds	2-hydroxy-5-(3-methylbut-2-enyl)benzaldehyde **(9)**	MIC: 20 µg/mL against *V. harveyi* MIC: 65 µg/mL against *V. anguillarum*	[80]

	2-hepta-1,5-dienyl-3,6-dihydroxy-5-(3-methylbut-2-enyl)benzaldehyde **(10)**	MIC: 32 µg/mL against *V. harveyi* MIC: 65 µg/mL against *V. anguillarum*	
Acetone extract of *Streptomyces* sp. K01-0509	Guadinomine B **(11)**	IC_{50}: 14 nM potent type III secretion system (TTSS) inhibitor	[128]
Ethyl acetate extract of *Streptomyces* sp. SCSIO 01689 derived from submarine sediment	Pyranosesquiterpene compound **(12)**	MIC: >100 µg/mL against *V. anguillarum*	[82]
	Cyclo(D)-Pro-(D)-Ile **(13)**	MIC: 0.05 µg/mL against *V. anguillarum*	
	Cyclo(D)-Pro-(D)-Leu **(14)**	MIC: 0.04 µg/mL against *V. anguillarum*	
	Cyclo(D)-trans-4-OH-Pro-(D)-Phe **(15)**	MIC: 0.07 µg/mL against *V. anguillarum*	

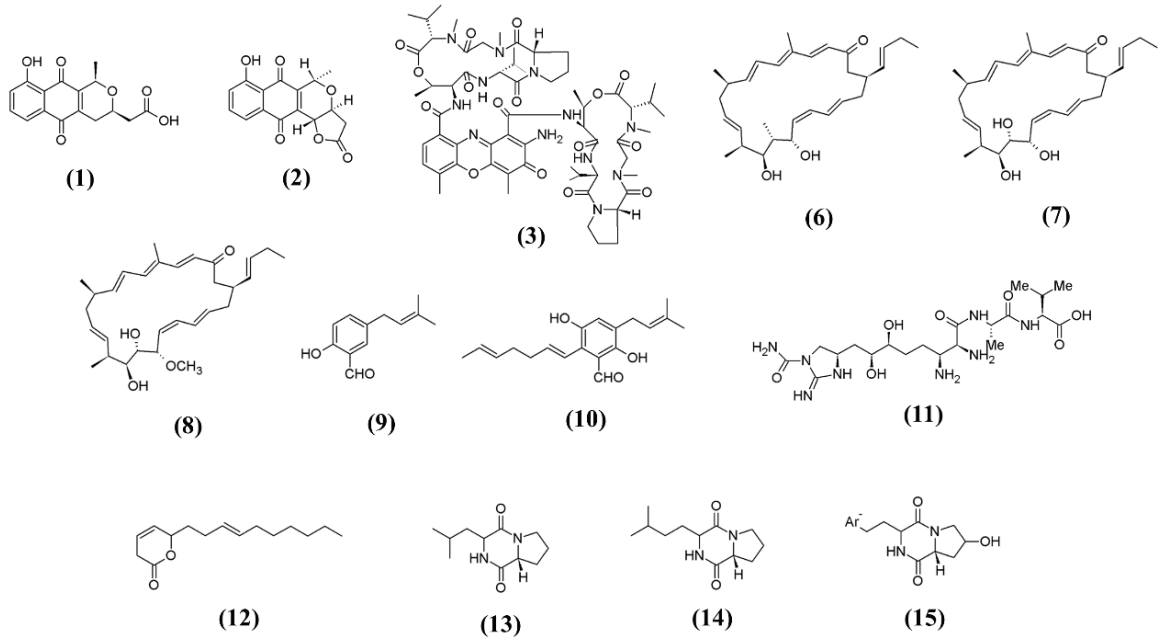

Figure 2. The chemical structures of anti-*Vibrio* secondary metabolites isolated from *Streptomyces* sp.

Based on the data, the commonly used solvents for the extraction of bioactive compounds include, methanol, acetone, chloroform, ethyl acetate, *n*-butanol, *n*-hexane and petroleum ether. From these studies, ethyl acetate (83.3%) was the most commonly used solvent. This may be due to the property of ethyl acetate which is only partially miscible with water, hence allowing easier recovery of the metabolites from the fermentation broth by liquid-liquid extraction methods. Besides that, methanol was the second (27.8%) most commonly utilized solvent among the reviewed studies. Usually, methanol is preferable for the extraction of unknown metabolites from new strains of bacteria. This is because methanol has been known to be efficient in extracting a wide range of metabolites from bacteria[129]. Eventually, the resulting extract is filtered, concentrated by vacuum evaporation before being used for bioactivity analysis. It is imperative to remove the solvent or extractant completely from the resulting extracts as their presence in the final product is undesirable and might affect the results of the bioactivity screening. Gas chromatography is a useful tool for the detection of residual solvents. This is because of the low detection limits allowing for the detection of trace organic compounds[130]. Furthermore, supercritical carbon dioxide at 200 atm and 35°C was shown to be effective in removing organic solvents from antibiotic without affecting the antibiotic activities[131].

Moreover, it is common to find that interesting compounds can be overlooked due to the presence of other molecules in a crude extract, or simply because of its low titers in an extract resulted overall low activity observed. Fractionation step after the extraction could be a way to overcome these issues. For instance, the fractionation of *Streptomyces* sp. C34 methanolic extracts with three other different solvents, *n*-hexane, dichloromethane and ethyl acetate and eventually identified the three novel macrolactones from the dichloromethane fraction with the most diverse metabolic profile[74].

Once a bioactive extract is identified, a more detailed analysis is performed, normally involving chromatography-based separation of the individual constituents, to identify the specific bioactive molecules and also structure elucidation with NMR analysis.

Subsequently, the bioactive compounds from these screening activities are tested in an *in vivo* model to examine efficacy and safety. Most of current clinically used antibiotics have been discovered using this approach. For instance, Barakat and Beltagy (2015)[114] demonstrated that the phthalic acid isolated from *S. ruber* EKH2 with antagonistic activity against *V. ordalii* is non-toxic toward *Artemia salina* (brine shrimp) up to 2800 µg/mL, suggesting that the compound is natural and minimum side effects. Furthermore, the conventional screening process also provides valuable information such as the potency of the antibiotic by determining the minimum inhibitory concentration (MIC) of the antibiotic toward specific pathogens, the spectrum of activity. Cho and Kim (2012)[80] determined the potency of benzaldehyde compounds isolated from *S. atrovirens* PK288-12, revealing a lower MIC displayed by both compounds as compared to ciprofloxacin against *V. harveyi.*

By referring to the studies which reported the isolation of *Streptomyces* with anti-*Vibrio* activity, most of them have focused on the preliminary screening and optimization of the various culture conditions. However, there is only limited number of the study that further analyzed and identified the bioactive compounds that displayed potent antibacterial activity against *Vibrio* sp. Hence, there is a need to improve the isolation and screening strategies, as the conventional methods of cultivation, extraction and bioac-tivity testing of anti-*Vibrio Streptomyces* are time consuming and prone to rediscovery of known compounds. New research strategies such as genome mining, which reveals the silence biosynthetic gene cluster, coupling with the advanced chemical separation and characterization techniques[132] have been developed to enhance the antibiotic production and discovery of new compounds in *Streptomyces*. Furthermore, more advanced extraction method could be employed to replace the conventional organic solvent extract method. For example, supercritical fluid extraction, pressurized solvent extraction and ultrasound-assisted extraction have been discussed as some of the better alternative extraction techniques to isolate bioactive natural products[133]. These advanced extraction methods are known for their higher selectivity, shorter extraction time, nontoxic organic solvents and more environmental friendly as compared to the conventional solvent extraction method[133]. Majority of these advanced extraction methods have been widely used to extract biologically active compounds with antioxidant and antimicrobial activity from plants[53,134]. Despite that, only a small portion of studies have utilized the advanced extraction methods to extract the bioactive compounds from the fermentation broth of microorganism. For instance, griseofulvin, which is one of the few examples of microbial antifungal antibiotic, was extracted with supercritical carbon dioxide extraction method[135]. Although the supercritical carbon dioxide is less effective in extracting highly polar compounds, this extraction method offers a better alternative to organic solvents because of its nontoxic property, inexpensive and most importantly can be easily removed from the final products[133]. This is because the residual organic solvent presents a major concern over the safety of food and pharmaceutical products over the years[136]. Therefore, future studies are encouraged to utilize one of these advanced extraction methods to improve the yield and purification of the biologically active compounds from *Streptomyces*.

CONCLUSION

Given the ever-increasing reports of antibiotic resistant *Vibrio* pathogens, there is a critical need to search for alternatives of major antibiotics. Numerous studies demonstrated the production of promising bioactive compounds with anti-*Vibrio* activity by *Streptomyces* sp. Fermentation parameters can have great impact on the secondary metabolism of *Streptomyces* and subsequently on production of different microbial products. The information and knowledge obtained in this review could help in the optimizing of suitable fermentation medium is important for better yield and antimicrobial activity from *Streptomyces* sp. We suggest that starch and yeast extract are both good carbon and nitrogen source for the secondary metabolites production by the anti-*Vibrio Streptomyces*. The temperature, concentrations of phosphate and sodium chloride are also important criteria should be taken into consideration when designing the fermentation medium and condition for the anti-*Vibrio* metabolite production in the genus *Streptomyces*. The limited findings on the bioactive compounds with anti-*Vibrio* activity from *Streptomyces* sp. suggesting that more studies should focus on identifying the potential bioactive compounds that specifically against *Vibrio* sp. Taken together, with optimal fermentation conditions and appropriate extraction techniques, future development of clinically important drugs is warranted from these *Streptomyces* sp. to treat infections inflicted by *Vibrio* pathogens.

Author Contributions

The literature review and manuscript writing were performed by LT-HT, L-HL and B-HG. L-HL and B-HG founded the research project.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Review Article

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Epidemiology of Chronic Kidney Diseases (CKD) in Malaysia and Pakistan, Pathophysiology of CKD-Associated Pruritus and Other CKD-Associated Dermatological Disorders

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Abstract: Almost 50–90% of chronic kidney disease patients undergoing haemodialysis have been reported to have Chronic kidney disease-associated pruritus (CKD-aP). The intensity of CKD-aP may vary from a mild itch to an unbearable pruritic sensation which interferes with the patient's quality of life. CKD-aP has become one of the upmost distressing cutaneous and most common symptom of chronic kidney disease which is often overlooked by nephrologists, primary care physicians, and other health-care professionals. Typically sleep disorders, mental and social well-being have been correlated with chronic kidney disease patients. With that this article presents vital comprehensive review which includes epidemiology of chronic kidney disease in Malaysia and Pakistan, CKD-associated pruritus and other dermatological disorders associated with chronic kidney diseases, pathophysiology of CKD-associated pruritus, clinical features of chronic kidney disease-associated pruritus, diagnosis of CKD-associated uremic pruritus, differential diagnosis of CKD-associated uremic pruritus, assessment and quantification of pruritus severity.

Keywords: Chronic Kidney Diseases (CKD); Malaysia; Pakistan; pathophysiology of CKD-associated pruritus

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INTRODUCTION

In Malaysia, the prevalence of CKD is on the rise from 13,479 per million populations in year 2004 to 20,589 per million populations in year 2008[1]. In Malaysia the exact estimation of CKD is unknown, nevertheless the expected incidence of chronic kidney disease in West Malaysia is 9.07%[2]. Additionally, its distribution stage-wise i.e. Stage 1, 2, 3, 4, and 5 are 4.16%, 2.05%, 2.26%, 0.24%, and 0.36%, respectively[2]. According to the 21st report of Malaysian dialysis and transplant registry, the number of patients on dialysis increased from 13,356 in year 2004 to 34,767 in year 2014[3].

In Pakistan, chronic kidney disease is progressing, and multiple factors are responsible for this epidemic such as poor health care facility, deficient primary health care system, no proper health education, insufficient funding and higher prevalence of diabetes and hypertension[4].

DEFINITION AND STAGES OF CHRONIC KIDNEY DISEASE (CKD)

Kidney Disease Outcome Quality Initiative (KDOQI) defines chronic kidney disease (CKD) as an immediate or continuing to decrease kidney function or efficiency for a duration exceeding three months[5]. The standards for assessing the disease initiation are mainly urinary outcome, proteinuria and hematuria[6,7]. In some cases, the initial presentations are temporary and can be solved by early initiation of drug therapy while in the majority

of conditions, the decrease in the creatinine clearance and accumulation of waste products like urea and uric acid occurs[7]. Glomerular filtration rate (GFR) and creatinine clearance (CC) are two parameters used for estimation of kidney function[5,8]. "Glomerular Filtration Rate (GFR) can be defined as the amount of blood that is filtered by Bowman's capsule per unit of time (mL/min/1.73m^2)". For a healthy human being the GFR values should be range from 120-130 mL/min/1.73m^2[8]. Listed here are two most common equations used in practice for estimating GFR based on serum creatinine (Scrt).

1. Cockcroft-Gault equation[9]
2. Modification of Diet in Renal Disease (MDRD) equation[10]

Cockcroft-Gault Equation

CC (mL/min) = ([140-Age × weight] / [72 × Scrt]) × 0.85 if female

MDRD Equation

eGFR (mL/min/1.73 m^2) = 186 × [Scrt] – 1.154 × [Age] – 0.203 × [0.742 if female]

Note: for African/ Black use the multiplication factor 1.21

Stages of kidney diseases:

On the basis of Creatinine Clearance (CC) or estimated Glomerular Filtration Rate (eGFR) kidney disease can be classified in five stages[5,11]:

i. Stage 1: Normal or increased GFR i.e. 90 or more mL/minute/1.73m^2
ii. Stage 2: Mild decrease in GFR i.e. 60–89 mL/ minute/1.73m^2
iii. Stage 3: Moderate decrease in GFR i.e. 30–59 mL/ minute/1.73m^2
iv. Stage 4: Severe decrease in GFR i.e. 15–29 mL/ minute/1.73m^2
v. Stage 5: Kidney failure i.e. Less than 15 mL/ minute/1.73m^{2}or on dialysis

Creatinine is basically a by-product that forms from protein metabolism. When the kidney function starts deteriorating, clearance from kidney reduces which leads to an elevated serum creatinine, uric acid and urea[9]. Majority of the patients at stage 4 and stage 5 of kidney diseases get frequent dialysis based on their renal reservoir. In general, most of the patients get dialysis three times a week. The main aim for performing dialysis is to eliminate the waste products from the blood such as urea, uric acid, nitrogen and excessive electrolytes.

Low GFR alone is not confirmatory for diagnosis of CKD, as it may be borderline normal or normal. In order to establish the diagnosis for CKD, the presence of one or more markers as listed is vital[12].

- Histological abnormalities
- Urine sediment abnormalities
- Albuminuria (albumin excretion >30 mg/24 hr)
- Electrolyte and other abnormalities owing to tubular disorders
- Renal transplantation history
- Structural abnormalities detected by imaging

EPIDEMIOLOGY OF CKD

CKD is currently one of most serious health crises. Epidemiological data suggests that CKD is a big threat globally for both developing and developed countries[13]. According to 2010 statistic of Global Burden of Disease (GBD), among the directory causes of global deaths, CKD was categorized 27th in 1990 but due to its higher prevalence, it climbed to 18th in 2010[14]. The 2013 statistic by Global Burden of Disease (GBD), comparing the mortality rate of CKD patients between 1990 and 2013, indicates mortality rate is increased by 134.6% in 2013 compared to 1990[15]. The prevalence of CKD is heterogeneous globally, with the incidence of CKD was higher in Indo-Asians population as compared to the European population. The statistics indicated CKD is more prevalent in Asian countries as compared to the rest of the world. Malaysia reported prevalence of 9.07%[2], while China and Nepal reported prevalence of 10-19%[16]and 10-20%[17] respectively. Whereas Pakistan, Bangladesh, and India reported prevalence of 20%[18-20]. One possible reasons for the increased in prevalence is the reduced access to preventative health care, which helps to reduce the progression of kidney diseases[21]. Furthermore the global increase in CKD cases and its progression toward end-stage renal disease due to global increase of diabetes and hypertension pandemics [22–24].

Chronic kidney diseases in Malaysia

In Malaysia, the prevalence of CKD is increasing from 13,479 per million populations in 2004 to 20,589 per million populations 2008[1]. In Malaysia the exact estimation of CKD is unknown, still the expected incidence of chronic kidney disease in West Malaysia is 9.07%[2]. Moreover, its distribution stage-wise i.e. stage 1, 2, 3, 4, and 5 are 4.16%, 2.05%, 2.26%, 0.24%, and 0.36%, respectively[2]. The 21st report of Malaysian dialysis and transplant registry stated the number of patients on dialysis increased from 13,356 in 2004 to 34,767 in 2014[3], as depicted in Figure 1. The acceptance rate and prevalence rate of dialysis are 210 and 1065 per million populations respectively[1]. The report stated that overall acceptance and prevalence rate of dialysis is almost doubled during the span of 10 years[1]. States in Malaysia which are economically advanced have a much higher rate of dialysis treatment compared to those states which are economically less advanced. Approximately 90% of new dialysis patients are accepted into haemodialysis center and the rest into the peritoneal dialysis program[1]. In 2013, the annual death rate on dialysis was reported as 11.3%, out of which 10.9% were of haemodialysis and 15.8% were of peritoneal dialysis[1]. Diabetes is considered to be the typical cause of CKD, with reported cases of CKD due

to diabetes at 53% in 2004 and 61% in 2013[1]. End stage renal disease caused by diabetes has increased dramatically and is accountable for 50% incidents in dialysis patients. The Malaysian National Registry estimated the prevalence of end-stage renal disease in 2007 will be 680 per million population[25]. According to National Renal Registry Malaysia (2015)[3], 32,026 patients were on dialysis; of whom 91% were on haemodialysis and 9% were on peritoneal dialysis. It has been estimated that by 2040, the number of patients with end stage renal disease would triple from existing 2014 cases[26]. Ibrahim *et al.* (2011)[27] reported the prevalence of CKD-aP in Malaysia by 64.2% and sleep disturbance with 61.7% in CKD patients.

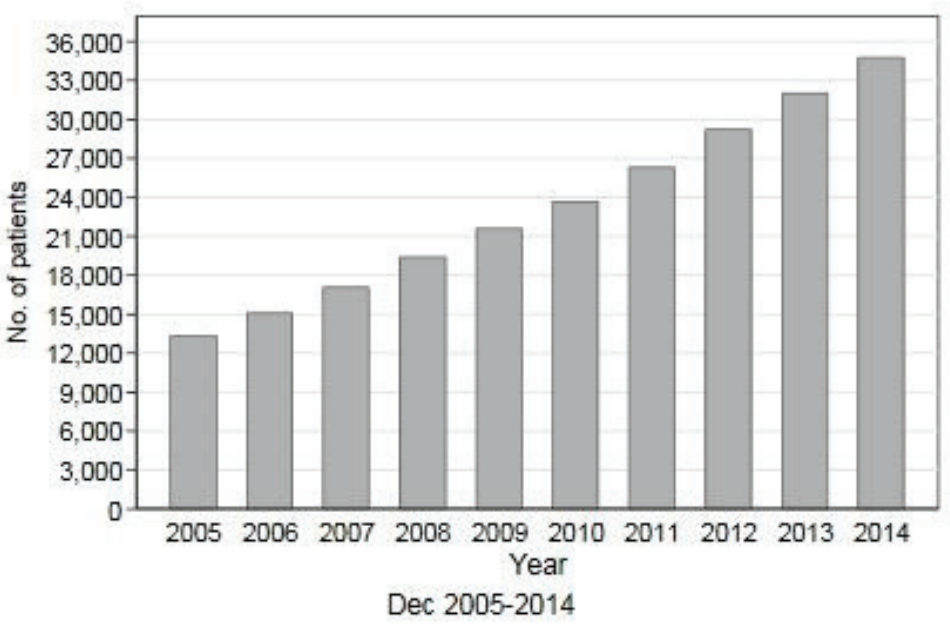

Figure 1. Statistic of increase in dialysis patients indicating an increase during December 2005- December 2014 in Malaysia.

Chronic kidney disease in Pakistan

In Pakistan, chronic kidney disease is progressing with multiple factors responsible for this epidemic such as poor health care facility due to deficient primary health care system, no proper health education, insufficient government funding and higher occurrence of diabetes and hypertension marked as a high-risk factor for CKD[4]. In Pakistan, the absence of periodically maintained central registry kidney diseases on a national level about its epidemiological and burden makes it extremely difficult to assess the cases of CKD, dialysis, mortality, and allocation of the fund in Pakistan[4]. Data from the Dialysis Registry of Pakistan 2007–2008 indicated the number of CKD patients increased from 4,393 in 2006-2007 to 6,127 in 2007–2008[28]. While current data indicated 16,000 Pakistanis suffer from CKD annually and Pakistan is ranked the eighth in the world on basis of highest number of epidemiology and prevalent cases of CKD[29]. Jessani *et al.* (2014)[19] reported that CKD prevalence in Karachi is 12.5%. While Luqman *et al.* (2012)[30] reported the prevalence of CKD in Pakistan as 64%.

CKD-ASSOCIATED PRURITUS (CKD-aP) AND OTHER DERMATOLOGICAL DISORDERS ASSOCIATED WITH CHRONIC KIDNEY DISEASES

Dermatological disorders are regularly observed in CKD patients. Skin-associated problems like CKD-aP, xerosis, pallor, and hyperpigmentation are very common in CKD patients[31]. Most often, in the case of renal failure, the skin becomes an excretory organ for the substances the kidney usually clears from the body. A study on dermatological problems associated with CKD indicated the prevalence of skin conditions observed were pallor 91.5%, xerosis 75.9%, pigmentary changes 65%, CKD-aP 60.2% and panniculitis 1.2%[31]. CKD-aP is a commonly found complication reported by majority of chronic kidney disease patients[32]. The prevalence of CKD-aP was found to be more in haemodialysis patients (68%) than in peritoneal dialysis patients (38%) [33]. The occurrence of CKD-aP in CKD patients differ extensively from 22% to 90%[34-38]. Normally, the whole body is affected while the back and forearms are most likely to get affected compared to other parts of the body. Severe CKD-aP has a major impact on the patient's life quality which leads to other disorders such as anxiety, disturbed sleep, and depression[39–43]. Recently two studies (a Japanese study and the DOPPS) demonstrated a relationship between CKD-aP and an amplified threat of mortality[34,38]. Several studies report a considerable prevalence of CKD-aP among dialysis patients, ranging from 10% to 70% in PD patients and 20% to 90% in HD patients[44]. Almost 50% or greater chronic kidney disease patients are reported of having the dermatological problem like dry skin and itching[45]. These associated dermatological problems have a significant impact on quality of life which negatively impacted on CKD patients' physical and mental health[31].

Pruritus is linked with some other metabolic changes which

trigger and potentiate it, such as xerosis, decreased transepidermal elimination of pruritogenic factors, hyperparathyroidism, hypercalcemia and hypophosphatemia, higher levels of histamine and transdermal mast cell proliferation, and uremic sensory neuropathy[46]. In parallel to internal factors, few external factors are also hypothesized to be related with pruritus, such as dehydration, excessive sweating, humid and hot weather, shower with cold/hot water and stress[46]. Also CKD-aP patients could experience abnormal skin pigmentation and low platelet counts as a result of prolonged bleeding[47]. Usually after a temporary relief from pruritus, the symptoms reappear within six months with much-intensified degree regardless of any demographic variables[46]. CKD-aP occurs intermittently in some cases that may last for few minutes while some patients suffer from lengthy phases of severe CKD-aP that may be present during both day and night[45]. CKD-aP onset, duration, and intensity can change over time and the itching is usually worsened at night time. Most normally affected body parts by CKD-aP are the back, limbs, chest, and head, however, approximately 20–50% of patients experience a generalized CKD-aP[38].

PATHOPHYSIOLOGY OF CKD-ASSOCIATED PRURITUS (CKD-aP)

The exact mechanism associated with the pathophysiology of CKD-aP is poorly understood. Several hypotheses are discussed in this article for the pathophysiology of CKD-aP.

Immune-mediated hypothesis

CKD-aP is potentially due to dysregulated systemic inflammation[48-50]. In CKD-aP patient's elevated levels of T-helper type-1 cells[49], interleukin-6[49], interleukin-2[50] and C-reactive protein[51] were observed. It is recommended that CKD-aP is associated with high white blood cell count, high ferritin level and low albumin level[51]. The immune hypothesis refers CKD-aP as overproduction of proinflammatory substances such as histamine (by mast cells), interleukin 2, tumor necrosis factor α and interferon γ by T Helper 1 lymphocytes[52,53]; elevated level of inflammatory markers such as C-reactive protein and interleukin 6[50,54,55]. The elevated levels of serine protease and Proteinase-Activated Receptor-2 could also play vital roles in the pathogenesis of CKD-aP[56].

Xerosis hypothesis

Xerosis (dry skin), is another common dermatological condition reported in patients with CKD[57,58]. The reduction of the eccrine sweat glands size and the atrophy of the sebaceous glands are assumed as the main reasons for xerosis[59]. Xerosis has been considered as a substantial contributor in CKD-aP severity[60].

Histamine hypothesis

The increased of mast cells and histamine levels[61–63], Serotonin level[64], eosinophil's and tryptase have been witnessed in patients with CKD-aP[61,62]. Nevertheless, Prasad et al. (2015) reported that despite increased mast cells and histamine levels there was no direct correlation between histamine level and CKD-aP.

Neuropathic hypothesis

The neuropathic hypothesis suggests that the somatic and autonomic neuropathy caused by lesion can result in neuropathic itch[65,66]. It is recognized that neuropathic pain and pruritus shared the same neuronal pathway[67]. The involvement of neuropathic mechanisms in the mediation of pruritus is further demonstrated with gabapentin and pregabalin that are effective in the improvement of the patient with pruritus[68,69]. This also advocated that afferent C-terminal nerve fibers that are GABA-aminobutyric acid (GABA) dependent is involved in the CKD-aP[70].

Opioid hypothesis

The opioid hypothesis suggests that the imbalance of endogenous opioidergic system plays an important role in the pathophysiological mechanism of pruritus by the ability of μ receptor antagonists and ϰ receptor agonists in relieving itchiness[67,71,72]. The μ-opioid receptor activation is involved in the intervention of pruritus while ϰ-opioid receptor activation has an inhibitory effect on μ-opioid receptor both peripherally and centrally[67,72–74].

Hyperparathyroidism hypothesis

The hyperparathyroidism is expected to play a role in pruritus through inducing mast cell secretion[75]. Secondary parathyroidectomy trigger elevation of divalent ions such as magnesium, phosphate, and calcium, this could result in micro-precipitation that is known to have affect the modulation of mast cells degranulation[76]. Low serum phosphorus level was recently found to be significantly lower in haemodialysis patients with severe and frequent CKD-aP as compared to those without CKD-aP[77].

Other factors

Other factors associated with CKD-aP include the production of pruritogenic substances such as abnormal growth, cytokines, and sprouting of "itch fibers" in the skin, also neuropathy that leads to the decreased of the threshold for itch sensation[78,79].

CLINICAL FEATURES OF CKD-aP

Clinical characteristic varies for each patient and over time among patients. As the CKD-aP onset, intensity and duration can change over time[37], and the itch is typically worsened at night time[45]. CKD-aP could be generalized or localized; however back, abdomen and forearms are most likely to be affected compared to other parts of the body[38,45]. Other characteristics include:

- Worsen in itch intensity at night and cause sleep disturbance[34,38,45,80,81].
- Xerosis (dry skin), skin scaling and epidermal cracking[60].
- Elevated blood urea nitrogen (BUN), calcium, phosphate, and magnesium and parathyroid hormone (PTH), levels[34].
- Fatigue and depression[38,82].

DIAGNOSIS OF CKD-aP

CKD-aP is one of the most common complication reported by CKD patients on haemodialysis, so the diagnosis is easy unless there is other compelling evidence of other causes. The most common suggestive diagnosis characteristic of CKD-aP is its occurrence after the imitation of haemodialysis. Also, elevated calcium, magnesium, phosphate, blood urea nitrogen (BUN) and parathyroid hormone (PTH) levels[34] were confirmed among CKD-aP patients on haemodialysis.

Differential diagnosis of CKD-aP

Several diseases could cause CKD-aP in patients with and without CKD. In order to confirm CKD-aP, a non-uremic cause of CKD-aP should be kept in thought among patients on haemodialysis with symptoms that are refractory to common treatments such as an oral antihistamines, gabapentin, analgesic agents and topical emollient[83].

ASSESSMENT AND QUANTIFICATION OF CKD-aP SEVERITY

CKD-aP has become one of the most distressing cutaneous, and the most common symptom of CKD that is commonly overlooked by nephrologists, primary care physicians, and other health-care professionals[46]. To assess the severity of CKD-aP, several instruments have been developed and these tools shall be illustrated in this review.

The literature showed that the rating scales are commonly used for assessment and quantification of CKD-aP. The rating scales/tools includes verbal rating scale (VRS), visual analogue scale (VAS), numerical rating scale (NRS), Eppendorf itch questionnaire (EIQ), Dermatology quality of life questionnaire (DLQI) and 5Ditch scale (5D-IS), Modified pruritus questionnaire for itch severity score, Skindex(Skindex-29 and Skindex-16)[84–91].

Visual analogue scale (VAS)

Visual analogue scale is a unidimensional scale that is an easy and rapid tool commonly used for assessment of pruritus severity[71]. The VAS consists of a 10 mm horizontal line indicating the severity of itch; “no itch” (o points) and ending with “worst itching”[92]. The evaluation of CKD-aP relying on a single measure is not sufficient, VAS is a useful tool for the assessment of pruritus intensity but it does not provide any further information on other aspects of CKD-aP[93].

Verbal rating scale (VRS)

Verbal rating scale (VRS) is another unidimensional tool for assessment of pruritus that assists the patient to verbally describe the degree of pruritus. This is possibly the most convenient method for assessment of pruritus; with four-point scale “none, mild, moderate and severe pruritus”[94] and five-item scale “none, mild, moderate, severe and very severe pruritus”[95] are used for assessment of pruritus. The variability in different versions of VRS point scale contributes toward a major limitation of this scale, thus making the comparison of these results very difficult[96]. However, VRS has the advantage as one of the most suitable tool for assessment of CKD-aP in certain populations such as elderly or patients with cognitive problems[96].

Eppendorf itch questionnaire (EIQ)

The Eppendorf itch questionnaire was developed by Darsow *et al.* (2001)[97], for the exact characteristic of pruritus using a comprehensive list of sensory and affective descriptors, and also collects evidence on the impact of pruritus on quality of life[97].

Dermatology quality of life questionnaire (DLQI)

The Dermatology Life Quality Index, the first dermatology was by Finlay and Khan (1994) [98], and contains 10 questions concerning “symptoms and feelings, daily activities, leisure, work, and school, personal relationships and treatment” over previous one week. The responses for this tool are ranged from 0 to 3 ‘‘not at all’’, ‘‘a little’’, ‘‘a lot’’ or ‘‘very much’’ respectively. Each response is scored from 0 to 3 and then summed up, score of 0 indicates no impairment of life quality while score of 30 indicates maximum impairment[98].

5D-itch scale (5D-IS)

A 5D-itch scale is a multidimensional tool, that comprises of five domains, addressing the “duration, degree, direction, disability, and distribution of itching”[87]. The duration, degree and direction domains each included one item, while the disability domain had four items. All items of the first four domains were measured on a five-point Likert scale (where 1 represented the lowest degree of pruritus, and 5 represented the highest degree). The 4th domain (disability) measured the effect of itching on daily activities, and its score was calculated by selecting the highest score. In the 5th domain, participants were asked to select which part of the body was most affected by CKD-aP, and participants could select as many body parts as they wished. If two body parts were affected, the score given was 1; 3–5 body parts affected was scored as 2, 6–10 body parts were scored as 3, 11–13 body parts were scored as 4, and 14–16 body parts were scored as 5. The overall score of the 5D-IS was calculated with all the five domains; 5 indicates no pruritus; whilst a score of 25 indicates severe pruritus[87].

Modified pruritus questionnaire for itch severity score

The Itch severity scale developed by[81] for evaluation of pruritus was modified by[88], as the questionnaire by Yosipovitch *et al.* (2001)[81] has no method of scoring for quantification of symptom severity. The modified questionnaire by Majeski *et al.* (2007)[88] consists of 7 components: “frequency, itch description, affected body surface area, intensity, the effect on mood, an effect on sexual desire/function and effect on sleep”. The responses to a different component of the questionnaire were separately summed and divided by a maximum score for the respective question. All the seven values achieved were then added and multiplied by 3 to get a total out of 21 scores. The yielding total scores ranging from 0 to 21[88].

Skindex questionnaire

Skindex is among the best dermatological instruments for the measurement of dermatological specific health related quality of life (QOL)[89]. Skindex was originally comprised of 61 questions that were later on divided into two brief versions i.e. Skindex 16[91] and Skindex 29[90]. The Skindex-29 is instruments of choice in dermatology[99], it is comprised of 7 items addressing the symptoms domain, 10 items for the emotional domain, and 12 items for the functioning domain. The responses of all domains were transformed to a linear scale of 100, varying from 0 (no effect) to 100 (effect experienced all the time)[100]. Whereas Skindex-16 is a single page questionnaire, that is an accurate and sensitive measurement for patients experience and widely used for skin-related quality of life[101,102]. The Skindex-16 comprised of 4 items related to symptoms, 7 items related to emotions and 5 items related to functioning scales and linear scale of 100, varying from 0 (no effect) to 100 (effect experienced all the time)[91].

CONCLUSION

Researchers reported the prevalence of CKD-aP was slightly higher in Pakistani patients when compared to Malaysian patients. Prevalence studies in Pakistan reported rates of 64.0% to 77.7%[30,103,104] while Malaysia studies reported rates of 58.6% to 64.2%[27,105]. The pruritus median score was also significantly higher in Pakistani 10.0 [8.0–12.0] compared to Malaysian patients 8.0 [6.0-9.0]; p=<0.001. The possible reasons for the variation in the prevalence of CKD-associated pruritus between Malaysian and Pakistani because patients in Pakistan receive haemodialysis twice/week, whereas in Malaysia, it is three times/week. Moreover, low to medium flux dialyzers were used in Pakistan, whilst high flux dialyzer (which removes average-sized molecules more effectively — thus reducing the severity of uremic pruritus) was used in Malaysia. CKD-aP is believed to be caused by middle-molecule uremic toxins which are not dialyzed properly when using low flux dialyzer[34]. Globally the high-flux haemodialysis is the most commonly used blood purification method. But in developing countries such as Pakistan, low-flux dialysis is the main method of extracorporeal blood purification therapy due to insufficient funds to purchase high flux dialyzers[106]. Haemodialysis machines are also limited in Pakistan as in 2004 there were 140 dialysis centers although in 2009 these machines increased to 175[107], alarmingly out of the available dialysis centers in Pakistan, 10–15% are non-functional and the patients have limited access to treatment[107].

The Pakistani patients were significantly younger than Malaysian patients and had a shorter median duration of CKD and being on haemodialysis. This could be due to poorer management of chronic conditions (such as hypertension or diabetes mellitus) which predisposes an individual to renal damage at younger age. Additionally, Pakistani patients may have less knowledge of the complications of their chronic conditions and therefore may not take any preventive measures such as control of hypertension and dietary modifications[108]. Malaysian population was graded higher when their knowledge was tested against diabetes and hypertension[109–112], these results were quite different for the Pakistani population[113–116]. Furthermore, awareness and attitude also have a decisive role in the actions of diabetic and hypertension patients. In Pakistan, many factors may account for poorer outcomes during dialysis; such as malnutrition, late referral, anaemia and lack of qualified nephrologists at dialysis centers[117–120].

In conclusion, this review provided vital insight on the epidemiology of chronic kidney diseases (CKD) in Malaysia and Pakistan, together with the pathophysiology of CKD-associated pruritus and other CKD-associated dermatological disorders. Both countries could improve the awareness of their populations and providing more support to the healthcare setting to increase the quality of life of CKD patients.

Authors Contributions

The literature review and manuscript writing were performed by I-UR and T-MK.

Conflict of interest

The authors declare that there is no conflict of interest in this work.

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Review Article

Progress in Microbes and Molecular Biology

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The Rising of "Modern *Actinobacteria*" Era

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Abstract: The term "Modern *Actinobacteria*" (MOD-ACTINO) was coined by a Malaysian Scientist Dr. Lee Learn-Han, who has great expertise and experience in the field of actinobacteria research. MOD-ACTINO is defined as a group of actinobacteria capable of producing compounds that can be explored for modern applications such as development of new drugs and cosmeceutics. MOD-ACTINO members consist of already identified or novel actinobacteria isolated from special environments: mangrove, desert, lake, hot spring, cave, mountain, Arctic and Antarctic regions. These actinobacteria are valuable sources for various industries which can contribute directly/indirectly towards the improvement in many aspects of our lives.

Keywords: modern; bioactive; actinobacteria; environment; bioprospecting

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INTRODUCTION

The *Actinobacteria* has a long evolutionary history for it has existed on earth around 2.7 billion years ago, anteceding the Great Oxidation event that occurred 2.3 billion years ago[1, 2]. In the *Bacteria* kingdom, ancient *Actinobacteria* is one of the major phyla associated with the early colonization of land and they play important roles in assisting Earth's ecosystems function[2]. As one of the most primitive lineages among prokaryotes, actinobacteria have extraordinary diversity of morphology and function[3,4]. This phylum consists of free-living Gram-positive bacteria with a variety of morphological features including coccus, rod, and complex fragmenting hyphal that develops into branched mycelium[3,5]. These bacteria can be found predominantly in terrestrial soil and marine ecosystems[6]. Actinobacteria have significant functions, for instances, they are important agents of global carbon and nitrogen cycles; agents of bioremediation; probiotics in humans and animals; pathogens of humans, animals and plants; producers of enzymes and clinically important metabolites[1,3,7].

Following the pioneering research led by Professor Waksman, the '52 Nobel laureate who revealed streptomycin antibiotic from *Streptomyces griseus*, actinobacteria have since become the "star" in the scientific community[8,9]. Essentially, the investigation of novel *Actinobacteria* (genera or species) and bioprospecting of active isolates have intensified around the world, often through random large-scale sampling of environment, selective isolation and subsequently bioactivity screening of isolates[6]. This resulted in the discovery and screening of over thousands of species of actinobacteria. Historically, the actinobacteria were documented as a controversial kind of microorganisms due to their diverse and unique appearances, for which, several of them resemble the appearance of fungi[10]. The taxonomy of phylum *Actinobacteria* has been revised over time and the recent roadmap has been proposed with 6 major classes in the phylum, namely: *Actinobacteria*, *Acidimicrobiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria*, and *Thermoleophilia*. Class *Actinobacteria* is the largest among others as it consists of 15 orders: *Actinomycetales*, *Actinopolysporales*, *Bifidobacteriales*, *Catenulispo-*

rales, *Corynebacteriales*, *Glycomycetales*, *Jiangellales*, *Kineosporiales*, *Micrococcales*, *Micromonosporales*, *Propionibacteriales*, *Pseudonocardiales*, *Streptomycetales*, *Streptosporangiales*, and *Frankiales*[11,12]. The genus *Streptomyces* (order: *Streptomycetales*, family: *Streptomycetaceae*) is the most famous actinobacteria as they have been greatly studied due to their tremendous bioactive potentials[7].

THE ERA OF MODERN *Actinobacteria* (MOD-ACTINO)

Actinobacteria have been distinguished for their prolific production of antibiotics. From the 1950s to 1970s, approximately 60% of new antibiotics were predominantly isolated from streptomycetes[13]. Eventually, researchers have further exposed the presence of actinobacteria in special and extreme environments with the increasing efforts to discover new metabolites from various microbial sources. This essentially leads to a significant paradigm shift in the exploration of *Actinobacteria*, such instances include the isolation of actinobacteria from underexplored unique habitats and the investigation of their secondary metabolites with different activities other than antimicrobials (e.g. antioxidant, anticancer)[14]. Furthermore, the non-*Streptomyces* genera (e.g. *Sinomonas*, *Microbacterium*, *Nocardia*) which referred as the "rare *Actinobacteria*" have shown growing importance as valuable sources in discovery of novel bioactive secondary metabolites[15]. Malaysia Research Star Award winner, Dr. Lee Learn Han — who has great expertise and experience in the field of actinobacteria research, coined the term "Modern *Actinobacteria*" (MOD-ACTINO) to define actinobacteria with modern applications (Figure 1). In this context, the term refers to actinobacteria that synthesize natural products with new interesting bioactivities in recent years, for examples, drug leads with anti-viral (HIV), anti-protozoa (malaria), antioxidant, and neuroprotection properties as well as compounds utilized for cosmetic formulation. In addition, this term covers actinobacteria which produce approved drugs and have been subjected to drug repurposing effort. MOD-ACTINO also inclusive of known or novel actinobacteria that have been discovered from special environments.

Figure 1. The ideas of "Modern *Actinobacteria*" (MOD-ACTINO) proposed by Dr. Lee Learn Han.

By the end of 20th century, actinobacterial natural products have been found to exert extensive biological activities comprising antibacterial (against antibiotic resistant strains), antifungal, antiparasitic, immunosuppressant, antioxidant, and anticancer agents[8,16–22], Numerous actinobacterial bioactive compounds are well-known for the treatment of plant, animal, and human diseases. For instances, kasugamycin is a marketed antifungal antibiotic produced from *Streptomyces kasugaensis* which used for the control of rice blast caused by phytopathogenic fungus *Magnaporthe oryzae*[23,24]. Moreover, several chemotherapeutic drugs such as bleomycin (from *Streptomyces verticillus*) and doxorubicin (from *Streptomyces peucetius*) that have been introduced into clinical use are of actinobacterial origin[25–27]. Another remarkable drug discovery event from genus *Streptomyces* is achieved by Professor William C. Campbell and Professor Satoshi Omura through the isolation of a new "miracle" drug avermectin from *Streptomyces avermitilis* (renamed as *Streptomyces avermectinius*)[28]. Avermectin was later being refined into the safest and most potent derivative known as ivermectin. Ivermectin is an antiparasitic drug effective against helminths, arachnids and insects. It was marketed in 1981 for veterinary use around the world and subsequently approved for human use in 1987. Ivermectin is administered for treatment of onchocerciasis and lymphatic filariasis in many parts of the world. This "miracle" drug has revolutionized the treatment of these devastating parasitic dis-

eases, thereby improving the health of millions of individuals. Resultantly, the 2015 Nobel Prize in Physiology or Medicine was awarded (with one half jointly) to Professor William C. Campbell and Professor Satoshi Omura[28,29].

Research on actinobacteria is still ongoing as they never cease to amaze us with their vast potential of bioactive secondary metabolite production. Studies conducted nowadays, towards the 21st century, have gradually revealed the immense ability of actinobacteria in producing compounds with new captivating bioactivities far more than expected. This is witnessed through findings of compounds with *in vitro* anti-human immunodeficiency virus (HIV) activity produced by actinobacteria[30–32]. One of the earliest research studies on this was reported by Chokekijchai *et al*. (1995)[33], for which a new anti-HIV polypeptide was obtained from a *Streptomyces* sp. isolated from soil sample collected in Japan. Besides, a recent study conducted by Ding *et al*. (2010)[34] had successfully isolated a novel pentacyclic indolosesquiterpene — xiamycin produced by mangrove-derived *Streptomyces* sp. GT2002/1503 which is active against HIV. Apart from anti-HIV activity, a number of actinobacteria were documented to produce compounds (e.g. borrelidin, metacycloprodigiosin, bafilomycin A_1) with promising activity against human malaria parasite (*Plasmodium falciparum*)[35–37]. Furthermore, studies also reported the production of neuroprotective substances by actinobacteria that may be potential medicines for brain ischemia and other neurodegenerative diseases such as multiple sclerosis, Parkinson's diseases, and Alzheimer's disease[38,39]. As an example, Hayakawa *et al*. (2013)[40] revealed a new neuroprotective compound isolated from *Streptomyces* sp. RAI20 - indanostatin, which is also the first reported 1,3-indanone from bacteria. The compound was found to partially protect C6 glioma cells (derived from rat neural tumors induced by N-nitrosomethylurea) against glutamate toxicity which could be useful as treatment for cerebral ischemic disorders.

Likewise, the possibility of incorporating actinobacterial bioactive metabolites in modern skin care cosmetics has further uplift the value of MOD-ACTINO. The human skin is the largest organ of our integumentary system which could face esthetic issues such as freckles, acne, and aging. Dahal *et al*. (2016)[41] proposed the addition of actinobacterial derived resources into cosmetics products for beneficial effects which could enhance the appearance of human skin such as anti-acne, anti-aging, skin whitening, and antioxidant effects. In the study, 12 strains of actinobacteria belonging to the genera *Streptomyces*, *Actinokineospora*, and *Calidifontibacter* exhibited antibacterial activity against skin pathogens *Staphylococcus epidermidis* and *Propionibacterium acnes*. The crude supernatant of these actinobacteria also demonstrated promising tyrosinase inhibition, elastase inhibition, and antioxidant activities. Another research conducted by Tan *et al*. (2019)[42] had reported the isolation of a mangrove *Streptomyces* sp. MUM273b which possessed antioxidant and UVB protective properties. Hence, actinobacterial derived resources can be added to cosmetics applications to improve skin conditions by providing skin whitening effects, acne vulgaris treatment, anti-aging effects, anti-oxidant effects, and anti-UV properties.

Interestingly, there is an increasing number of studies that support the concept of using actinobacteria as probiotics in animal feed especially for aquaculture[43]. Probiotics in aquaculture are expected to confer health benefits to the host such as growth enhancement, improvement in nutrient digestion and immune response, also, to assist in prevention of bacterial infection through production of inhibitory compounds[43,44]. A few number of studies have suggested the utilization of actinobacteria as potential probiotic strains against shrimp and fish pathogenic *Vibrio* spp.[45–49]. Meanwhile, the members of *Streptomyces* and *Bacillus* are also compelling probiotic strains as they have been shown to be capable of promoting growth and increasing resistance against bacterial infections in fishes and shrimps[50–52]. Most studies recommended the genus *Streptomyces* as the most potent actinobacteria probiotic for aquaculture mainly due to their ability to produce a multitude of extracellular enzymes and antibiotics, and to form heat- and desiccation-resistant spores[44,50]. Therefore, these MOD-ACTINO will be a great asset to the biopharmaceutical, agriculture, aquaculture, and cosmetic industries.

Aside from the exploration of actinobacteria-derived compounds for development of novel drugs, research also emphasizes on the investigation of drug repurposing. Drug repurposing (drug repositioning/reprofiling/retasking) is defined as an approach to search for new applications of approved or investigational drugs that are beyond the scope of the original medical indication[53]. Previously approved actinobacteria-derived drugs such as rapamycin (sirolimus; produced by *Streptomyces hygroscopicus*) was initially known as an antifungal agent[54]. Rapamycin was approved as an immunosuppressant for the prevention of allograft rejection in 1999 due to its strong suppression of interleukin-2 (IL-2)-stimulated T cell proloferation[55]. It is a macrolide and an allosteric inhibitor of mammalian target of rapamycin (mTOR)[55,56]. The mTOR is a serine/threonine protein kinase and it is often upregulated in different types of cancers. As a result, researchers are determined to examine its anticancer potentials. Rapamycin has been verified to be a potent immunosuppressant and a promising anticancer/antitumor agent that can be used as a single agent or in drug combination[57–59]. Thus, this demonstrated one of the criteria of MOD-ACTINO where the actinobacterial compounds exhibited different bioactivities from their originally identified bioactivity.

PRESENCE OF MOD-ACTINO IN SPECIAL ENVIRONMENTS

Actinobacteria are sporulating organisms that possessed astonishing capability to generate extraordinary properties[60–62]. This is often associated with their complex morphological changes in their multicellular life cycle and their large genome size as observed particularly in streptomycetes[3,11,63]. The complexity of these organisms has enabled them to thrive in extreme and special environments[15] such as the Arctic and Antarctic regions[64,65], mountain plantations[66], glaciers[67], caves[68], deserts[69], hot springs[70], and mangroves[71–75]. These environments are special in terms of physical parameters (e.g. unusually high/low temperature,

radiation, pressure) or chemical conditions (e.g. acidic/alkaline pH, high salinity, low levels of nutrients and moisture)[76,77]. The actinobacteria evolved by developing unique defense mechanism that enables them to survive under hostile and extreme conditions. Consequently, actinobacteria from special and extreme environments may be thermotolerant, acidtolerant, alkalitolerant, psychrotolerant, halotolerant, haloalkalitolerant or xerophilous[76].

In addition, several novel genera/species have been discovered from these special environments. For instances, *Mumia flava* gen. nov., sp. nov. (family *Nocardioidaceae*)[78], *Barrientosiimonas humi* gen. nov., sp. nov. (family *Dermacoccaceae*)[79], and *Monashia flava* gen. nov., sp. nov. (family *Intrasporangiaceae*)[80] were each novel species of a new genus isolated from mangroves in Malaysia; *Actinocrinis puniceicyclus* gen. nov., sp. nov. (family *Actinospicaceae*)[81] isolated from acidic spring; and *Desertiactinospora gelatinilytica* gen. nov., sp. nov. (family *Streptosporangiaceae*) isolated from desert[82]. Besides, other novel species of rare actinobacteria were also identified such as *Microbacterium mangrovi* sp. nov.[83] and *Sinomonas humi* sp. nov.[84] from mangroves; *Rhodococcus kroppenstedtii* sp. nov.[85] and *Micromonospora acroterricola* sp. nov.[86] from desert; and *Nonomuraea monospora* sp. nov.[87] from cave soil. In fact, recent studies also uncovered many novel bioactive actinobacteria which originated from these unique niches. There are multiple novel *Streptomyces* strains recovered from mangrove environments with useful bioactivities, for examples, *Streptomyces colonosanans* sp. nov. (antioxidant and anticancer)[88], *Streptomyces monashensis* (antioxidant and anticancer)[27,89], *Streptomyces mangrovisoli* sp. nov. (antioxidant)[90], *Streptomyces pluripotens* sp. nov. (antibacterial)[91], and *Streptomyces malaysiense* sp. nov. (antioxidant and anticancer)[92]. Many compounds produced by MOD-ACTINO exhibit important properties which can be developed into new drugs/drug leads with higher efficacy in the near future.

HARNESSING THE POTENTIALS OF MOD-ACTINO AND CONCLUSIONS

With the growing importance of actinobacteria in various fields, the advancement in molecular biology especially in this post-genomic era can assist us to reach a higher level of understanding of these organisms by studying their genome. The availability of next generation sequencing (NGS) technologies and the -omics methods (metagenomics, metaproteomics) have greatly assisted in overcoming the issue on detection of unculturable bacteria as well as contributed to the research on actinobacteria biosynthetic gene clusters and their secondary metabolites production[93]. Lately, there is an increase in the number of new genome sequences of actinobacteria which have been made available to the public. Majority of them were resulted from projects aimed to understand the connection of secondary metabolites productions or to evaluate new actinobacterial natural products to their biosynthetic pathways via genome mining[94]. In particular, the bioactive actinobacteria strains have been subjected to whole genome sequencing to further appreciate their biological importance in bioactive metabolites or enzyme production[95–104]. It is anticipated that the accessibility to large sets of actinobacterial genome sequences will provide us a more thorough understanding of actinobacteria phylogeny and facilitate in the identification of medically useful new natural products[105]. Members of MOD-ACTINO are valuable sources for various industries which can contribute directly/indirectly towards the improvement in many aspects of our lives. MOD-ACTINO will be the "key" microorganisms to further improve human health and wellbeing in the modern society.

Authors Contributions

The research and manuscript writing were performed by JW-FL, VL and L-HL. LT-HT, H-LS and B-HG provided vital guidance of the research and proof of the writing. The research project was founded by JW-FL and L-HL.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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Review Article

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HH PUBLISHER

Effect of chronic kidney diseases-associated pruritus on patients' sleep quality, well-being and its management

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Abstract: Chronic kidney diseases-associated pruritus **(**CKD-aP) affects the patients' mental and physical health, potentially resulting in fatigue, depression, and directly affecting quality of sleep. Hemodialysis patients were reported to experiencing moderate to extreme CKD-aP, thus exhibited higher possibilities of remaining awake at night while sleeping in the day. Therefore, CKD-aP is attributed toward nocturnal awakenings and difficulty falling asleep. This condition (CKD-aP) significantly impacts the quality of life (QOL), triggering sleep disturbance, mood changes, and uncontrollable scratching. CKD-aP patients have a compromised QOL that is generally linked to limited personal freedom and control due to lengthy treatment time. Overall, the loss of freedom has wider implications, such as altering marital, family, and social relationships. Thus, this writing highlights the vital effect of chronic kidney diseases-associated pruritus on patients' sleep quality, social and mental well-being and providing comprehensive management and treatment options to improve patients' quality of life.

Keywords: chronic kidney diseases-associated pruritus; Malaysia; Pakistan; sleep quality; well-being; management

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INTRODUCTION

Chronic kidney diseases-associated pruritus **(**CKD-aP) influences the patients' mental and physical capacity, resulting in fatigue, depression, and quality of sleep[1–6]. Hemodialysis patients, experiencing moderate to extreme CKD-aP, exhibit higher chances of being awake at night while sleeping in the day. Hence, CKD-aP is attributed toward nocturnal awakenings and difficulty to sleep[2,3,7,8]. Pruritus is an undesirable disorder that stimulates itching and could negatively affect sleep quality and affecting the quality of life[9]. CKD-aP significantly influences patients' quality of life, causing sleep disturbance, mood changes, and uncontrollable scratching[10]. CKD-aP could cause serious problems such as discomfort, anxiety, depression, sleep disorders, and an overall negative effect on one's physical and mental health. About 42% of chronic kidney disease patients on dialysis experienced CKD-aP with intensity from moderate to severe, and also correlated with other health-related complications such as poor sleeping quality and poor quality of life[1]. Sleep disorders account for chronic fatigue which is connected with disturbed day and night rhythm, causing a negative impact on physical and mental ability[11]. Furthermore, CKD-aP is associated to higher risk of mortality in dialysis patients[10].

Chronic inflammatory skin diseases such as pruritus, psoriasis, and atopic eczema have a considerable impact on CKD patients QOL, including psychological health, physical well-being, family relationships and social development[12]. Among all the psychological complications, depression is common and has a serious impact on the quality of life of CKD patient and their caregivers.

It has a negative impact on social, economic, and psychological well-being[13]. CKD-aP has a substantial effect on patients QOL as it may cause serious discomfort, depression, anxiety[11] Depression is more frequently seen in CKD patients mainly between the 3rd to 9th years of treatment, with mostly female patients being affected. Depression is exhibited mainly in the form of sadness, anxiety, depressed mood, poor self-esteem, pessimism about the future, decreased libido, sleeps disorders, and reduced appetite[14].

Quality of life of patients having CKD is adversely affected by rising intensity of CKD-aP; and is correlated with higher mortality risk[15]. CKD-aP patients have a compromised QOL that is mostly linked to limited personal freedom and control due to lengthy treatment time. Generally, the loss of freedom has wider implications, altering marital, family, and social relationships[16]. CKD-aP has a negative impact on the social well-being of female patients on hemodialysis as compared to male patients[17]. A strong association exist between QOL score and CKD-aP intensity; therefore treating CKD-aP may improve the QOL of CKD patients[15]. CKD-aP should be frequently assessed and effectively managed to reduce associated morbidity, mortality and to improve the overall quality of life.

GUIDELINES FOR THE MANAGEMENT OF CKD-AP

CKD-aP is graded as one of the most common dermatological complications among patients on hemodialysis. Due to the refractory nature of CKD-aP and its unknown pathophysiology, there is no definitive cure for its management. Even though a wide range of therapeutic agents has been utilized for its management, however no therapy has been established for proper management of CKD-aP. Based on the proposed hypothesis for the pathogenesis of CKD-aP, different treatment options shall be discussed in this writing.

Alteration in hemodialysis techniques

Studies have suggested the role of optimum dialysis rates along with the use of dialyzer membranes can play a role in CKD-aP. The increasing dose of dialysis may improve CKD-aP[18–20]. Shaldon (1993)[21] suggested that short dialysis sessions and underdialysis could lead to patient malnutrition and death. Indeed, dialysis time of fewer than three hours and 30 minutes has been associated with a doubled rate of patient mortality as compared to patients dialyzed for four hours and being dialyzed thrice weekly. Likewise, among dialysis patients, clearance was strongly correlated with an increased duration time of dialysis[22]. High-flux hemodialysis is one the most frequent blood purification method used worldwide, but in developing countries, low-flux dialysis is the main method of extracorporeal blood purification therapy due to poor economic conditions. This method is not effective in removing the middle-molecule uremic toxins that contributes toward CKD-aP[23]. Ko *et al.* (2013)[24] also supports the notion that use of low-flux dialyzer has significant association with the aggravation of CKD-aP. As the high flux dialyzers efficiently remove average-sized molecules[25]. The occurrence of CKD-aP can be reduced by use of high flux hemodialysis, as it significantly contributes toward better improvement in patients' CKD-aP intensity[26,27]. Chen *et al.* (2009)[28] reported the use of high permeability hemodialysis (ultrafiltrate coefficient, 40 mL/h/mm Hg) in having significant improvement in CKD-aP with high-permeability as compared to conventional hemodialysis (ultrafiltrate coefficient, 5.5 mL/h/mm Hg). The use of hemodiafiltration with hemoperfusion is also effective in relieving CKD-aP[29]. The intensity of CKD-aP is also reduced by the use of biocompatible dialysis membrane (polymethylmethacrylate [PMMA])[30,31].

Local pharmacological therapies (Topical treatments)

Emollients and topical analgesic agents

Emollients such as high-water-content emollient[32,33], glycerol and paraffin[34] are the favored topical treatment of CKD-aP if xerosis (dry skin) is present. Aqueous gels with higher water content (containing 80g of water and 20g of aloe vera extract, squalane, naturally-derived vitamin E, silk powder with no artificial and synthetic substances) can help to relief discomfort of CKD-aP[33]. Topical analgesic agents are also useful in the treatment of CKD-aP such as Pramoxine HCl 1% lotion is reported to be useful in relief of CKD-aP[35]. Multiple studies showed that Topical Capsaicin 0.025% cream were effective for localized CKD-aP[36–38]. Suzuki *et al.* (2015)[39] stated that capsaicin act by desensitization of nociceptive nerve endings depletion of substance P causing blocking of the conductor of pruritus.

Tacrolimus ointment

The effects of Tacrolimus ointment in relieving CKD-aP is uncertain. With some studies indicating that it is effective in relieving CKD-aP[40,41], nevertheless, in a randomized control trial, Tacrolimus 0.1% ointment showed no effect of among patients on hemodialysis over control group[42].

Topical cromolyn sodium

The use of topical cromolyn sodium 4% was reported as more effective in decreasing CKD-aP as compared to placebo[43].

Gamma linolenic acid (GLA) enriched Cream

Chen *et al.* (2006)[44] reported that Gamma linolenic acid enriched cream contributes significantly improvement in CKD-aP severity.

Sarna and Eurax Lotions

Both Sarna lotion (0.5% of each camphor, menthol, and phenol) and Eurax lotion (10% crotamiton) has been reported to be effective in improving CKD-aP[45].

Systemic therapies

Although local pharmacological therapies are effective for the management and treatment of localized CKD-aP, yet for the management of generalized CKD-aP, systemic

therapies are used and shall be discussed here:

Oral histamines

Antihistamines are a widely used to relieve itch. They are classified into 2 categories: "histamine receptor antagonists such as hydroxyzine, diphenhydramine, loratadine, or cetirizine and medications that prevent the release of histamine like the mast cell stabilizers cromolyn sodium and ketotifen"[46].

Researchers reported that the used of histamine receptor antagonist for the management of pruritus and antipruritic activity have been generally unsuccessful[47–49]. Furthermore oral antihistamnes cannot be recommended as first line option for treatment of pruritus due to dangerous side effect[46]. While mast cell stabilizers are reported to be effective in the management of pruritus. It is stated that CKD-aP severity is reduced by using Ketotifen therapy[50,51], Cromolyn sodium[52,53], Zinc sulfate[54,55] and Nicotinamide[56].

Gabapentin and pregabalin

The use of neuroleptic agents such as gabapentin and pregabalin to manage CKD-aP has increased. But patients should be monitored closely for potential side effects from these agents.

Many studies reported favorable effects of gabapentin in treating CKD-aP. Studies showed that intervention with Gabapentin 100mg[51,57]; gabapentin 300mg[58–60] and gabapentin 400mg[61] could significantly improve CKD-aP intensity. Kobrin (2017)[62] stated that Gabapentin 100mg is the preferred initiating dose after each dialysis session, and the dose may be gradually increased to 350mg daily. However, a dose greater than 350mg daily are not recommended in dialysis patients.

Pregabalin could be used in patients who are unable to tolerate Gabapentin[63]. Pregabalin 25mg daily is the preferred initiating dose and can be gradually increased to 75mg daily. Dose greater than 75mg daily are not recommended in dialysis patients[64]. While Pregabalin 50mg[65] and Pregabalin 75mg[66] were reported to improve CKD-aP intensity significantly.

Opioid imbalance treatment

The overstimulation of central mu-opioid receptors or antagonism of kappa-opioid receptors is a contributing factor in CKD-aP. Therapies treating the opioid imbalance are employed to improve CKD-aP among patients.

Studies showed that Mu-opioid receptor antagonists such as Naltrexone 50mg were effective in the relief of pruritus[67]. But a study by Pauli-Magnus *et al.* (2000)[68] indicated no effect of Naltrexone in the relief of pruritus [68]. Kappa opioid receptor agonists indicated good result in CKD-aP patients on hemodialysis, and the widely used kappa opioid receptor agonist is Nalfurafine[69]. Nalfurafine 2.5μg[70] and Nalfurafine 5μg[69,70] displayed effectiveness in management of CKD-aP. Furthermore Nalbuphine hydrochloride 60 mg and 120mg extended-release tablet (mu-opioid receptor antagonist and kappa opioid receptor agonist) were reported as effective in the management of CKD-aP[71,72].

Other Systemic Treatments

Thalidomide 100mg[73], Montelukast 10mg[74], Cholestyramine 5gm[75], sertraline (selective serotonin reuptake inhibitor)[76–78] were reported as effective in the management and reduction of CKD-aP.

PHOTOTHERAPY

Studies were conducted and indicated potential effects of phototherapy on CKD-aP. The narrowband ultraviolet B phototherapy[79,80] was reported to be effective in the management of CKD-aP. However, study by Ko *et al.* (2011) [81] indicated that narrowband ultraviolet B phototherapy showed no significant improvement in CKD-aP. Nevertheless the potential carcinogenic effect of ultraviolet radiation requires serious consideration[82]. While Hsu *et al.* (2009)[83] reported that thermal therapy with far-infrared rays could effectively improving CKD-aP intensity.

ALTERNATIVE TREATMENT

Alternative therapies such as acupressure, acupuncture and homeopathic verum medication were used for treatment and management of CKD-aP. Acupressure therapy at LI-L11 point[84] and auricular acupressure[85] were stated to be effective in the management of CKD-aP. Acupuncture therapy which block spinal cord release of opioid-like substances, if applied at Quchi (LI11) acupoint is an easy, safe and effective ways in relieving CKD-aP[86]. A systematic review on acupuncture for treatment of CKD-aP in end-stage renal disease patients reported the beneficial effect of acupuncture intervention but also reported the high risk of bias[87].

ASSESSMENT OF SLEEP QUALITY AND QUALITY OF LIFE

Several validated and self-designed questionnaires were used to assess the sleep quality and quality of life among patients having CKD-aP undergoing dialysis.

Validated questionnaires for sleep assessment

The Pittsburgh sleep quality index (PSQI)

Pittsburgh sleep quality index (PSQI) is one of the most commonly used questionnaires for assessment of sleep quality among CKD-aP patients[88–91]. It assess the self-rated sleep quality over the past one month. This questionnaire consists of "19 items and seven domains: subjective sleep quality, sleep duration, sleep latency, sleep disturbances, habitual sleep efficiency, use of sleep medication, and daytime dysfunction", and responses were rated on a 4-point Likert scale[92,93]. The overall score was calculated by totalling the scores of the seven domains (range: 0 to 21)[94]. PSQI score of 5 and ≥ 5 were classified as bad sleepers and PSQI < 5 classified were as good sleepers[94].

Epworth Sleepiness Scale

The Epworth Sleepiness Scale (ESS) is a simple and inexpensive measure for evaluation of daytime sleepiness, it is a questionnaire comprised of 8 items. The questionnaire rates

the responses of sleepiness in 8 daily situations ranging from 0 to 3, giving a total score of 0 (no daytime sleepiness) to 24 (the most excessive daytime sleepiness). The score equal to or greater than 10 is the cutoff point for excessive daytime[95,96].

Sleep and Health Questionnaire

The Sleep and Health Questionnaire (SHQ) comprised of 16 questions that were grouped into 5 factors "self-reported breathing disturbances, functional impact of sleepiness, roommate-observed breathing disturbances, driving impairment, and insomnia"[97]. Most of the responses to the questionnaire utilized either a 5-point frequency scale "never", "rarely", "sometimes", "frequently" and "always" ; or by the use of a 6-point Likert scale which graded the severity of the symptoms "1–2 points (not affected); 3–4 points (mild); 5 points (moderate); 6 points (severe)"[97].

Itch Medical outcome study (Itch MOS)

The Itch Medical outcome study (Itch MOS) was developed from the Medical Outcomes Study sleep questionnaire[98]. The itch MOS instrument contained 10 questions assessing the effect of itch on sleep disruption, sleep latency and daytime somnolence[46].

Validated questionnaires for quality of life and sleep assessment combine

Kidney Disease Quality of Life Short Form (KDQOL)

Kidney Disease Quality of Life Short Form (KDQOLSF) is one most valid and reliable questionnaire for assessment of the QOL of CKD patients. It encompassed 3 domains: "Kidney disease component score (KDCS) comprising of effect of kidney disease, symptoms, work status, burden of kidney disease, sleep, cognitive function, sexual function, social support, quality of social interaction, patient satisfaction and dialysis staff encouragement". The Physical Component Score (PCS) included "physical functioning, role functioning, general health perceptions and pain", while Mental Component Score (MCS) consist of "energy/fatigue, social function, role emotional and emotional well-being"[99].

Short-Form Health Survey (SF-12 and SF-36)

The Short-Form Health Survey (SF-12) is one of the most widely used tools for assessing health-related quality of life, it is originally developed from the Medical Outcomes Study (MOS) 36-item Short-Form Health Survey SF-36[100]. The SF-12 is a health-related quality of life questionnaire containing 12 questions measuring 8 health domains to assess physical and mental health. "Physical health-related domains include General Health (GH), Physical Functioning (PF), Body Pain (BP) and Role Physical (RP). Mental health-related scales include Vitality (VT), Social Functioning (SF), Role Emotional (RE), and Mental Health (MH)"[101].

WHO-Quality of life BREF (WHOQOL-BREF)

WHOQOL- BREF is a 26-item instrument with 4 domains: "psychological health (6 items), physical health (7 items), social relationships (3 items) and environmental health (8 items). It also encompasses QOL and general health items. Each individual response is scored from 1 to 5 and then transformed linearly to a 0–100-scale"[102].

MANAGEMENT OF SLEEP DISTURBANCE AMONG CKD-AP PATIENTS

As sleep disturbance in CKD patients was mainly caused by CKD-aP, therefore the primary objective is mainly to treat the CKD-aP and eventually improves sleep quality. However, due to the refractory nature of CKD-aP, no absolute treatment is available for its management. So far, no reports on improving sleep quality using pharmacological or non-pharmacological treatment among CKD-aP patients. Nevertheless, for dialysis patients the therapeutic treatment options for sleep disturbance is available, these options include pharmacotherapy with hypnotic agents[103], pharmacotherapy with wide range of the rapeutic agents for treatment and relief of CKD-aP[104] and improvement of sleep; cognitive behavioral therapy[105] e.g., relaxation[106] and sleep hygiene[107]. Non-benzodiazepine hypnotics are considered to be alternative hypnotic agents in dialysis centers due to no physical dependence, good effects, no active metabolites and no or least adverse effects of inducing sleep apnea[108–110]. For non-pharmacological interventions, acupressure is applied at specific meridians or acupoints in Traditional Chinese Medicine to improve sleep quality[111–114]. Unlike pharmacological and other interventions, acupressure is a non-invasive therapy that has low risk of side effect profile[115].

CONCLUSION

In conclusion, acupressure and zolpidem tablets were able to improve sleep quality among CKD-aP patients on hemodialysis. With an overall improvement in sleep quality among CKD-aP patients observed in both control and intervention group. Healthcare practitioners should consider acupressure therapy as an alternate method to improve the quality of sleep among CKD-aP patients on hemodialysis. However, more studies are needed to establish suitable data using specific tools to determine CKD-aP and sleep quality for quantitative analysis. CKD-aP should be treated with equal importance as other complications to improve patient's quality of life, and to avoid secondary infections due to persistent scratching for relieve of the itch.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

Authors Contributions

The literature review and manuscript writing were performed by I-UR and T-MK.

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Review Article

Progress in Microbes and Molecular Biology

HH PUBLISHER

Novel coronavirus 2019-nCoV: Could this virus become a possible global pandemic

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Abstract: In December 2019, the emerging of a new coronavirus-induced pneumonia created a distress among people in China and the international community. This virus was identified as novel coronavirus 2019-nCoV. At the time this Review went to press, the virus has spread to across 27 countries, infecting 17,488 people and caused 362 deaths. The transmission of 2019-nCoV to individuals of different countries is predominantly through close contact with an infected person. Based on the available data, there is a rising trend of infected and death cases. At this point, there is no specific drug or vaccine to treat infected patients besides supportive care. Nevertheless, researchers and doctors around the world have reported the success of using existing antiviral drugs to treat patients.

Keywords: Novel Coronavirus, 2019-nCoV, supportive care, global pandemic, treatment

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INTRODUCTION

A new year and a new decade kicked off with a global health threat and challenge, the novel coronavirus 2019-nCoV. News emerged in early December 2019 of pneumonia outbreak by an unknown aetiology, where it is believed to be contracted at Wuhan's Huanan Seafood Wholesale Market[1]. On 31st December 2019, the World Health Organization (WHO)'s Chinese office was notified of pneumonia cluster cases, which led to the closing of the market on 1st January 2020[1]. The Chinese authorities and WHO identified it as a new type of coronavirus, better known as 2019-nCoV[2]. The 2019-nCoV virus belongs to same coronavirus family which includes the Severe Acute Respiratory Syndrome (SARS) — the virus that caused an eventual 8,096 cases and 744 deaths in 2002-2003[3]. The virus became an instant hot news and raised the fear of the international community.

The health organizations along with scientists and clinicians worked around the clock to uncover the information of 2019-nCoV. Within days of detecting the initial cluster of cases, the complete genome sequence of 2019-nCoV with a sequence length of 29,903bp was deposited on GenBank and Global Initiative on Sharing All Influenza Data (GISAID)[4,5]. The genome sequence information is beneficial in the development of primers for surveillance tests and diagnostic kits[3]. Pharmaceutical companies and researchers around the world have been working on different approaches to produce an effective drug or vaccine for 2019-nCoV[6]. These efforts accelerated after over 30 genome sequence of 2019-nCoV have been publicly deposited in GenBank (Table 1)[7].

Table 1. Genome sequence of 2019-nCoV deposited in GenBank.

GenBank Accession	Gene Region	Locality
MT008023	M	Italy, Rome
MT008022	M	Italy, Rome
MT007544	complete	Australia
MN997409	complete	USA
MN996531	complete	Wuhan
MN996530	complete	Wuhan

MN996529	complete	Wuhan
MN996528	complete	Wuhan
MN996527	complete	Wuhan
MN994468	complete	USA
MN994467	complete	USA
MN988713	complete	USA
MN988669	complete	China
MN988668	complete	China
MN985325	complete	USA
MN975268	S	China
MN975267	S	China
MN975266	S	China
MN975265	RdRP	China
MN975264	RdRP	China
MN975263	RdRP	China
MN975262	complete	China
MN970004	RdRP	Thailand
MN970003	RdRP	Thailand
MN938390	S	Shenzhen

RdRP:RNA-dependent RNApolymerase; S: Surface

EPIDEMIOLOGY

2019-nCoV reservoir

Researchers compared the genome sequence of 2019-nCoV with a viral database and suggest it belongs to betacoronavirus originated from bats. However, the specific wildlife host as virus reservoir is yet to be confirmed since various live animals was sold at the market[8,9]. A comprehensive genome sequence analysis was carried out by researchers from Beijing, China to determine the possible animal reservoir. Their evolutionary analysis suggested that snakes are the most likely wildlife animal as virus reservoir for 2019-nCoV[10]. Conversely, scientists affiliated with UK-based universities disputed the findings by Chinese researchers, and affirmed that 2019-nCoV is most closely related to bats[11]. Zhou and colleagues from China also reported this virus is potentially bat origin[12]. Pushing aside on the debate whether bats or snakes are the real culprit of 2019-nCoV, this zoonotic virus has spread to over twenty-seven countries worldwide.

The spreading

Wuhan, the epicenter has been left deserted amid the deadly coronavirus-induced pneumonia outbreak. In aim to contain the virus spread, the local authorities ordered a city-wide lockdown in Wuhan, the sprawling capital of Central China's Hubei province on 23 January 2020[1,13]. Although, early reports revealed limited human to human spread, but the recent drastic increase of infected cases proven the latter assumption was wrong. It is indeed a human-to-human transmission and the virus is spreading in a fast pace to other Chinese cities, Asian countries and other parts of the world[14,15] (Table 2). This fast-spreading virus has claimed 362 lives and infected 17,488 people in 27 countries at the time this Editorial went to press (Figure 1). China tops all the countries with over 17,000 infected patients and 361 deaths (Table 3), this figure increases daily with an estimated transmission rate/basic reproductive number (Ro) 3.0-4.0 (3–4 newly infected cases from 1 case)[16]. On 2nd February 2020, a death of a 44-year-old Chinese man from Wuhan in Philippines marked the first death that occurred outside of China. The patient showed initial signs of improvement, however his condition deteriorated within the last 24 hours and he succumbed[17]. The concern raises as transmission of 2019-nCoV to other countries besides China is mainly by people who have travelled from the epicenter of the outbreak or had close contact with an infected person. For instance, in Malaysia, eight confirmed cases are patients of China nationality, and they have been under isolation hospitalization until fully recover from the infection. Of note, based on the existing epidemiological data, the incubation period of this virus is 14 days.

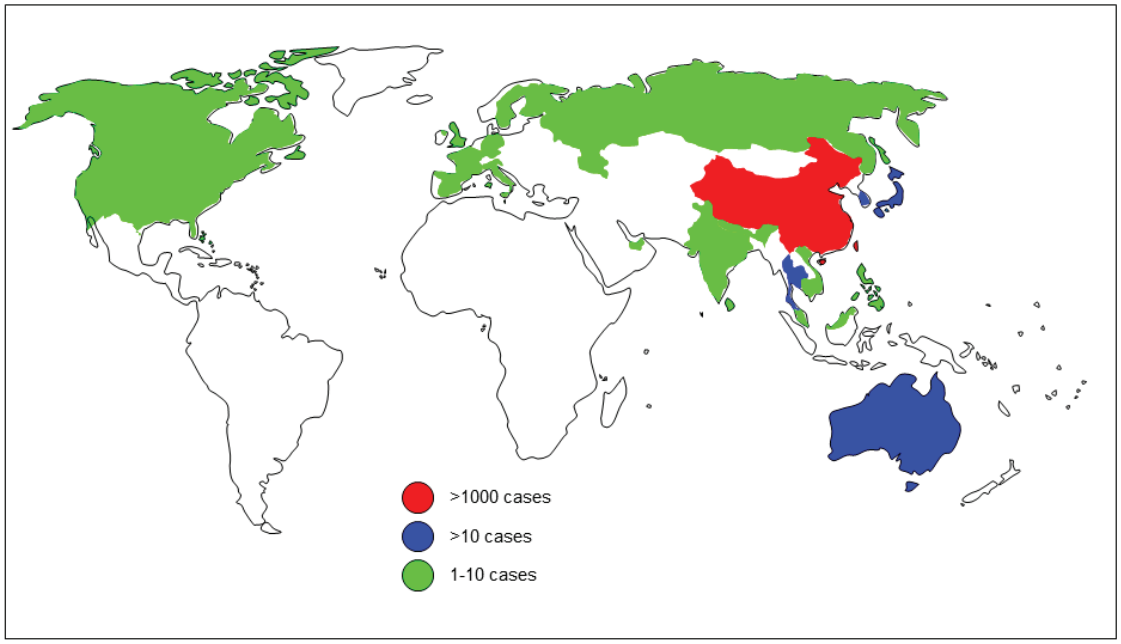

Figure 1. Illustration of novel coronavirus 2019-nCoV transmission across the global. As of 3 February 2020 , this virus has spread to over 27 countries and alarming a fear to the international community.

Table 2. The worldwide spread of coronavirus 2019-nCoV.

Countries	Confirmed Cases	Death	Cured
China	17,302	361	512
Hong Kong	15		
Taiwan	10		
Japan	20		1
Thailand	19		7
Singapore	18		
Korea	15		
Australia	12		2
Germany	10		
Malaysia	8		
US	11		
Vietnam	8		1
France	6		
United Arab Emirates	5		
Canada	4		
India	3		
Italy	2		
Philippines	2	1	
Russia	2		
UK	2		
Cambodia	1		
Finland	1		
Nepal	1		
Spain	1		
Sri Lanka	1		1
Sweden	1		
Macau	8		

Source: National Health Commission of the People's Republic of China and Worldometer on Wuhan coronavirus outbreak - https://www.worldometers.info/coronavirus/.

Table 3. The spread of coronavirus 2019-nCoV in China.

Province	Confirmed Cases	Death	Cured
Hubei	11177	350	295
Zhejiang	724		36
Guangdong	725		14
Henan	566	2	15
Hunan	521		17
Anhui	408		8
Jiangxi	391		18
Chongqing	312	2	8
Jiangsu	271		7
Szechuan	254	1	13
Shandong	259		7
Beijing	212	1	12
Shanghai	203	1	10
Fujian	179		
Shaanxi	128		
Guangxi	127		7
Yunnan	114		5
Hebei	113	1	3
Heilongjiang	118	2	2
Liaoning	74		1
Hainan	70	1	4
Shanxi	66		2
Tianjin	56		1
Ganxu	51		3
Guizhou	46		2
Ningxia	31		
Inner Mongolia	34		1
Jilin	31		1
Xinjiang	24		
Hong Kong	15		
Qinghai	13		
Taiwan	10		
Macau	8		
Tibet	1		

Source: National Health Commission of the People's Republic of China, as of 3rd February 2020

MANAGEMENT MEASURES

Lock-down and medical facilities

The occurrence of this virus has placed the Chinese government in a strong crisis management and preparedness for a pandemic. The government imposed a complete lock-down of Wuhan and about a dozen of other cities, in an attempt to prevent the virus from spreading further within China. The effectiveness of this implementation has been disputed by many experts globally. Professor Chandy John, a former president of America Society of Tropical Medicine and Hygiene mentioned that a number of potential issues may arise including basic human rights concern, inadequate healthcare facilities or medication for the sick people, and people who aren't sick — unable to leave and may fall ill after exposure to the virus. However, he still agrees that prevention of travel is one the effective way to contain the virus and reduce the transmission[18]. Likewise, a bioethicist at New York University also expressed an agreement for Wuhan quarantine and added that it is a prudent action implemented by the government[18].

Since the majority of infected cases are in Wuhan, the Chinese government has invested huge capital in building two new hospitals: Huoshenshan Hospital and Leishenshan Hospital that would be operational in early February 2020[19,20]. In addition, the Chinese government has approved the sale of two virus detection kits and a sequencing system by genomic company BGI Group, to aid and enhance the identification of this novel virus. According to BGI sources, their test kit is able to identify the novel 2019-nCoV within three hours, while the other kit can assist to differentiate and the diagnose infections. One hundred thousand test kits have been dispatched to the country's worst hit regions[21].

Global effort and protection measures

In other parts of the nation and several countries has taken precaution measures to control the spread of coronavirus. Health screening in all entry and exit points (airports, port terminals, train stations) have been beef-up in effort to contain the virus[22]. Passengers who present any clinical symptoms of fever, flu and cough, are placed in an isolation and quarantine for further evaluation and treatments. Many airlines have also drawn-up preventive measures by not providing hot meals, blankets and newspapers in the plane, in aim to contain the virus and reduce the personal contact among passengers[23]. The healthcare organization has been promoting awareness and preventive measures that should be followed by the members of the public. People should adopt good hygiene practice by washing their hands with water and soap, or use hand sanitizer, always wear a mask, avoid crowded public locations, and avoid close contact with any infected person. A person should directly report to their respective healthcare centers if they are presenting any symptoms and other risk factors of 2019-nCoV infection including had a travel history or close contact with a confirmed patient.

The novel coronavirus 2019-nCoV has created an impact to the public healthcare and to our community. In a recent interview, Dr Huang Chaolin, the vice director of Wuhan Jinyintan Hospital, revealed that there might be multiple places where the virus was first originated and transmitted to humans[24]. It is because the first few patients who was admitted at the hospital had no direct exposure to Wuhan's Huanan Seafood Wholesale Market, which was considered the primary source of the epidemic. Judging from the whole situation, there could be multi-sources, nonetheless the authorities have no clue about other sources. The Chinese Center for Disease Control and Prevention have collected environmental samples from Wuhan's Huanan Seafood Wholesale Market, and over thirty samples were found to contain the nucleic acid of the virus. If the virus is from multi-sources, experts warned the situation would become worsen in the coming days if efforts taken to contain the virus are ineffective[24].

Treatment options

Currently, there is no specific drug or vaccine available to treat this virus infection besides supportive care. A patient will present symptoms of fever, fatigue, dry cough, shortness of breath, runny nose and sore throat [25]. Patients with underlying comorbidities for example diabetes, hypertension, chronic lung disease, asthma, cardiovascular diseases, and immunocompromised are prone for severe complication of 2019-nCoV infection [25,26]. Hence, the older group and children are advice to take extra precaution measures to avoid been contract with 2019-nCoV.

In hospitals, doctors are doing their best to treat and save the lives of their patients, without the effective drug or vaccine. Doctors from Thailand successfully treated a 71-year-old patient with a combination of antivirals that used to treat flu (oseltamivir) and HIV (lopinavir and ritonavir) [27]. Where else, in The United States (US), doctors treated a 2019-nCoV positive patient with experimental Gilead Sciences drug - remdesivir. Remdesivir was given to the 35-year-old patient and his symptoms improved without any side effects [28]. Although the combinations of antiviral treatments have shown positive effect in treating 2019-CoV patients, further tests should be done to confirm the effectiveness of these antivirals. Hopefully, this novel coronavirus does not exhibit any resistance phenotype towards flu antivirals e.g. oseltamivir-resistance, which was observed previously in influenza A H7N9 virus [29].

Conclusion

The rapid rising of infected cases and deaths alarm experts, who fear the virus 2019-nCoV would likely become a pandemic across the globe. The lethally of this new virus is yet to be known, but now findings suggest that the virus may be spreading from person to person via the digestive system [30,31]. There is an increasing trend of infected cases in China and around the worldwide countries. Nevertheless, the China government, WHO and respective healthcare bodies are doing their best to understand and manage the coronavirus outbreak. On 30th January 2020, WHO declared the novel 2019-nCoV coronavirus outbreak as a Public Health Emergency of International Concern (PHEIC), and this brings an impact to China and international community

[32,33]. The PHEIC will see the mobilization of international response to work to together to contain the virus threat. Hopefully, this announcement would help with controlling the spread of the virus.

In view of the present virus outbreak, the community should adapt to the new lifestyle changes. Social distancing must be a part of their daily life. Avoid being proximity with people, mass gatherings, and functions. In certain countries, their public has the tendency to greet others by hugging or a handshake. Without realizing, they probably might be spreading or contracting the virus by their actions. Now, we may want to stop this culture for the time being. Another aspect is self-quarantine if one exhibit any symptoms of infection or had a close contact with a confirmed patient. This measure is proven to be an effective tool to contain the spread of any airborne virus.

The nation may feel stressed and unpleasant if a lock-down is imposed in their country or asked to wear mask. Looking back at history, lock-down and wearing mask was seen to be an effective step implemented by Dr Wu Lien-teh in effort to control the spread of pneumonic plague virus in 1910. Dr Wu Lien-teh, a Malaysian born doctor gained fame by ending the pneumonic plague in China and encouraging people at that time to wear gauze-and-cotton masks, controlled the people's movement, instructed to hospitalize all infected patients, carried out disinfection in open areas, mass burials of the deaths, and prohibited close contact [34]. His endless effort managed to contain the plague within seven months (March 1911).

In summary, the novel coronavirus 2019-nCoV outbreak is a reminder to the international community of an emerging virus infection that need constant surveillance, rapid diagnosis, and robust research to understand the biology features of this novel virus. There is a possibility this novel coronavirus would become a global pandemic which the severity and people's susceptibility is yet to be forecasted by the experts. If it becomes a pandemic, the developing countries may not have enough resources to cope this deadly virus. In addition, countries may experience severe setbacks as the world economic may paralyze and will take months for the recovery. Hence, the world should get prepared with adequate preventive measures to contain the spread of this deadly virus. The international community should have the preparedness for a possible pandemic outbreak and adhere to all the precaution measures. The healthcare sector should develop effective countermeasures and ensure all medical resources like masks. PPEs, and ventilators are readily available in all hospitals. Well, it is rather safer to be over prepared than being under prepared on facing this novel coronavirus 2019-nCoV.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

Author Contributions

VL and N-SAM performed the literature search, critical data analysis and writing of this review. Technical support and proofreading was contributed by B-HG and L-HL. This review writing was founded by L-HL.

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Review Article

Progress in Microbes and Molecular Biology

HH PUBLISHER

Role of Low FODMAP Diet and Probiotics on Gut Microbiome in Irritable Bowel Syndrome (IBS)

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Abstract: Irritable bowel syndrome (IBS) is a chronic disease prevalent in today's society and diet remains the most common aggravator of IBS symptoms. Existing literature suggest that IBS patients are dysbiotic as evidence indicates decreased levels of *Bifidobacteria*, *Bacteroidetes* and *Faecalibacterium prausnitzii* and increased levels of *Firmicutes* in comparison to healthy individuals. Studies suggest that changes in diet can modulate gut microbiota and therefore improve IBS symptoms. The two diets being investigated are the low FODMAP diet and the use of probiotics. A low FODMAP diet implements a reduction in the amount of poorly absorbed carbohydrates and probiotics are live microorganisms that have been proven beneficial when consumed appropriately. Based on the literature acquired from PubMed, a positive correlation appears to exist between the low FODMAP diet and IBS symptoms; 57% report symptom relief. There is also a notable effect on the gut microbiome after changing to low FODMAP diet, noted with a significant decrease in levels of *Bifidobacterium*, *Clostridium*, *F. prausnitzii* and *Actinobacteria*. This poses a concern as bacteria such as *Bifidobacteria* and *F. prausnitzii* are beneficial for health. When probiotics are taken amongst IBS patients a reduction in symptoms is also observed. Additionally, there is an increase in the abundance of *Bifidobacterium* and *Lactobacilli*. It is suggested that co-administration of probiotics with a low FODMAP diet may ensure beneficial levels of *Bifidobacterium* while IBS symptoms ameliorate.

Keywords: FODMAP; gut microbiome; probiotics; irritable bowel syndrome (IBS).

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Introduction

Irritable bowel syndrome (IBS) is a functional bowel disorder and it is more prevalent than one may think. In fact, IBS has a global prevalence of 11% and accounts for up to 60% of outpatient appointments in gastroenterology (Figure 1)[1]. It is a disease that affects between 10–15% of individuals in Western countries, while slightly lower rates are seen in Asia, 2.3–11.5%[2]. While the prognosis for this condition is non-fatal, the impact on an individual's quality of life remains significant; IBS patients are 7.93 times more likely to experience severe pain and discomfort, 2.83 times more likely to complain of issues relating to mobility and 2.39 times more likely to experience anxiety and depression[3].

IBS requires an effective and long-term treatment and existing literature has made it abundantly clear that food is a crucial factor correlating with the presentation and severity of gastrointestinal symptoms; 80% of all IBS patients report being able to identify a minimum of one food item that aggravates their symptoms[4]. Furthermore, this disorder is also known to be influenced by a range of factors such as diet, anxiety, gastrointestinal inflammation and family history, thus may require a more holistic approach in its management. The current practice of management of IBS mainly focuses on relief of symptoms such as abdominal pain in combination with dietary modification including the introduction

of low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) diet. On the other hand, some studies have unveiled the relationship between gut microbiome and IBS, suggesting that the imbalance in microbial population may aggravate IBS symptoms[5]. Therefore, these evidence then lead to the idea of probiotics intake as a means to reinstate balance in the microbial balance. Probiotics is thought to prevent bacterial overgrowth by improving gut barrier function and receptor interactions, while at the same time producing a range of protective substance include short chain fatty acids (SCFAs)[6]. Thus, the directive of the current study aims to provide an overview on the role of gut microbiome in IBS, while consolidating the data on how diet modifications, particularly low FODMAP diet and/or intake of probiotics improve IBS via actions on the gut microbiome.

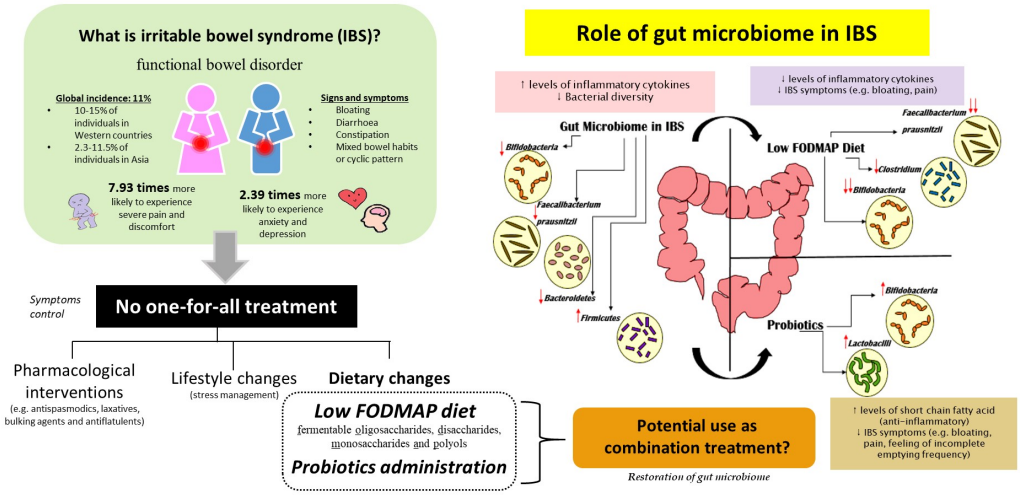

Figure 1: Targeting gut microbiome for the treatment of IBS.

What is irritable bowel syndrome (IBS)?

As one of the most common functional bowel disorders (FBD), the pathogenesis of IBS remains convoluted. Based on the Rome IV criteria, IBS is defined as a FBD which is distinguished by recurrent abdominal pain accompanied with changes in bowel habits and defecation pattern, in addition to symptoms such as bloating[7]. The criteria classifies IBS into subtypes according to the Bristol Stool Scale; IBS with diarrhea (IBS-D), IBS with constipation (IBS-C) and IBS with mixed bowel habits or cyclic pattern (IBS-M)[8]. IBS has been associated with a range of intestinal and extra-intestinal presentations; there is often an overlap between the following three conditions—functional dyspepsia, gastroesophageal reflux disease and IBS[9]. Urological and gynaecological problems also arise in patients with IBS. On top of the associations made with physical health, mental health has been affected in IBS, with cases of depression and anxiety co-existing. The diagnosis of IBS is commonly suspected through extensive history taking; a pattern of intermittent or continuous abdominal pain, relief with defecation, bloating, flatulence and a lack of alarming features (loss of weight, loss of appetite or an abdominal mass) is suggestive of IBS.

The aetiology of IBS is multifactorial and the pathogenesis of the disease is not fully understood. However, some evidence showed the potential role of gut-brain axis in development of IBS. Known as the"second brain", the huge network of neurons lining the gut forms the enteric nervous system which consists of more than 100 million neuronal cells[10]. Evidence suggests that symptoms such as diarrhea and/or pain result from exaggerated responses of the intestine to food or stress and that abnormal mucosal secretions and serotonin uptake by enterocytes cause alterations in gastrointestinal motility[11,12].

Visceral hypersensitivity or pain/discomfort occurs as a result of heightened perception of mechanical triggers applied to the bowel is commonly thought to be the conditions leading to IBS; up to 50% of patients have been found to have increased visceral perception[13,14]. As a matter of fact, gut-brain axis exists as a bidirectional interaction and psychological distress can cause IBS symptoms flare-up and exaggeration[15]. Besides causing activation in immune system, stress could further enhance dysbiosis or imbalance in the microbial population residing in the gut, which then lead to the development of vicious cycle of inflammation[16–20]. Inflammation is another factor in the pathogenesis of IBS as there is increasing evidence of the association between enteric infection and the development of post infectious IBS (PI-IBS) along with studies demonstrating a greater abundance of pro-inflammatory cytokines like IL-1β and IL-8 in IBS patients[20, 21]. An eight-year follow up study conducted by Marshall *et al*. showed that 15.4% of patients (n = 488) were diagnosed with IBS using the Rome I criteria, suggesting an association between enteric infections and persistent IBS symptoms[20]. Nevertheless, the study also noted other significant risk factors for the development of PI-IBS such as anxiety and depression, which is in-line with other evidences proposing participation of gut-brain axis in IBS[21, 22]. Although the exact mechanism remains unclear, the authors suggested an association between enteric infections and persistent IBS symptoms, particularly caused by prolonged alteration in gut microbiota composition and chronic inflammation.

Above all, the current treatment for IBS patients is mainly to relieve symptoms, whereby the first line management primarily attempts to control diarrhea and constipation along with some over-the-counter medications (e.g. non-

steroidal anti-inflammatory drugs, anti-flatulent, bulking agent for diarrhea)[8,23]. On top of that, patients are often advised to change their lifestyles, including stress management and dietary modifications such as high fiber intake, probiotics administration and/or adaptation of a special diet known as the low FODMAP diet. FODMAP refers to a group of carbohydrates, with 1–10 sugars, that are poorly absorbed[24,25]. When ingested, they have an osmotic effect; drawing water into the lumen of the small intestine. In the distal ileum and colon, FODMAPs are fermented to produce short-chain fatty acids and gases such as methane, carbon dioxide and hydrogen. Such products of fermentation result in gastrointestinal symptoms in individuals with existing issues of visceral hypersensitivity and gut motility[23,26]. Another proposed mechanism through which FODMAPs generate gastrointestinal symptoms is through immune activation. Urinary metabolomic profiles of IBS patients were analysed after low and high FODMAP diets. Such an intervention indicated that patients who took a low FODMAP diet demonstrated a slight decrease in urinary histamine levels. In addition, a decrease in inflammatory cytokines IL-6 and IL-8 have also been identified in patients undergoing a low FODMAP diet[27,28]. Nonetheless, FODMAPs may act as prebiotics, inducing growth and/or activities of bacterial in the gastrointestinal tract thereby changing the composition of the gut microbiome[27,29]. So, what is the role of gut microbiome in IBS and more importantly can we tackle the symptoms of this chronic disease by targeting gut microbiome?

Gut Health: IBS and Microbiome

Out of the 38 trillion bacterial cells living in the human body, more than one-third (33%) of them are residing in the gastrointestinal tract[30,31]. Gut microbiota has multiple functions and proves beneficial for a range of reasons. For instance, gut microbiome serves like a"physical barrier, preventing colonization by harmful pathogen[32]. In reality, gut microbiome was thought to be sterile before birth, but this dogma was challenged numerous times in the past 10 years with studies identifying bacterial DNA and/or their product present in the placenta, amniotic fluid or even meconium[33]. Having said that, the gut microbiome also plays an indispensable role in the development of the immune system; the intestinal mucosa comes in contact with external antigens, presenting antigens and "educating" immune cells on when to attack via binding on receptors (e.g. Toll-like receptor recognition) [32,34]. Several observations in rodent models showed that germ free animals (i.e. absence of microbiota) displayed immunological defects with reduced number of immune cells and abnormal functioning[35].

The connection between the gut and health has been well established for centuries; in 400 B.C. Hippocrates stated that death is in the bowels"and implied that indigestion would be the cause of all illnesses. Thousands of years have passed since then and later in 1916, Ali Metchnikoff suggested that diseases originated from the gut, when bad"bacteria can no longer be controlled. The ideal state in gut microbiome or eubiosis is achieved when there is a balance in bacterial abundance, particularly relative abundance of pathogens that may pose serious infectious risk[36]. In the gastrointestinal (GI) tract, predominant bacterial phyla comprise of *Bacteroidetes* and *Firmicutes*, along with smaller populations of *Fusobacteria*, *Actinobacteria* and *Verrucomicrobia*[37]. Typically, the complex microbial community in gut stabilizes after two to three years of age[38]. Nevertheless, it should be highlighted that variations exist between individuals as a multitude of factors contribute to the microbial composition such as age and diet to name a few[37,39,40].

Dysbiosis in the gut microbiome has been suggested to be associated with a combination of both acute and chronic diseases, or, increase the risk of its development. The different potential mechanisms on how dysbiosis and/or certain metabolites can lead to mucosal leakiness in the gut and promote inflammation milleu in entire body has been actively conversed over the past ten years[41–46]. In the recent years, many researchers have witnessed the changes in gut microbiome and its relationship to the pathogenesis of illnesses, particularly in those with IBS[30,47–51]. In patients with PI-IBS, it is simply obvious that those with history of enteric infection have a higher risk of developing IBS, given that the abundance of microbial population in the gut changed with the colonization of pathogens [51,52]. These patients have been shown to have decreased levels of *Bifidobacteria*, *Bacteroidetes* and *F. prausnitzii* and an increase in the abundance of *Firmicutes*. A 2-fold decrease in *Bifidobacteria* is found in IBS patients compared to healthy controls; *Bifidobacteria* is beneficial to the host as it produces acetic and lactic acid, thereby preventing the growth of pathogenic bacteria and maintaining the immune system[52–54].

Apart from the differences in microbial taxa, studies have also suggested a reduction in microbial diversity, richness and temporal stability. Tap *et al.* found that the severity of IBS symptoms is negatively associated with microbial richness and enterotypes enriched with *Clostridiales*/*Prevotella* species[55]. This was further emphasized by Halkjaer *et al.* whereby IBS patients have a lower stool microbial biodiversity, suggesting an association between IBS and gut microbiome[56]. In conjunction with this, there are some studies suggest that microbial composition varies according to IBS subtypes. In a study conducted by Jeffery and team, *Lactobacilli* was found in greater abundance in IBS-D patients in comparison to IBS-C patients[52]. Additionally, the luminal microbiota of patients with PI-IBS was distinguishable from non-PI-IBS patients, but like those with IBS-D. Furthermore, Kroguis-Kirikk *et al.* found a reduction in microbial diversity in IBS-D patients and this can be related to the reduction in microbial composition identified in patients with acute diarrhea[57]. Conversely, similar results were obtained in animal models of IBS, whereby fecal transplantation using stool from IBS patients induced visceral hypersensitivity in germ-free rats[58].

Relationship between Low FODMAP Diet and Gut Microbiome

Following the diagnosis of IBS, dietary changes are often crucial steps to control unwanted symptoms such as diarrhea and bloating. The traditional IBS diet focuses a great deal on when and how to eat. Commonly, patients are told to eat 3 meals and 3 snacks a day and to reduce their intake of foods such as coffee, fatty foods, alcohol and spicy dishes[59]. Along with that, since the knowledge of saccharides causes

gastrointestinal symptoms came to light, IBS patients are introduced to a meal plan"with low composition of FODMAP content as these are considered potential triggers[25,30]. In a low FODMAP diet, the daily intake of FODMAPs in an IBS patient is reduced from 15–30 g/d to 5–18 g/d. The execution of this diet requires global restriction for 4–8 weeks followed by reintroduction of food according to a patient's tolerance. As a consequence, this diet is not only "therapeutic" food avoidance plan but can also use as a diagnostic tool to test food intolerance.

The low FODMAP diet can be deemed clinically effective as a total of 50–70% of patients with IBS reported adequate symptom relief, marked by a lower IBS symptom severity score after implementing a low FODMAP diet[1,27,60]. Evidence suggests that gastrointestinal symptoms ameliorate, with reported reductions in bloating, borborygmi, stool frequency, urgency and improvement in consistency[1,59,61]. Taking a closer look at the microbial composition, Hustoft *et al.* indicated that reduction in FODMAP intake (50%) produced a significant change in microbial composition; a 6-fold reduction was observed in those following low FODMAP diet in the relative abundance of *Bifidobacteria*, in comparison to individuals on normal diet (Table 1)[28]. Similarly, Silk *et al.* found that a significant reduction in *Bifidobacteria* abundance was seen following a 3-week implementation of a low FODMAP diet. Differing from Histoft *et al*., this study also found a 47% overall reduction in total bacterial count and a reduction in abundance of other bacterial groups[62]. A significant reduction was found in *Clostridium*, *F. prausnitzii*, *Bifidobacterium*, *Megasphaera*, *Pediococcus* and *Actinobacteria* and patients presented more dysbiotic gut microbiome. Likewise, a study by McIntosh *et al*. highlighted an increase in the relative abundance of bacteria involved in gas consumption in patients on low FODMAP diet[27]. Members of genus *Adlercreutzia* are described as hydrogen "consumers" for equol production, while reducing gas formation and eventually preventing symptoms of bloating and pain simultaneously[27,63].

Table 1. Effect of low FODMAP diet on IBS symptoms and gut microbiota.

Study Design	Methods	Impact of Low FODMAP Diet on IBS symptoms	References
Randomised Controlled Trial	IBS Symptom Scoring System (IBS-SSS)	57% had adequate pain relief vs control (38%) 73% achieved > 50 reduction of IBS-SSS score vs control No significant difference for IBS-QOL	[61]
Randomised Controlled Trial	IBS Symptom Scoring System (IBS-SSS)	Mean decrease of 28% vs. control High FODMAP group had a mean increase of 7% in symptoms 52% reduction in abdominal pain Positive correlation between IBS symptom severity and level of FODMAP consumption	[27]
Randomised Controlled Trial	IBS Symptom Scoring System (IBS-SSS)	Symptom severity was reduced in both groups There was no significant difference between the IBS-SSS scores in both groups compared to baseline. The number of bowel movements reduced in the low FODMAP group compared to the traditional diet group.	[59]
Study Design	**Methods**	**Impact of Low FODMAP Diet on Gut Microbiota**	**References**
Randomised Controlled Trial	16S rRNA	Increase in the number of dysbiotic patients after the low FODMAP diet (60%) Reduction in *Clostridium*, *Faecalibacterium prausnizii*, *Bifidobacterium*, *Megasphaera*, *Pediococcus* and *Actinobacteria*.	[28]
Randomised Cross Over Controlled Trial	FISH	No difference in the relative proportion of each bacterial group at baseline and post intervention. Higher proportions of *Bifidobacterium* spp. Lower proportions of *C. perfingens* subgroup *histolyticum* and *Bacteroides*/*Prevotella* spp.	[62]
Randomised Controlled Trial	16S rRNA Operational Taxonomic Units (OTU)	No difference between α-diversity or β-diversity. Higher *Actinobacteria* richness and diversity IBS-M and IBS-D had higher bacterial richness (*Firmicutes*, *Clostridiales* and *Actinobacteria*). *Actinobacteria* richness was increased	[27]
Randomised Controlled Trial	16S rRNA	Responders had high levels of: *Bacteroidaceae*, Clostridiales (*Ruminococcaceae*, *Dorea* and *Faecalibacterum prausnitzii*) and *Erysipilotrichaceae*	[64]
Randomised Controlled Trial	GA-map™ Dysbiosis Test	Responders has a high level of: *Bacteriodes fragilis, Acinetobacter, Ruminiclostridium, Streptococcus* and *Eubacterium.* Responders had lower levels of: Clostridials, *Shigella/Escherichia,* Unable to identify a significant change in dysbiosis.	[65]

Even so, some researchers argued that the effectiveness of the low FODMAP diet may be dependent upon individual gut microbial composition. Chumpitazi *et al*. conducted a study among children and concluded that 24% of patients who experienced symptom response had a high abundance of *Bacteroides*, *Ruminococcaceae* and *F. prausnitzii*[64]. These findings therefore proposed that the greatest benefit of a low FODMAP diet can be seen in patients who have a high concentration of microbiota with saccharolytic potential. Amongst adults, patients who respond significantly to the diet have been found to have higher levels of *Bacteroides fragilis*, *Acinetobacter*, *Ruminiclostridium*, *Streptococcus* and *Eubacterium*[65].

Administration of Probiotics in IBS Patients

In IBS patients, several studies have indicated a drop in abundance of certain beneficial microbes or probiotics, particularly those under the phyla *Bifidobacterium* and *Actinobacteria*[51,52,66]. Besides producing lactic acid from digested dietary sugars, some probiotics such as *Lactobacillus*, *Lactococcus*, *Bifidobacterium* and *Streptococcus* are capable of producing small chain fatty acids (SCFAs) like butyrate, propionate and acetate through fermentation of ingested food[67]. SCFAs, in particular butyrate is the primary energy source for colon cells and plays a central role in intestinal maintenance through anti-inflammatory actions[68–71]. In addition to that, one of the most traditional theory on how probiotics confer protection to the host is by displacing pathogenic GI bacteria, preventing their colonization and subsequently re-establishing the balance in gut microbiome.

In the light of current research on IBS, many researchers have reported the benefits of probiotics in IBS patients, particularly in the reduction of IBS symptoms such as abdominal pain and bloating[72–76]. A multicenter studies conducted in India by Ducrotte and team showed that 78.1% of patients consider treatment with *Lactobacillus plantarum* 299v to be effective (Table 2)[73].

Table 2. The effect of probiotics on IBS symptoms and gut microbiota.

Study Design	Methods		Probiotics intervention (Form: single/multi-strains; Dose; Duration)	Impact of Probiotics on Gut Microbiota	References
	Monitoring IBS symptoms and quality of life	Monitoring of microbiome			
Randomised Controlled Trial	Patient Diary; RAND-36	N.R.	Capsule: *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bb99 and *P. freudenreichii* ssp. *shermanii* JS (Valio Ltd, Helsinki, Finland. Equal amount of strain: each $8–9\times10^9$ CFU/day Duration: 6 months	a. 42% reduction in symptom score b. Probiotics appeared to be beneficial for all symptoms. c. Significant reduction in scores for borborygmi	[78]
Randomised Controlled Trial	Patient Diary; RAND-36	Human Intestinal Tract chip (HITChip, Agilent Technologies)	Drink: *L. rhamnosus* GG (ATCC 53103, LGG (Valio Ltd, Helsinki, Finland)), *L. rhamnosus* Lc705 (DSM 7061), *P. freudenreichii* ssp. *shermanii* JS (DSM 7067) and *B. animalis* ssp. *lactis* Bb12 (DSM 15954) Equal amount of strain: each 1×10^7 CFU/mL Duration: 5 months	a. Higher mean reduction in IBS score in the probiotic group, recorded at 37%, compared to only 9% reduction in the placebo group b. Health-related quality of life analysis showed improvement in the probiotic group for the domain describing bowel symptoms	[72]
Randomised Controlled Trial	Visual Analogue Scale (VAS)	N.R.	Capsule: *L. plantarum* 299v (DSM 9843) Amount: 1×10^{10} CFU/day Duration: 4 weeks	a. 45.2% reduction in mean severity of abdominal pain. b. 78.1% found the treatment good/excellent compared to placebo (8.1%) c. No significant side effects	[73]
Randomised Controlled Trial	N.R.	qPCR (Selected bacterial group only)	Capsule: *L. rhamnosus* GG (ATCC 53103), *L. rhamnosus* Lc705 (DSM 7061), *P. freudenreichii* ssp. *shermanii* JS (DSM 7067) and *B. breve* Bb99 (DSM 13692) Equal amount of strain: each $8–9\times10^9$ CFU/day Duration: 6 months	a. Intestinal microbiota remained stable except for *Bifidobacterium* spp. b. Number of *Bifidobacterium* spp decreased	[76]

Randomised Controlled Trial	Clinical assessment at baseline and end of treatment Telephone interview (1 month after end of treatment)	qPCR	VSL-3: 9.3 × 10^{10} CFU/g of *Bifidobacterium* (*B. longum* Y10, *B. infantis* Y1 and *B. breve* Y8), 2.7×10^{9} CFU/g of *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum*) and 2× 10^{11} CFU/g of *Streptococcus salivarius* subsp. *thermophilus.* Duration: 20 days	a. Significant increase in Lactobacilli, *Bifidobacteria* and *Streptococcus thermophilus* b. No significant changes in enterococci, coliforms, *Bacteroides* and *Clostridium perfingens*.	[82]
Randomised Controlled Trial	N.A.	qPCR (Selected bacterial group only)	Capsule: *Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* sp. *shermanii* JS and *Bifidobacterium breve* Bb99 Equal amount of strain: each 8–9×10^{9} CFU/day Duration: 6 months	a. Group receiving probiotics appeared to be display lesser monitored IBS-related GI symptoms. b. Significant decrease in the amount of *R. torques* 94%, increase in *C. thermosuccinogenes* 85%. c. Increased abundance of *Ruminococcus torques* 93%. d. *Bifidobacterium* decreased in the probiotic group	[79]

IBS patients reported overall reductions in stool frequency, bloating and feeling of incomplete emptying frequency after 4-weeks administration of probiotics. Furthermore, there are studies emphasized that multi-strain probiotics have shown a more significant impact on improving gastrointestinal symptoms as compared to single strain probiotics. This is likely due to the fact that more niches are colonized, thus having a greater effect on gut motility[76,77]. A study using a probiotic mixture containing *Lactobacillus rhamnosus* GG*, L. rhamnosus* LC705*, Bifidobacterium breve Bb99 and Propionibacterium freudenreichii* sp. *shermanii* revealed that IBS patients experienced lesser symptoms, achieving a reduction rate as high as 42% in symptom score, whereas the placebo group only reported a 6% reduction[78]. Also, the gut microbiome of IBS patient receiving probiotics was indeed different compared to the placebo as examined using similarity index which compared the stability of the microbiota composition at baseline and post-intervention. Similar observation was obtained by Lyra and team where they observed that probiotics intake reduced amount of *Ruminococcus torques* 94%, which has been associated with gastrointestinal diseases like Crohn's Disease as well as IBS[79–81].

After all these years, the use of probiotics in IBS remains as a compelling topic as some studies did not produce obvious long-term efficacy compared to placebo. For instance, using another commercially available probiotics capsule, VSL-3 which contains *Bifidobacterium*, *Lactobacillus*, *Streptococcus salivarius* subsp. *thermophiles*, Brigidi and team observed an increase in these probiotics, but not other bacterial population such as enterococci, coliforms, *Clostridium perfringens* and *Bacteroides*[82]. The same team also noted that the gut microbial composition returned to initial values once the probiotic had been suspended, indicating that the effects do not persist long term. However, bearing in mind that most of the previous studies investigated these using conventional qPCR targeting genes like 16S–23S ribosomal RNA may not provide a full glimpse of which population changes after intervention. Additionally, most studies have also highlighted that patients can continue with their normal diet, regardless of whether they are taking probiotics or placebo, which may explain for the inconsistencies in data. There are some concerns over the form of probiotics used such as capsule, liquid or powdered form, as studies have pointed out that the delivery systems vary greatly in effectiveness[83]. Nonetheless, with the advancement in next generation sequencing and enhanced computational power, it may be easier to visualize how gut microbiome changes after probiotics intake in IBS patients, and at the same time answer the doubts on whether the observed effect is due to specific strains or a synergistic effect of these probiotics. Consequently, this method would also assist the development of biomarkers to facilitate the detection and/or diagnosis of IBS on top of classic clinical assessment.

Conclusion and Future Recommendations

It is evident that gut microbiome plays an important role in maintenance of gut health, including IBS. Even though some medications may alleviate symptoms for IBS patients, there is still no one-for-all drug or treatment plan that would work for all of them[59,60]. The low FODMAP diet has shown to be one of the clinically effective strategy by reducing overall symptom severity in multiple studies. By adhering to the low FODMAP diet, patients may not experience symptoms flare-up but it is a challenging diet to adopt in certain regions of the world, particularly limitation of food choices as well as insufficient knowledge in delivery of the diet plan among medical practitioners[84,85]. Some clinicians may doubt its implementation in the long run as a significant reduction in carbohydrates, iron and fiber can lead to other issues like calcium deficiency. While this may be concerning, concurrent use of probiotics along with

low FODMAP diet may really help to ensure beneficial levels of *Bifidobacteria* are maintained. Theoretically, the gut microbiome patients receiving probiotics could make up for the reduced diversity following the restrictive diet, particularly by increasing the abundances of these probiotics[1,76,77]. In actual fact, there are still much to do before researchers could unravel the role of gut microbiome or even specific microbial population in IBS. Amongst existing studies, there is a lack of consistency and clarity which may be attributed by the heterogeneity between them. This heterogeneity exists in variations in individual baseline microbiota, potential microbial differences according to the IBS subtypes, study designs, the methods of analyzing the microbiota composition, the definition of clinical response and the population of selected individuals for the study[4,52]. Above and all, an innovative approach like combining probiotics and low FODMAP diet in combating IBS is clinically applicable with no or minimal side effects. Further investigations incorporating high throughput next generation sequencing technologies, analytical tools like chromatography and mass spectrometry techniques as well as improvement in delivery methods of probiotics may expedite the development of the next effective regime for the management of IBS.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

Author Contributions

The literature review and manuscript writing were performed by SMS and H-LS. SHW and L-HL provided vital guidance of the research and proof of the writing. L-HL and H-LS founded the research project.

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Review Article

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Comprehensive Insight of Neurodegenerative Diseases and The Role of Neurotoxin Agents — Glutamate

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Abstract: The increased concentration of extracellular glutamate has been reported to play a key role in most of the neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, even though its importance as an amino acid neurotransmitter in mammalian. Glutamate toxicity, which can be caused by excessive intake of monosodium glutamate (MSG), is the major contributor to pathological neuronal cell death. It causes neuronal dysfunction and degeneration in the central nervous system (CNS). Glutamate neurotoxicity can be categorized into two forms, which are receptor-mediated glutamate excitotoxicity and non-receptor mediated glutamate oxidative toxicity. The receptor-mediated glutamate excitotoxicity involved excessive stimulation of glutamate receptors (GluRs) which lead to excessive ion calcium (Ca^{2+}) influx and activates a cell death cascade involving the accumulation of mitochondrially generated reactive oxygen species (ROS). Studies showed excessive extracellular glutamate leads to nerve cell death via the activation of *N*-methyl-D-aspartate (NMDA) receptors in the cases of trauma or stroke. Whereas non-receptor mediated oxidative toxicity involved the breakdown of the cystine/glutamate antiporter (x_c^-) mechanism, which leads to the depletion of glutathione (GSH) and causes oxidative stress and cell death. The cystine/glutamate antiporter couples the import of cystine to the export of glutamate. The increased concentration of extracellular glutamate could inhibit the uptake of cystine, which is required for the synthesis of the intracellular antioxidant GSH. GSH plays an important role in the disposal of peroxides by brain cells and in the protection against ROS. Depletion of GSH renders the cell to oxidative stress and ultimately leading to cell death. This article aims to provide a comprehensive review of neurodegenerative diseases and the role of neurotoxin agents, glutamate in these diseases.

Keywords: neurodegenerative diseases; Alzheimer's disease; Parkinson's disease; neurotoxin agents; glutamate

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INTRODUCTION

Nervous System

Nervous system is the major controlling, regulatory and communicating system in the body responsible for receiving and transmitting stimuli throughout the body[1]. Nervous system also monitors and coordinates internal organ function and initiates response to the changes in the external environment. Nervous system can be divided into central nervous system (CNS) and peripheral nervous system (PNS). CNS is composed of the brain and spinal cord, which serves as the main processing center for the entire nervous system[2]. It receives information from and sends information to the PNS. PNS is made up of all the nerves connecting the body to the brain and spinal cord. Sensory neuron is responsible in sending information from internal organ or from external stimuli to CNS, whereas motor neuron transmits information from CNS to organs, muscles and glands[2]. The motor nervous system can be further divided into two systems; autonomic nervous system and somatic nervous system. Autonomic nervous system is responsible for involuntary or visceral bodily function while somatic nervous system is mediating voluntary reflexes for instance skeletal muscle. In general, activity of the nervous system depends on the connectivity among two cell types, which are neurons and glia[3–5].

Neuronal Cell

Neuronal cell is an excitable nerve cell that processes and transmits information by electrochemical signaling. Most neurons cannot divide; thus, neurons cannot be replaced if lost due to injury or disease[1]. There are 3 types of neurons based on functions, which are sensory neurons, motor neurons, and interneuron. Interneurons carry information between sensory neurons and motor neurons and can be found in CNS.

Generally, neuronal cell is comprised of neuronal cell body (soma), axon and dendrite. The axon has a terminal end that constitutes the pre-synaptic site of the synapse while the dendrite has an apical site and multiple branches. Neuron communicates with each other at synaptic cleft (synapse), which involved release of neurotransmitter by pre-synaptic cell and binding of neurotransmitter to the receptor on post-synaptic cell. Neurotransmitter will trigger an electrical response or a secondary messenger pathway that may either excite or inhibit postsynaptic neuron[4]. Excitatory neurotransmitter causes changes that generate action potential in the responding neuron while inhibitory neurotransmitter will block the changes[6,7]. Major neurotransmitters found in the body include glutamate, dopamine, glycine, gamma-aminobutyric acid (GABA) and serotonin.

Glial Cell

The glial cells provide vital support for the proper functioning of neurons. Glial cells are also known as nerve glue, are non-neuronal cells of the nervous system. It does not transmit electrical impulses, but it appears to be the most abundant cell types in the CNS. Glial cell can be divided into macroglia (astrocytes, oligodendroglia and Schwann cells) and microglia. Different types of glial cell provide different support to neurons. Microglia cells in CNS protect neurons from bacteria invasion. Oligodendrocytes and Schwann cells provide myelination to axons in CNS and PNS respectively. Myelin sheath provides insulation to the axon that allows electrical signals to be transmitted efficiently[8]. Astrocytes not only provide physical support to neurons, but also support neurons by providing antioxidant protection, substrates for neuronal metabolism via neurovascular coupling, digest dead neurons and glutamate clearances[4,9]. Also, astrocytes have interaction with the cerebral endothelium in determining blood brain barrier function, morphology and protein expression[10].

Neuron-astrocyte interaction in the brain

Astrocytes are the most numerous and diverse glial cells in the CNS. Many studies demonstrating astrocyte dysfunction throughout the neurodegenerative process as a prominent determinant for survival for both neuronal cell and the entire organism. Neuronal activity leads to increase of potassium ion (K^+) and glutamate levels in extracellular space. As a supportive cell, astrocyte plays important role in maintaining the extracellular K^+ homeostasis through potassium channels expressed by astrocytes[11]. Moreover, astrocytes are known to express high level of glutamate transporter to remove glutamate from extracellular space[12]. The increased concentration of extracellular K^+ and glutamate will trigger astrocyte glycolysis and further enhance lactate and pyruvate production to fuel neuronal tricarboxylic acid cycle (TCA cycle)[13,14]. Astrocyte and neuronal cell also coupled by glutamate-glutamine cycle. Astrocytes take up glutamate and convert it to glutamine. This glutamine is then shuttled back to presynaptic terminals (neuronal cells) and used by neuronal cell to replenish the neurotransmitter glutamate[15]. In addition, neurotransmitters released by neuronal cells induce transient elevations of internal Ca^{2+} levels in astrocytes due to the activation of metabotropic glutamate receptors on astrocytes[16,17]. The increased level of astrocytic Ca^{2+} triggers the release of chemical transmitters from astrocytes, which can cause sustained modulatory actions on neighbouring neuronal cells[17].

NEURODEGENERATIVE DISEASES

Neurodegenerative diseases are becoming huge public health challenge due to their increasing medical and social impact. It is one of the major global health burdens in the Western world and is often found in aging population[18]. These diseases can be life-threatening and have great impacts on both the patients and caregivers. Neurodegenerative diseases can be illustrated by progressive nervous system dysfunction that causes deterioration of many of human body's activities, including speech, movement, balance, heart function and breathing[19]. The causes of neurodegenerative diseases comprise genetic factors, infections or autoimmune diseases, brain trauma or injury and environmental toxins[20–23]. Most of the neurodegenerative disorders are incurable; thus, treatments are applied with the hope to relieve pain, improve symptoms, and increase mobility. Neurodegenerative diseases are categorized based on primary brain region or type of affected brain cell, clinical symptoms, and type of protein aggregated in brain[24]. Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS) are the most common age-related neurodegenerative diseases. Some forms of these diseases could be inherited. Moreover, morphological, biochemical, genetic, cell and animal model studies reveal that mitochondria play roles in the neurodegeneration through rendering vulnerable neurons susceptible to stress, cellular aging and genetic variations[25].

Alzheimer's Disease (AD)

Alzheimer's disease (AD) can be categorized by progressive degenerative disorders of the brain which causes irreversible loss of neurons and memory. It is one of the common causes leading to dementia. Late-onset of Alzheimer's disease (LOAD) with age 65 years or older is representing the majority of AD. Senile plaques, intracellular neurofibrillary tangles (NFTs), extracellular degenerating neurons, dystrophic neuritis, and activated

astrocytes or microglial found in the brain underlie the pathogenesis of AD[26]. Senile plaque is mainly composed of β-amyloid (Aβ) while NFTs consist of hyperphosphorylated and aggregated forms of the tau protein. Tau is a microtubule-binding protein that acts as neuronal cytoskeleton stabilizer and participates in vesicular transport and axonal polarity. The deposition of hyperphosphorylated tau protein impairs axonal transport therefore affecting the nutrition of dendrites and axon terminals. While the accumulation of Aβ activates cellular stress responses and disrupts signal transduction pathways[27]. Studies indicated that Aβ also induces inflammatory reaction in the brain and causes release of various cytokine such as TNF-α (tumor necrosis factor-alpha)[28]. TNF-α could induce infiltration of leukocytes and possible T-cells into the central nervous system through its capacity in enhancing blood brain barrier permeability[29]. The increased permeability of blood brain barrier causes influx of neurotoxin, such as glutamate and arachidonic acid, which could lead to substantial damage to the brain. Cholinergic and glutamatergic neurotransmission systems, which play role in cognition, were affecting in patients with Alzheimer's disease[30].

Parkinson's Disease (PD)

Parkinson's disease (PD) is the second most common late-life neurodegenerative disease after Alzheimer's disease. It can be characterized by progressive dopaminergic cell loss in the substantia nigra pars compacta which leads to dysfunction of the basal ganglia system and motor symptoms and subsequently causing tremors, slow movement, rigidity and gait impairment[31,32]. Another pathological feature of PD is the accumulation of Lewy bodies in neurons of the brainstem nuclei, substantia nigra, hippocampus, cerebral cortex, myenteric plexus, and olfactory bulb. Lewy bodies are accumulations of microscopic protein deposits in the brain, mainly alpha-synuclein. The pathology of PD is not yet fully understood. Some genetic and toxin-based animal models suggest that inflammation, oxidative stress, mitochondrial dysfunction, aberrant processing of proteins by the ubiquitin-proteasome system, and activation of apoptotic pathways are playing roles in dopaminergic cell death[33]. Furthermore, some studies indicated the increased glutamatergic transmission may contribute an excitotoxic component to the cellular insults that lead to degeneration in the substantia nigra pars compacta as well as the glial response that arises in the striatum and the substantia nigra[34].

Other Neurodegenerative Diseases

Other neurodegenerative disorders include Huntington's disease (HD) and amyotropic lateral sclerosis (ALS). HD can be categorized by cognitive decline, psychiatric disturbances and motor dysfunction, which will lead to dementia and death approximately 15–20 years after the onset of the disease[35]. HD is an autosomal-dominant disorder caused by the expansion of a CAG trinucleotide repeat encoding an abnormally long polyglutamine track in the N terminus of the huntingtin protein[36]. This protein may inhibit mitochondrial function and proteasome activity[37]. HD has a prevalence of 5–10 cases per 100,000. Thus, HD is known as one the most common inherited neurodegenerative disease[35].

While amyotrophic lateral sclerosis seemed to be a progressive and usually fatal disorder caused by motor-neuron degeneration in the brain and spinal cord. Motor-neuron degeneration in the brain and spinal cord will lead to paralysis of voluntary muscles. The mutation in superoxide dismutase 1 (SOD1) seems to be associated with amyotrophic lateral sclerosis. This mutation will form aggregates in mitochondria which could interfere anti-apoptotic function of B-cell lymphoma 2 (Bcl-2), mitochondrial import and generate free radicals. Reactive oxygen species produced will inhibit the function of glial glutamate transporter (EAAT2) and lead to the increase of extracellular glutamate, next triggering motor neuron death[38].

FACTORS INVOLVED IN NEURODEGENERATIVE DISEASES

Neurodegeneration is due to the death of neuronal cells through the delayed process of apoptosis or necrosis. Among the known risk factors for neurodegenerative diseases, such as genetic polymorphism, infection and head trauma, the role of neurotoxin agents such as glutamate and an oxidative stress received huge attention recently. This is because most of the pathologies of neurodegenerative diseases involve oxidative stress triggered by neurotoxin agents or imbalance in pro-oxidant/antioxidant homeostasis[39,40].

Astrocytes in Neurodegenerative Diseases

Astrocytes have many housekeeping functions, such as the maintenance of the extracellular environment and synaptic function in the CNS. Also, astrocytes play a vital role in the maintenance of neurotransmitter synthesis and neuronal metabolism. Astrocytes become activated (reactive) in response to a variety of brain insults, such as trauma, stroke and neurodegenerative diseases[41]. Reactive gliosis can be characterized by the increase expression of vimentin (Vim), glial fibrillary acidic protein (GFAP), S100β and proliferation that could probable occur in neurodegenerative diseases[42]. Astrocytes will be activated under stress and injury, leading to an upregulation of proinflammatory cytokines and chemokines, which are related to the pathogenesis of AD[43]. Recent studies suggested that activated astrocytes play a role in the clearance of the Aβ-peptide and thus preventing the plaque formation in AD. Furthermore, astrocytosis (abnormal increase number of astrocytes) was discovered in affected brain region of HD patients. The Huntingtin protein was also observed to be co-localized with reactive astrocytes[15].

GLUTAMATE

L-glutamate is a non-essential amino acid which can be gained from alimentary protein, endogenous protein and

also monosodium glutamate (MSG) that is used as food additive[44]. It is one of the most abundant neurotransmitter in mammalian central nervous system and it is involved in numerous normal brain functions, such as memory, cognition and learning[45]. Furthermore, glutamate has an essential role in regulating bio-energetic processes such as reactions of glycolysis, gluconeogenesis, citric acid cycle and synthesis of ketone bodies. It is the precursor for γ-aminobutyric acid (GABA) and glutathione (GSH) [46]. Glutamate is one of the key factors in central pain transduction mechanisms and excitotoxic neuronal cell injury[45].

Glutamate receptors

Neurotransmission occurs at synapses and it involved processes of neurotransmitters carrying signals released from presynaptic neuron terminals to synaptic cleft followed by binding of neurotransmitter to the receptors on the postsynaptic neuron. Binding of excitatory neurotransmitters, such as glutamate, to the receptor proteins on the postsynaptic neuron will cause sodium ion (Na^+) channels to open and allow influx of Na^+ to neuronal cell, and causes the neuron membrane to depolarize due to an action potential. The excitation of glutamate can be mediated via activation of metabotropic glutamate receptors and ionotropic glutamate receptors[47]. There are 3 families of ionotropic glutamate receptors; *N*-Methyl-D-Aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate receptors. The ionotropic glutamate receptors are coupled directly to membrane ion channels which conducting only Na^+ or both Na^+ and Ca^{2+}. Metabotropic glutamate receptors comprise of G-protein coupled receptors (mGluR1–8) that are important in regulating ion channels and enzymes producing second messenger[48]. It is categorized into 3 groups based on the sequence, function and pharmacology. Group I mGluRs (mGluR1 and 5) activates phospholipase C via Gq proteins and initiate an inositol triphosphate/ diacylglycerol (IP_3/DAG) second messenger cascade. Whereas Group II mGluRs (mGluR2 and 3) and Group III mGluRs (mGluR4, 6, 7 and 8) inhibit adenylyl cyclase via $G_{i/o}$ proteins. Reports showed that group II and III were mainly localized on presynaptic neuron while group III was localized on postsynaptic neuron[49]. Metabotropic and Ionotropic glutamate receptors present on both neuronal and glial cells[50,51]. Nevertheless, the involvement of glutamate receptors of glial cells in neurotransmission remains unknown. While the binding of inhibitory neurotransmitters, such as serotonin or GABA, to the receptor proteins on the postsynaptic neuron will lead to hyperpolarization and efflux of potassium ion (K^+) from neuronal cell[52].

Glutamate Transporters

The level of extracellular glutamate must remain low to prevent excessive excitation that can injure or kill neuronal cells due to the deficiency of enzyme at extracellular space to metabolize glutamate. The excessive glutamate can only be removed by cellular uptake through glutamate transporters on the plasma membrane of astrocytes and neuronal cells. Glutamate transporter can be discovered in the plasma membranes and intracellular membrane[53]. Glutamate transporters in the plasma membranes can be categorized into high affinity glutamate transporters and low affinity glutamate transporters. There are 5 high affinity glutamate transporters, also known as sodium-dependent glutamate transporters, have been cloned, namely excitatory-amino acid transporter 1-5 (EAAT1-5). These 5 glutamate transporters catalyze Na^+- and K^+-coupled transport of L-glutamate and also L- and D-aspartate[54]. Each glutamate is co-transported with two or three Na^+ and counter-transport of one K^+[55]. EAAT3, EAAT4 and EAAT5 are known as neuronal glutamate transporters. However, there were studies that showed EAAT4 was expressed in astrocytes as well[56]. EAAT1 and EAAT2, also known as glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) respectively, are expressed in astrocytes[57]. These astrocytic glutamate transporters are responsible for the majority of glutamate uptake near excitatory synapse[58,59].

Cystine/glutamate antiporter (system x_c^-) is a low affinity glutamate transporter which also known as sodium-independent glutamate transporter. System x_c^- is composed of two subunits, which are xCT light chain and 4F2 heavy chain. It is also known as cystine transporter which carries cystine into the cell in exchange for internal glutamate by using the transmembrane gradient of glutamate as driving force[54,60]. This system is suggested to mediate the cellular cystine uptake for the synthesis of GSH and maintenance of cystine—cysteine redox balance in the extracellular compartment, which is vital for the cellular protection from oxidative stress and cell death[61].

Glutamate Metabolism

When glutamate taken up by cells, it is not only used as transmitter, but also used for metabolic purposes such as protein synthesis, energy metabolism and ammonia fixation[62]. In the nerve terminal, cytoplasmic glutamate is transported into synaptic vesicles by vesicular glutamate transporters (VGLUTs) and then released to the synaptic cleft by exocytosis. As neurotransmitter, the binding of a transmitter to postsynaptic ionotropic glutamate receptors (AMPA, NMDA and kainate receptors) will cause an ionic influx that depolarizes the neuron (excitation)[63]. Whereas, a slower second messenger-mediated affects occur following the activation of the metabotropic glutamate receptors by glutamate[64]. To avoid excessive excitation that could injure and damage neurons, glutamate in the synapse should be removed rapidly. Glutamate can be removed via the uptake into the postsynaptic compartment, uptake into nonneuronal compartment (astrocyte) and reuptake into the presynaptic compartment. Glutamate uptake into presynaptic and postsynaptic neurons seems to be less important than astrocytic transport due to the membrane potential of astrocytes lower than neurons[65]. In astrocytes, glutamate taken up from extracellular fluid is metabolized by 2 different pathways, which are

tricarboxylic acid cycle (TCA cycle) and glutamate-glutamine cycle. Astrocytes convert glutamate and ammonia to glutamine (non-neuroactive species) via ATP-dependent glutamine synthetase. This pathway further supports the buffering of ammonia, which is a potential neurotoxic species. Glutamine synthetase in the brain is located mostly in astrocytes[66]. Glutamine produced is then released to the extracellular fluid and taken up by neuronal cell. Glutamine taken up into neuronal cell could be hydrolyzed to glutamate and ammonia via the action of phosphate-dependent glutaminase. The regenerated glutamate will be reused as neurotransmitter or potential fuel by being oxidized to α-ketoglutarate for neuronal cells[67,68].

Meanwhile, glutamate taken up by astrocyte could be converted to α-ketoglutarate and ammonia via reversible dehydrogenation by glutamate dehydrogenase (GDH) with either NAD^+ or $NADP^+$ as a cofactor[69]. Alpha-ketoglutarate is metabolized through TCA cycle to succinate, fumarate and malate. Malate is further decarboxylated to pyruvate and reduced to lactate. Both pyruvate and lactate are exported from astrocytes to the extracellular fluid and taken up by neuronal cell. Furthermore, glutamate uptake into astrocytes will stimulate glycolysis, that is glucose utilization and lactate production. Glutamate activates astrocytic Na^+/K^+ ATPase by cotransport Na^+ into the cell via Na^+-dependent glutamate uptake by glutamate transporters. The resulted lactate will provide the fuel for neuronal TCA cycle[70]. Figure 1 denotes the metabolism of glutamate in neuronal cell and astrocyte.

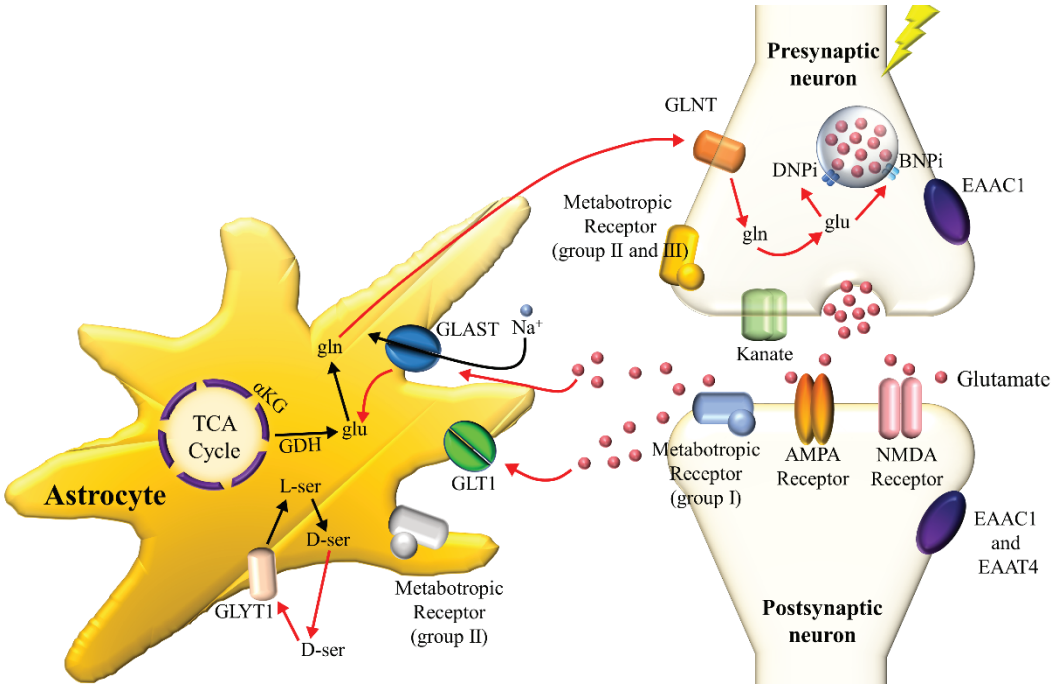

Figure 1. Glutamate metabolism in neuronal cell and astrocyte.

Glutamate Neurotoxicity

The maintenance of a low level (1–3 μM) of extracellular glutamate is vital for normal brain function to prevent excessive stimulation of receptors, excessive formation of ammonia by glutamate dehydrogenase and breakdown of glutamate/cystine antiporter. The excessive accumulation of extracellular glutamate is the main contributor to neuronal cell death under pathological conditions for instance ischemic insults and traumatic brain damage which could be associated to the disruption of glutamate metabolism[71,72]. The disruptions of glutamate metabolism underlie many neurodegenerative pathologies, for instance Alzheimer's disease, Parkinson's disease, Huntington's disease, brain ischemia and amyotrophic lateral sclerosis. These may be associated to variation of glutamate metabolism enzyme activities, disruption in mitochondrial homeostasis, and oxidation or redox imbalance in cells[73,74]. Glutamate neurotoxicity can be classified into receptor-mediated glutamate excitotoxicity and non-receptor mediated oxidative glutamate toxicity.

Receptor-Mediated Glutamate Excitotoxicity

Hayashi was the first to discover the excitatory action of glutamate in 1952 by injecting glutamate into mammalian brain able to produce convulsion[75]. Toxicity of glutamate was then observed when the injection of monosodium glutamate destroyed the neurons in the inner layers of the retina and induced acute neuronal necrosis in brain and term as excitotoxicity[76–78]. Elevated concentration of extracellular glutamate will cause excessive stimulation of glutamate receptors at postsynaptic membrane, which will further induce excessive influx of Ca^{2+} and Na^+. Ionotropic glutamate receptors directly activate channels that allow influx of extracellular Ca^{2+} and Na^+ while metabotropic glutamate receptor stimulates calcium released from internal store (endoplasmic reticulum and mitochondria)[79,80]. The influx of Ca^{2+} and Na^+ through glutamate receptor activation will result in membrane depolarizations which further activate voltage-dependent calcium channels. Plasma membrane calcium transporters regulate the calcium homeostasis by maintaining low concentration of

free intracellular calcium relative to free extracellular. Basically, there are 3 types of membrane transporters, namely antiporters, symporters and ATP-coupled active transporters. Calcium is controlled by the antiporters and Ca^{2+}-ATPase. Antiporters and Ca^{2+}-ATPase move Ca^{2+} out of the neuron and restore Ca^{2+} to mitochondria and endoplasmic reticulum[81]. Antiporter transport Ca^{2+} by a sodium-calcium exchange mechanism while the Ca^{2+}-ATPase transports one Ca^{2+} for each ATP hydrolyzed with a resultant increase in ATP demand. In addition, the direct uptake of Ca^{2+} into the matrix utilizes the proton circuit for oxidation phosphorylation and competes with mitochondrial ATP synthesis[82]. Therefore, mitochondrial ATP synthesis in mitochondria may cease as the Ca^{2+} flood from cytoplasm to the matrix[83]. Moreover, there are studies that showed Ca^{2+} influx from extracellular space activates cytosolic phospholipase A2 and lead to production of arachidonic acid[84,85]. ROS will be created during arachidonic acid oxidative metabolism[86].

Ca^{2+} excess of mitochondrial matrix triggers a mitochondrial permeability transition pore opens in the mitochondrial membrane, which will lead to collapse of mitochondrial membrane potential (MMP) [87]. Furthermore, reactive oxygen species formed from oxidation phosphorylation also contribute to the opening of the mitochondrial permeability transition pore[88]. The maintenance of MMP is vital for ATP synthesis. The collapse of MMP is related to a secondary increase in cytosolic Ca^{2+} concentration and the release of cytochrome *c* followed by caspase activation and apoptosis[89]. Moreover, caspase-independent death can also be induced by MMP through release of caspase-independent death effectors such as apoptosis-inducing factor and endonuclease G[90].

Non-Receptor Mediated Glutamate Oxidative Toxicity

The increased concentration of extracellular glutamate will lead to a prolonged cell death by oxidative stress known as glutamate oxidative toxicity. In glutamate oxidative toxicity, increased concentration of extracellular glutamate interferes with cystine uptake by blocking the glutamate gradient driven cystine/glutamate antiporter (system x_c^-)[91]. In facilitating the uptake of cystine, it is vital to exchange with glutamate at an equimolar ratio. The breakdown of system x_c^- will lead to the depletion of cystine and glutathione (GSH). In normal situation, imported cystine (oxidized form of 2 cysteines) is reduced to cysteine, which primarily acts as a precursor for GSH synthesis. The depletion of GSH renders the cells to oxidative stress and cell death[92]. Studies indicated that GSH depletion lead to activation of 12-lipoxygenase (12-LOX) and 15-lipoxygenase (15-LOX) and also causes inactivation of GSH peroxidase 4 (Gpx4), which damage mitochondria and resulted to cytochrome *c* and apoptosis-inducing factor (AIF) release, followed by reactive oxygen species accumulation and cell death[93–96].

OXIDATIVE STRESS

Oxidative stress built up caused by an imbalance of free radicals (pro-oxidant) and antioxidants in favor of the former. Free radicals are atoms or groups of atoms that have one or more unpaired electrons which make it extremely reactive[97]. These extremely reactive molecules tend to react quickly with adjacent molecules by donating or capturing an electron in order to gain stability and start the free radical reaction chain. It usually arises during body normal metabolism and increase with age. Also, free radicals can be generated by the immune system to destroy bacteria and viruses during infection. Environmental factors for instance pollution and radiation can further increase the production of free radicals. The most common cellular free radicals include hydrogen peroxide (H_2O_2), hydroxyl ($OH\cdot$), superoxide (O_2^-) and nitric monoxide ($NO\cdot$). High level of free radicals leads to redox imbalance and oxidative damage to biomolecules, such as protein, lipids and DNA, then lead to many chronic diseases in human such as cancer, diabetics, atherosclerosis, post-ischemic perfusion injury, cardiovascular diseases, stroke, chronic inflammation and other degenerative diseases[98].

Reactive oxygen species (ROS) and the reactive nitrogen species (RNS) are extremely reactive among free radicals, which acts as mediators in biological processes for instance respiration, cellular metabolism, gene translation and transcription, neurotransmission and inflammatory-type reactions[99]. Mitochondria are the main contributor to ROS production, and the electron transport chain that is found in the inner mitochondrial membrane uses oxygen to produce adenosine triphosphate (ATP)[100]. Cytochrome oxidase functions as the terminal enzyme of the chain by accepting an electron from cytochrome *c* and transferring it to oxygen (the final electron acceptor in the chain). The reduction of oxygen by one electron at a time by cytochrome oxidase make it becomes highly reactive. Superoxide (O_2^-), the product of one-electron reduction of oxygen, occurs mainly within the mitochondria of a cell[101]. It also functions as the precursor of ROS (such as hydroxyl radical) and as a mediator in oxidative chain reactions[102]. ROS is known to initiate lipid peroxidation by targeting the carbon-carbon double bond of polyunsaturated fatty acids (PUFAs) which are ample in cellular membranes and low-density lipoproteins. High level of oxygen requirement and unsaturated lipids in brain make it particularly susceptible to oxidative damage and lipid peroxidation[103,104]. Neuronal cells are most vulnerable to oxidative damage due to their low antioxidant activity[105]. Furthermore, the metabolism of excitatory amino acids and neurotransmitters in neuronal cells further increase the production of ROS and oxidative stress. Therefore, oxidative stress plays vital role in the pathogenesis of neurodegenerative diseases[106].

MODE OF CELL DEATH

In neuronal cells, calcium ions overload caused by glutamate excitotoxicity activates damaging enzymes for instance phospholipases that produce ROS. ROS induces neuronal injury such as cell membrane disruption, damage to intracellular proteins and DNA, and induction of mitochondrial release of cytochrome *c*, and caspase

activation. The release of cytochrome *c*, caspase activation and DNA fragmentation are the molecular hallmarks of apoptosis[107–110]. Furthermore, cystein protease or calpains are also activated by the elevation of intracellular Ca^{2+}. Calpains cleave various intracellular proteins, such as regulatory protein, membrane channels and cytoskeletal that lead to neuronal cell death by necrosis[111,112].

Apoptosis and necrosis signify 2 significant different cell death mechanisms. Apoptosis, also well-known as programmed cell death or cell suicide, happens under normal physiological conditions. It is an important mechanism in maintaining the homeostasis and regulation of tissue size during postnatal life. Apoptosis is signified by the activation of endogenous endonucleases that are activated directly or indirectly by a cascade of biochemical reactions, which involve cutting of DNA into fragments and consequently causing cell death. Apparently, cells continue to produce proteins and ATP during apoptosis. As a result, each apoptotic body is surrounded by cell membrane which contains intact and functional cell components. Apoptosis causes morphological changes that can be characterized by a deep chromatin condensation and formation of apoptotic bodies, followed by nuclear fragmentation and secondary necrosis that occur at the final stage.

In contrast to apoptosis, necrosis occurs when cells are exposed to extreme variance from physiological condition. It starts with an impairment of the cell's ability to maintain homeostasis. Necrosis can be signified by the loss of metabolic functions and the loss of cell membrane integrity. The cells terminate their proteins and ATP production during necrosis. The morphological changes of necrosis are signified by cell membrane rupture starts with swelling of cytoplasm and mitochondria. Cell membrane ruptures results in the release of the cell's components into the surrounding tissue. This process is recognized as cytolysis. The released cell's components will induce an intense inflammatory response[113–115].

CONCLUSION

The excessive glutamate-induced neurotoxicity had been associated to many chronic neurodegenerative disorders in CNS. Under normal situation, the astrocyte is responsible in clearing up glutamate from synaptic cleft. Hence, minimizes the neuronal injury from glutamate toxicity. However, excessive glutamate will induce oxidative stress in both astrocyte and neuronal cell and lead to cell death. Studies demonstrated that radical scavengers like vitamin E, in the form of tocopherol and tocotrienol, can protect the glutamate-injured cells from oxidative stress. Other than vitamin E, other sources such as microbial resources were also demonstrating strong antioxidants[116–126] and neuroprotective properties[127–132] such as metal chelating and radical scavenging potentials[133–140]. In conclusion, neuronal cell seemed to be the most vulnerable brain cells toward glutamate toxicity. In the absence of astrocyte, neuronal cells are 100-fold more vulnerable to glutamate toxicity. Thus, more studies should be conducted to explore the protective effect of astrocyte on neuronal cell against glutamate toxicity.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

Author Contributions

The literature review and manuscript writing were performed by H-MY, K-LL and LT-HT.

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Review Article

Progress in Microbes and Molecular Biology

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An Insight of Vitamin E as Neuroprotective Agents

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Abstract: Nervous system is the network of nerve cells that transmits nerve impulses throughout the body. It is rich in both unsaturated fats and irons, making it predominantly susceptible to oxidative stress and damage. Oxidative stress reflects the disruption of the redox balance between the formation and clearance of highly free radicals, for instance reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative stress will further damage the cell lipid, protein and DNA. Oxidative stress has a role in the modulation of critical cellular functions, such as apoptosis program activation, ion transport and calcium mobilization which lead to cell death. Many studies were conducted to prevent neuronal cell death caused by oxidative stress through administration of free radical scavenging antioxidant, such as vitamin E. Vitamin E is known as a chain-breaking antioxidant that showed the capability to increase the viability of neuronal cells that had undergone glutamate injury by inhibiting glutamate-induced pp60 (c-Src) kinase activation. Vitamin E occurs in 8 forms, namely α-, β-, γ- and δ-tocopherols and α-, β-, γ-and δ-tocotrienols. Tocotrienols differ from tocopherols by possessing an unsaturated isoprenoid side chain instead of a saturated phytyl tail. Tocotrienols, compared to tocopherols, are lightly studied due to the abundance of α-tocopherol in the human body and its antioxidant properties. Nevertheless, recent studies showed that α-tocotrienol is more effective in preventing lipid peroxidation compared to α-tocopherol. Furthermore, tocotrienol was discovered to protect neuronal cell through antioxidant-independent activities. The tocotrienol-rich fraction (TRF) is an extract that consists of 75% tocotrienol and 25% α-tocopherol. TRF was reported to possess potent antioxidant, anti-inflammation, anticancer and cholesterol-lowering properties. Thus, this writing highlights the significant neuroprotective effects of tocotrienol and tocopherol.

Keywords: neuroprotective agents; vitamin E; oxidative stress; tocotrienols; tocopherols.

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INTRODUCTION

Antioxidant

It known that aerobic organisms have developed a series of defense mechanisms, which involve antioxidants, in response to free radical production in order to maintain free radicals' level compatible with cellular functions and metabolic processes[1]. Antioxidant defense mechanism can be classified into enzymatic and non-enzymatic. The enzymatic defense mechanism includes superoxide dismutase (SOD), GSH peroxidase (GPx) and catalase (CAT), whereas non-enzymatic antioxidant defenses include vitamin E, ascorbic acid (vitamin C), glutathione (GSH) and other antioxidants[2].

GLUTATHIONE (GSH)

Glutathione (GSH) is the main thiol antioxidant and redox buffer of the cell[3,4]. It is a tripeptide comprised of glutamate, glycine and cysteine. It is synthesized in the cytosol by 2 enzymes that utilize ATP, that is GSH synthetase and gamma-glutamylcysteine (γ-GluCys) synthetase[5]. The gamma-glutamylcystein synthetase forms dipeptide gamma-glutamylcysteine by utilizing cysteine and glutamate as substrates. Gamma-glutamylcysteine is then merged with glycine in a reaction catalyzed by GSH synthetase thus forming GSH. GSH production is controlled by feedback inhibition of the γ-GluCys synthetase reaction by the end product GSH[6]. Total GSH in the cells can be free or bound to protein. The free GSH is present in reduced form, which will be converted to the oxidized form (GSSG) during oxidative stress and can be restored to the reduced form by the action of glutathione reductase (GR)[7]. The oxidation-reduction pathway of GSH is shown in Figure 1.

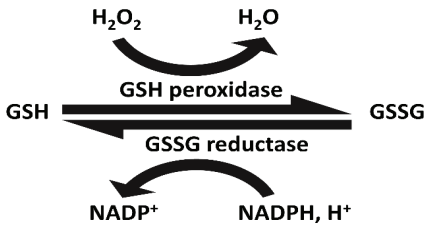

Figure 1. Oxidation-reduction pathway of reduced glutathione (GSH) and oxidized glutathione (GSSG).

GSH exerts its protection against oxidative stress by several approaches. Firstly, GSH directly scavenges reactive hydroxyl free radicals, ROS and radical centers on DNA and other biomolecules. Also, GSH functions as the cofactor for several detoxifying enzymes, for instance glutathione-S-transferases (GSTs) and glutathione peroxidase (GPx), which are vital in protection against oxidative stress. GPx is an enzymatic antioxidant which is predominantly responsible for the intracellular reduction of hydrogen peroxide (H_2O_2) to water with the support of GSH as electron donor[8]. Thus, GSH is oxidized to glutathione disulfide (GSSG) through GPx activity and quickly restored to GSH by the reaction catalyzed by glutathione reductase (GR). Whereas, glutathione-S-transferases conjugate GSH to free radicals, for instance hydrogen peroxides, thus reducing the deleterious interactions between free radical and essential cellular components[9,10]. Furthermore, GSH provides reducing capacity for the formation of deoxynucleotides by ribonucleotide reductase, the reduction of dehydroascorbate to ascorbate and restoration of vitamin E from radical form[2,11].

Also GSH has been suggested to function as neurohormone based on the presence of extracellular GSH in brain, the release of GSH from brain slices upon stimulation, the specific binding of GSH to extracellular receptors and the induction of sodium currents in neocortex, and the stimulation of a signal cascade in astrocytes[12,13]. Furthermore, some studies suggested that astrocytes support neuronal cells by means of providing GSH and cysteinylglycine (CysGly), which is the GSH precursor to neuronal cells[6].

VITAMIN E

Vitamin E is a lipid-soluble vitamin vital for human nutrition and health. The term 'vitamin E' was first introduced by Evans and Bishop (1922)[14] in describing a dietary factor in rat reproduction. The vitamin E family includes 8 different isomers, namely α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols. Tocopherols and tocotrienols, as a group known as tocochromanols comprise of a chromanol ring system and a polyprenyl side chain. The 8 isomers of vitamin E vary in the degree of antioxidant and biological activities. All tocochromanols are amphipathic molecules, with the lipophilic isoprenoic side chain of tocochromanol is anchoring the membrane lipids, whereas the polar chromanol ring is exposed to the membrane surface[15].

Vitamin E is the major component that present amongst the lipid elements of cell membranes and lipoproteins[16]. Vitamin E is exclusively synthesized by photosynthetic eukaryotes and other oxygenic photosynthetic organisms for instance cyanobacteria. Therefore, vitamin E is ingested along with fat-containing food, like nut oil seeds, egg yolk, vegetable oils, margarine, soya bean, wheat, avocados and germ[15].

Vitamin E has numerous biological functions. The pharmacologic use of vitamin E, in doses 10 to 50 times the daily requirement, was recommended in 1947 for the treatment of an array of cardiovascular disorders[17]. The chain-breaking antioxidant properties of vitamin E was detected in the 1950s and consequently proved to be useful in preventing lipid peroxidation by scavenging chain-carrying peroxyl radicals and generates an induction period[18,19,20]. Furthermore, studies reported that severe vitamin E deficiency in human will leads to neuromuscular abnormalities because of free radical damage to the nerve cells[21,22]. Vitamin E deficiency seldom occurs in human as a result of dietary deficiencies but occurs as a result of genetic abnormalities in the α-tocopherol transfer protein (α-TTP)[23]. Vitamin E also possesses non-antioxidant functions, with vital role in cellular signaling by regulating protein kinase C[24]. Moreover, vitamin E in combination with selenium were exhibiting ability to prevent loss of spermatogenesis in males[25]. Some studies also indicated that vitamin E prevents most of the glutamate-induced neuronal cell death[26]. Furthermore, dietary of vitamin E can enhance immune responses in numerous animal models[27].

Metabolism of vitamin E

The hydrophobic nature of vitamin E make it preferentially located in oil storage organs, fat deposits and in cell membranes. It is transported around the body as an element of plasma lipoproteins. After ingestion of dietary vitamin E, it will be absorbed into the enterocyte, followed by packaging into chylomicrons. These nascent chylomicrons are then secreted into the lymphatic circulation. During the chylomicron catabolism in the circulation, the absorbed vitamin E is transferred to circulating lipoproteins and drained into the bloodstream. The high-density lipoprotein (HDL) in the bloodstream donates apolipoprotein C-II (APOCII) and apolipoprotein E (APOE) to the nascent chylomicron and thus converts it to a mature chylomicron. Lipoprotein lipase (LPL) is bound to the endothelial lining of capillary walls. During the lipolysis by LPL, various form of vitamin E could be transferred to tissues. Also, vitamin E could be exchanged between HDLs and other circulating lipoproteins, which could deliver vitamin E to the peripheral tissues. The resultant chylomicron remnant from lipolysis are primarily taken up by the liver through the chylomicron remnant receptors[28]. In liver, remnant chylomicron-associated vitamin E is incorporated into nascent very-low density lipoproteins (VLDL) via the action of α-TTP[29]. One of the vital determinants of vitamin E biological activity is the affinity of its analogues for α-TTP. The α-TTP has higher preference to α-tocopherol compared to other vitamin E isomers[30]. When the VLDL are secreted into the plasma circulation, VLDL are converted into intermediate density lipoprotein (IDL) and low density

lipoprotein (LDL) via the action of LPL[31,32]. Vitamin E is then transferred from plasma to cells through uptake facilitated by receptor-mediated lipoprotein endocytosis, lipid transfer proteins and lipases, and selective lipid uptake[33]. Studies demonstrated that LDL receptor were facilitating the tissue incorporation of plasma vitamin E as part of LDL, while LPL and phospholipid transfer protein enable the tissue incorporation of plasma vitamin E as part of triglyceride-rich lipoprotein[34–36]. Furthermore, IDL and LDL have LDL receptor-binding domains which allow receptor-mediated lipoprotein endocytosis to facilitate uptake of vitamin E into the peripheral tissue[37]. The pathways of vitamin E absorption and distribution are depicted in Figure 2.

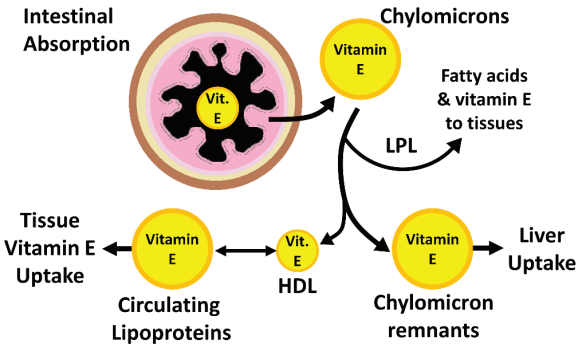

Figure 2. Pathways for vitamin E absorption and distribution.

Vitamin E is one of the most vital lipid-soluble antioxidants that protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction[31]. As an antioxidant agent, vitamin E does not work independently in scavenging free radicals. It is a part of the redox antioxidant system. Vitamin E is efficiently reduced from its free radical form (tocotrienoxyl or tocopheroxyl) back to its reduced native form via enzymatic or non-enzymatic mechanisms. Vitamin C can directly restore vitamin E and thiol antioxidant, for instance GSH, and indirectly restore vitamin E via redox antioxidant network. This system maintains the concentration of vitamin E radicals low. Hence, the loss or consumption of vitamin E is prevented[38].

ISOMER OF VITAMIN E

Tocopherol

Tocopherol contains a chromanol ring and a saturated phytyl side chain[39]. The structural formulae of tocopherols are shown in Figure 3. Tocopherol is primarily found in sunflower and olive oils. Among 8 isomers of vitamin E, α-tocopherol was firstly derived from wheat germ oil and named in 1936 by Evan *et al.*[40]. The α-tocopherol have the highest bioavailability among the isomers because of the recognition of α-TTP[41]. The core function of α-tocopherol is terminating the chain reaction of lipid peroxidation to inhibit cell membrane and LDL from oxidative disintegration[42]. Tocopherol also provides protection against peroxynitrite-induced lipid oxidation. Other than antioxidant function, vitamin E has functions in cell signaling activities, for instance regulation of protein kinase C, inhibition of cyclooxygenase-2 activity and modulation of phospholipase A2 activity were due to the present of α-tocopherol. The α-tocopherol could dilate blood vessels and interferes with aggregation of platelets[43]. Osakada *et al.*[44] reported that 1-10 μM α-tocopherol effectively protects striatal neurons against cytotoxicity induced by a L-buthionine-S,R-sulfoximine (BSO) via the reduction of oxidative stress. Study indicated that α-tocopherol can effectively relieve neuronal damage induced by oxygen-centered free radicals[45]. Also, α-tocopherol functions in regulating inflammation by reducing the release of cytokine interleukin-1β (IL-1β) via inhibition of 5-lipoxygenase pathway[45].

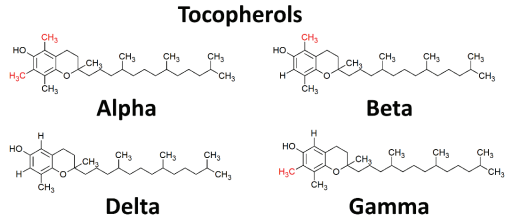

Figure 3. Structural formulae of Tocopherols.

Tocotrienol

Tocotrienols vary from tocopherols by having 3 double bonds in the hydrophobic tridecyl side chain[42]. Figure 4 depicted the structural formulae of tocotrienols. Tocotrienols are rich in barley oil and palm oil. More than 95% of studies on vitamin E focusing on α-tocopherol due to its richness in the human body and its antioxidant functions. Nevertheless, recent studies exhibited that tocotrienol possesses health-promoting properties such as vital neuroprotective effect, cholesterol lowering and anti-cancer properties that are usually not displayed by tocopherols[46]. Even though tocotrienols have low bioavailability, its antioxidant activity is higher than tocopherols[47]. The α-tocotrienol exhibited better peroxyl radical scavenging potency than α-tocopherol in liposomal membrane[48]. The unsaturated side chain of tocotrienol allowing even distribution of tocotrienol in the membrane bilayer that further enhance the interaction of chromanol ring of α-tocotrienol with lipid radicals. Tocotrienols also moves between lipid vesicles much faster than α-tocopherol. Furthermore, the chromanoxyl radical of α-tocotrienol (α-tocotrienoxyl) was to be recycled in membranes and lipoproteins more rapidly compared to α-tocopheroxyl radical[49,50].

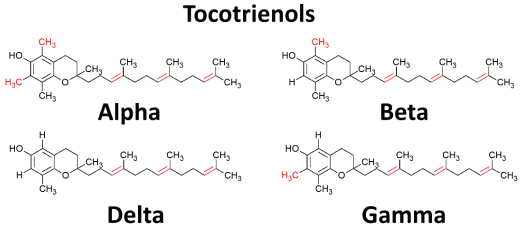

Figure 4. Structural formulae of Tocotrienols.

FUNTIONS OF VITAMIN E

Antioxidant

Vitamin E efficiently inhibits lipid peroxidation and scavenges the chain-propagating peroxyl radical. The scavenging outcome of α-tocotrienol was 1.5-fold higher than α-tocopherol in liposomes[49]. Moreover, α-tocotrienol was 6.5 times more effective in protecting cytochrome P-450 against oxidative damage. The tocotrienol-rich fraction (TRF) from palm oil is significantly more effective than α-tocopherol in inhibiting oxidative damage in rat brain mitochondria induced by ascorbate-Fe^{2+}, the free radical initiator azobis (2-amidopropane) dihydrochloride (AAPH) and photosensitization[51]. Furthermore, palm TRF at micromolar concentration providing better protection against copper-induced oxidation of plasma low density lipoprotein and also lipid peroxidation in human umbilical vein endothelial cells (HUVEC), as compared with α-tocopherol[52]. Moreover, the efficacy of α-tocotrienol in protection against Fe^{2+} NADPH-induced lipid peroxidation in rat liver microsome was 40 times higher than α-tocopherol[49]. This could strongly suggest that α-tocotrienol has greater scavenging effect compared to α-tocopherol.

Neuroprotection

Recent studies demonstrated that vitamin E have health benefit properties which go beyond their known antioxidant activity. Studies indicated that α-tocotrienol prevented both oxidative stress-dependent and oxidative stress-independent apoptosis, whereas δ- and γ-tocotrienol only inhibited oxidative stress-dependent apoptosis. This displays that neuroprotective effect of α-tocotrienol could be mediated via non-antioxidant anti-apoptotic actions in addition to its antioxidant property[53]. Moreover, nanomolar concentrations of α-tocotrienol could block glutamate-induced neuronal cell death, while α-tocopherol did not exhibit this property[54]. Furthermore, nanomolar concentration of α-tocotrienol could protect glutamate-induced cell death in mouse neuroblastoma HT4 cell via inhibition of 12-lipoxygenase and phospholipase A2 activation that further interfere the state of phosphorylation[26,54,55]. Additionally, tocotrienols effectively inhibited the activation of pp60 c-src kinase, a kinase that centrally involved in glutamate-induced cell death[26]. For neuroprotection properties, studies reported that other sources (e.g. microbial resources) were also demonstrating strong antioxidants[56–66] and neuroprotective properties[67–72], for instance radical scavenging and metal chelating potentials[73–80].

Other Beneficial Properties of Vitamin E

Numerous studies indicated that tocotrienols could suppress proliferation and induce apoptosis of several tumor cells such as breast, liver, lung, colon, stomach, skin, pancreas and prostate cancer cells[81–87]. The γ-tocotrienol and δ-tocotrienol were reported to have anti-tumor activity in breast cancer cell irrespective of estrogen receptor status[88,89]. The γ-tocotrienol also prevents cholesterol synthesis by suppressing 3-hydroxy-3-methylglutaryl-CoA reductase activity via a post-transcriptional mechanism[90]. The cardioprotective effects of tocotrienol are also facilitated via their ability to suppress inflammation thus reduce the expression of adhesion molecules and monocyte-endothelial cell adhesion[91].

BIOMARKER OF NEURONAL CELL INJURY

The continuous supply of oxygen and glucose is extremely important for brain energy metabolism. The disruption of this supply for a few minutes can introduces a sequence of biochemical event that lead to cell swelling, leakage and damage leading to neuronal cell death[92]. Intracellular components, such as neuron specific enolase (NSE), can be detected in the extracellular fluid and celebrospinal fluid (CSF) upon neuronal damage[93]. Among various intracellular proteins, the concentrations of NSE, S100β, glial fibrillary protein (GFAP) and myelin basic protein (MBP) exhibited positive correlation to the severity of the brain damage[94]. The NSE catalyzes the conversion of 2-phospho-D glycerate to phosphoenolpyruvate in glycolytic pathway and localized predominantly in neuronal cytoplasm[95]. The level of NSE in the cerebrospinal fluid has been used as markers of neuronal damage in patients with a variety of neurologic condition including status epilepticus and metastatic lung cancer. Furthermore, positive correlation was reported between the glutamate-induced changes of the neuron-specific enolase efflux fraction[96]. NSE is highly expressed as a glycolytic enzyme to replenish the ATP supply when energy depletion occurs, which could be due to neurotoxin agents for instance glutamate[97]. Meanwhile, S100β is a calcium-binding protein localized in astrocytes. The S100β levels were increased after central nervous system lesions. Furthermore, high level of NSE and S100β were reported in the CSF of infants and children after traumatic brain injury[98].

CONCLUSION

Vitamin E, which made up of tocotrienols and tocopherol isomers, is a known chain-breaking antioxidant. Studies demonstrated that vitamin E have health benefit properties beyond their known antioxidant activity. With the α-tocotrienol preventing both oxidative stress-dependent and oxidative stress-independent apoptosis, while δ- and γ-tocotrienol only inhibited oxidative stress-dependent apoptosis. These findings demonstrated that neuroprotective effect of α-tocotrienol could be mediated via non-antioxidant anti-apoptotic actions in addition to its antioxidant property[53]. Furthermore, TRF and α-tocopherol at concentration of 100 to 300 ng/mL demonstrated minor prophylactic properties but significant recovery ability in improving the glutamate-injured cell viabilities in both mono-culture and co-culture model. TRF at nanomolar concentration also exhibited better protection to neuronal cell against glutamate toxicity compared to α-tocopherol. Therefore, the putative mechanism of TRF and α-tocopherol action in protecting and recovering glutamate-injured cells was of great interest and warrant further research. More *in vivo* studies should be performed

to further understand the recovery mechanism of TRF and α-tocopherol in a complete body system.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

Authors Contributions

The literature review and manuscript writing were performed by H-MY and K-LL.

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Review Article

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Microbes from Peat Swamp Forest — The Hidden Reservoir for Secondary Metabolites?

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Abstract: Antimicrobial resistance is a significant threat to the healthcare sector. For the past century, there has been a decline in the discovery of new antibiotics. This has urged researchers to bio-prospect for new bioactive agents from microbes originating from untapped environments, as well as to explore the potential of other microbial genera apart from the well-known *Streptomyces*. Tropical peat swamp forests are an example of such an environment. Two novel antimicrobial-producing bacteria from the genera *Burkholderia* and *Paenibacillus* have been identified to produce potent antimicrobials. These two genera of bacteria have recently gained tremendous interest due to their genome complexity. They are known as multifaceted organisms not only because of their genetic content, but also due to their positive interactions with the environment along with a plethora of organisms including plants and animals. The interactions observed are attributed to their genomes and to their production of secondary metabolites including antimicrobials. Hence, this review provides an overview of the nature of tropical peat swamp forests, taxonomy and production of secondary metabolites of both *Burkholderia* and *Paenibacillus*, as well as discussing the future perspective of isolating antimicrobial-producing microbes from tropical peat swamp forests.

Keywords: Antimicrobials; *Burkholderia*; *Paenibacillus*; resistance; secondary metabolite; tropical peat swamp forest

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INTRODUCTION

Antimicrobials have been used for generations as prophylaxis to prevent initial or recurrence of infection, and as agents to destroy, inhibit or prevent pathogenic action of microbes[1]. Over time, use of antimicrobials has created an inevitable selective pressure leading to the evolution of microbes to resist the action of antimicrobials. Microbes can gain resistance towards antimicrobials intrinsically through mutations or by acquiring the ability via conjugation[2]. This phenomenon is further exacerbated by the extensive use of antimicrobials to control infections which has unprecedentedly accelerated the process and emergence of resistant microbes[3]. This is an alarming issue as the rapid emergence of antimicrobial-resistant pathogens limits treatment options and increases mortality. According to the Director General of the World Health Organization (WHO), we are heading towards a post-antibiotic era in which common infections and injuries could once again kill[4]. Therefore, there is an urgent need for alternative measures to tackle the crisis of antimicrobial resistance. One of the methods is by bioprospecting for new antimicrobials from untapped resources such as the tropical peat swamp forests where extreme conditions and low nutrients promote competition among microbes. The review will discuss the nature of tropical peat swamp forests, taxonomy and production of secondary metabolites of both *Burkholderia* and *Paenibacillus*, as well as discuss the future prospects of isolating antimicrobial-producing microbes from tropical peat swamp forests.

TROPICAL PEAT SWAMP FORESTS

Tropical peat swamp forests (TPSFs) are unique wetland ecosystems periodically flooded by fresh water

from rainfall[5]. Out of 30–45 million hectares of global wetlands in the world, about 18.1 million hectares are TPSFs widely distributed around Southeast Asia[6]. TPSFs contribute to almost 20% of the total global terrestrial organic carbon and form one of the largest terrestrial organic carbon sinks. Carbon is stored in the forest biomass with trees up to 70m tall, but most of it is sequestered peat layers up to 25m deep. Disturbance of TPSFs due to drainage and fire causes the release of greenhouse gases such as methane and carbon dioxide to the atmosphere. Burning of TPSFs results in about 25% of the total global greenhouse gas emissions from deforestation and forest degradation which result in an estimated 3% of total global anthropogenic greenhouse gas emissions. Furthermore, TPSFs hold a key role in regional hydrology (movement and distribution of water). This is because peat can hold 5–10 times its weight in water. This is important as the water stored can act as a buffer in reducing water velocity thus minimizing the impact of downstream flooding[5].

TPSFs grow on a substrate formed by the accumulation of layers of peat (partially decomposed organic matter) up to 25m deep. They are usually dome-shaped due to the buildup of peat, and are permanently waterlogged with a pH range of 2.9 to 4.5[8]. The leaching of tannic acids (20.2 ± 2.3 mg/l) from the leaves of endemic plants causes the dark brown, acidic water in TPSFs[9]. The dark brown water reduces light penetration and impedes photosynthesis by algae, which together with the slow flow rates and high temperatures create an anoxic environment, while toxic secondary compounds leach from the leaf litter which then reduces the microbial decomposition of organic matter (decay rates of 0.0006–0.0016 k day-1 for endemic plants), hindering nutrient recycling thus creating a highly concentrated carbon reservoir[5]. In addition, TPSFs are known to be ombrotrophic, receiving nutrients and water solely from rainfall and dust. The lack of nutrient input and slow decomposition rate results in a low nutrient environment in TPSFs[8].

BACTERIAL COMMUNITY IN TPSFS

It was previously thought that such extreme environmental conditions (acidic, waterlogged and low nutrient availability) meant low bacterial diversity. However, metagenomic studies have revealed the complexity of the genetic information of the bacterial community and high diversity[10]. Kanokratana *et al.* (2011)[10] deduced that bacteria constituted the most abundant microbial group in a Thailand TPSF. From the bacterial sequences identified, *Proteobacteria* was the largest species group (37.9% of total bacteria), which comprised mostly Alpha-proteobacteria, followed by *Acidobacteria* (35.0% of total bacteria). Other key minor bacterial phyla include *Verrumicrobia* (5.7%), *Planctomycetes* (9.6%), *Actinobacteria* (2.5%), *Bacteroidetes* (1.1%), *Nitrospirae* (1.8%), *Firmicutes* (0.4%) and others unclassified bacteria (6.0%). Moreover, the bacterial population (determined using next generation sequencing) in a Malaysian TPSF showed a similar pattern[11] as the Thailand TPSFs and was consistent with a previous study conducted by Jackson *et al.* (2009)[12] which showed that TPSFs are dominated by *Proteobacteria* and *Acidobacteria* (more than 50% of the total bacteria population).

Antimicrobial-producing bacteria from TPSFs

The slow degradation of organic matter in TPSF results in low levels of nutrients which creates a highly competitive environment[13]. Consequently, it is likely that bacteria would produce secondary metabolites such as antimicrobial compounds to secure their niche and resources. Such phenomena are consistent with the isolation of antimicrobial producing bacteria from two different TPSF: the southeast Pahang and Selangor TPSF. These antimicrobial producing isolates were identified via a polyphasic taxonomic approach to be novel species from the genus *Burkholderia* and *Paenibacillus*, namely *Burkholderia paludis* sp. nov. (from Pahang TPSF)[14] and *Paenibacillus tyrfis* sp. nov. (from Selangor TPSF)[15].

Burkholderia and their secondary metabolites

The *Burkholderia* genus consists of a group of ubiquitous bacteria that occur in aquatic environments, soil, plant rhizospheres and animals. *Burkholderia* are mesophilic Gram-negative rods, oxidase positive, motile microorganisms. *Burkholderia* can be characterized phenotypically by their pigmentation, presence of hydroxyl fatty acids of 14, 16 and 18 carbon atoms, possession of distinct polar lipids, and by having Q8 cellular respiratory quinones[14].

The genus can be divided into three groups: *Burkholderia* sensu stricto, *Paraburkholderia* and *Caballeronia*[16]. *Burkholderia* sensu stricto is a group of closely related *Burkholderia* species that share a high degree of 16S rRNA (98–100%) and recA (94–95%) gene sequence similarity which makes them difficult to be differentiated using conventional molecular techniques. To differentiate different species of *Burkholderia* sensu stricto, multilocus sequence analysis (MLSA) is usually adopted as the technique provides the discriminatory power needed for both identification and differentiation[17]. *Burkholderia* sensu stricto species have diverse ecological roles and have been used in biocontrol and bioremediation. Several *Burkholderia* sensu stricto species can be used for biocontrol agents as they can produce secondary metabolites to repress soil borne pathogens. Some *Burkholderia* sensu stricto species can act as plant growth promoters. They can also be used for bioremediation of recalcitrant xenobiotics, for instance, *Burkholderia xenovorans* can degrade chlorinated toxic phenolic compounds commonly found in pesticides and herbicides[18].

In contrast, nearly all *Paraburkholderia* species (e.g. *Paraburkholderia bryophila*, *Paraburkholderia tropica* and *Paraburkholderia nodosa*) and *Caballeronia* (e.g. *Caballeronia ginsengisoli*, *Caballeronia terrestris* and *Caballeronia humi*) are plant growth promoters as they are able to fix nitrogen and supply nutrients to their plant hosts[16, 19]. Many secondary metabolites with antimicrobial activity are produced by the *Burkholderia* species have been identified. They usually possess antifungal and/or antibacterial activity (Table 1).

Table 1. Antimicrobials produced by *Burkholderia* species.

Compounds	*Burkholderia* species	Bioactivity	References
2-pyrrolidone-5-carboxylic acid	*Burkholderia* sp. HD05	Antifungal	Zhang *et al.* (2019)[20]
Bis-(2-ethylhexyl) phthalate	*Burkholderia gladioli* OR1	Antibacterial	Bharti *et al.* (2015)[21]
Burkholdines	*Burkholderia ambifaria* 2.2N	Antifungal	Tawfik *et al.* (2010)[22]
Cepacidin A	*Burkholderia cepacia*	Antifungal	Lee *et al.* (1994)[23]
Cepacins A and B	*Burkholderia cepacia* SC 11	Antibacterial	Parker *et al.* (1984)[24]
Cepafungin	*Burkholderia* sp.	Antifungal	Shoji *et al.* (1990)[25]
Cepalycin	*Burkholderia cepacia*	Antifungal	Abe and Nakazawa (1994)[26]
Enacyloxins	*Burkholderia ambifaria AMMD*	Antibacterial	Mahenthiralingam *et al.* (2011)[27]
Gladiolin	*Burkholderia gladioli*	Anti-mycobacterium	Song *et al.* (2017)[28]
Icosalide	*Burkholderia gladioli*	Antibacterial	Dose *et al.* (2018)[29]
Iminopyrrolidines	*Burkholderia plantari* #9424 ICMP	Antibacterial	Mitchell and Teh (2005)[30]
Occidiofungin	*Burkholderia contaminans* MS14	Antifungal	Lu *et al.* (2009)[31]
Phencomycin	*Burkholderia glumae* 411gr-6	Antibacterial	Han *et al.* (2014)[32]
Pyochelin	*Burkholderia paludis*	Antibacterial	Ong *et al.* (2017)[33]
Pyrazoles derivatives	*Burkholderia glumae* #3729 ICMP	Antibacterial	Mitchell *et al.* (2008)[34]
Pyrrolnitrin	*Burkholderia cepacia*	Antifungal, antibacterial	El-Banna and Winkelmann (1998)[35]
Vietnamycin	*Burkholderia vietnamiensis*	Antibacterial	Rowe *et al.* (2016)[36]
Xylocandin	*Burkholderia cepacia*	Antifungal	Meyers *et al.* (1987)[37]

Paenibacillus and their secondary metabolites

The genus *Paenibacillus* comprises aerobic/facultative anaerobic and endospore-forming bacteria, with a majority of them typically showing Gram-positive cell wall structures[38,39]. This genus was initially included in the genus *Bacillus* based on morphological characteristics prior to reclassification. The genus *Paenibacillus* — which means "almost a *Bacillus*" was then proposed by Ash et. al (1993)[40] using phylogenetic classification[39]. The *Paenibacillus* spp. have MK-7 as major quinone, anteiso-$C_{15:0}$ as major cellular fatty acid, DNA G + C content which ranges from 39 to 59 mol%, and genome size ranges from 3.02 Mbp (eg. *Paenibacillus darwinianus*) to 8.82 Mbp (eg. *Paenibacillus mucilaginosus*)[38,39].

This group of bacteria can be isolated from a variety of environments, mainly from soil. They are often associated with humans, animals, and plants. The majority of the *Paenibacillus* spp. are producers of antimicrobial compounds and enzymes that are useful for bioremediation (Table 2). Furthermore, some of these compounds can be utilized as bio-fertilizers for plant growth promotion or bio-pesticides against root pathogens[39].

Table 2. Antimicrobials produced by *Paenibacillus* species.

Compounds	*Paenibacillus* species	Bioactivity	References
Paenibacillin	*Paenibacillus polymyxa* OSY-DF	Antibacterial	He *et al.* (2008)[41]
Paenicidin A	*Paenibacillus polymyxa* NRRL B-30509	Antibacterial	Lohans *et al.* (2012)[42]
Penisin	*Paenibacillus* sp. A3	Antibacterial	Baindara *et al.* (2016)[43]
Polymyxin	*Paenibacillus polymyxa*	Antibacterial	Nation and Li (2017)[44]
Colistin	*Paenibacillus polymyxa*	Antibacterial	Tambadou *et al.* (2015)[45]
Octapeptin	*Paenibacillus tianmuensis*	Antibacterial	Qian *et al.* (2012)[46]
Paenibacterin	*Paenibacillus tiaminolyticus* OSY-SE	Antibacterial	Huang *et al.* (2014)[47]
Pelgipeptin	*Paenibacillus elgii* B69	Antibacterial	Ding *et al.* (2011)[48]
Gavaserin	*Paenibacillus polymyxa*	Antibacterial	Pichard *et al.* (1995)[49]
Fusaricidins	*Paenibacillus polymyxa* KT-8	Antibacterial	Kajimura and Kaneda (1997)[50]

FUTURE PERSPECTIVES IN TPSF MICROBIAL CULTIVATION

Thus far, only two bacteria (*Burkholderia paludis* sp. nov and *Paenibacillus tyrfis* sp. nov) with antimicrobial-producing ability from TPSF have been successfully cultivated and identified. These bacteria such as *Burkholderia* from Proteobacteria and *Paenibacillus* from Firmicutes are common phyla of bacteria dominating the TPSFs. Hence, what needs to be improved in order to isolate the uncommon bacteria with antimicrobial-producing ability from TPSF? The possible reasons for isolating those common bacteria are the suitability of media used and the incubation period.

The use of alternative culture media

The failure in cultivating peat-inhabiting bacteria using culture dependent techniques is often due to the usage of conventional media such as nutrient and tryptone soy media. Such media contain near-neutral pH with high mineral salt content that do not simulate the acidic, low nutrient conditions of TPSFs[8]. Besides that, it favors fast growing bacteria and these fast-growing bacteria will outgrow the other slow growing bacteria[51]. Therefore, there is a need to use diluted acidic media with low salt content such as MM1, Medium M1, Medium M2, Medium M31, nitrate mineral salt media, peat extract medium and R2A in order to cultivate various peat-inhabiting bacteria at the same time suppressing the fast-growing bacteria (Table 3).

Table 3. Examples of media with low salt content.

Type of Media	Media Compositions	References
MM1	100 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 8mM KH_2PO_4, 16 mM K_2HPO_4 and micronutrient	Mehta and Rosato (2005)[52]; Schulte and Bonas (1992)[53]
Medium M1	0.25 g/L KNO_3, 0.1 g/L KH_2PO_4, 0.1 g/L $MgSO_4$, 0.02 g/L $CaCl_2.2H_2O$, 0.1 g/L yeast extract, 0.005 g/L Na_2MoO_4 and 0.05% (w/v) carbon source	Dedysh *et al.* (2006)[54]
Medium M2	0.1 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4$, 0.02 g/L $CaCl_2.2H_2O$ and 0.05% (w/v) carbon source	Dedysh *et al.* (2006)[54]
Medium M31	0.1 g/L KH_2PO_4, 20 mL Hutner's basal salt, 1 g/L *N*-acetylglucosamine, 0.1 g/L peptone and 0.1 g/L yeast extract	Kulichevskaya *et al.* (2012b)[55]
Nitrate mineral salt media	1 g/L KNO_3, 1 g/L $MgSO_4.7H_2O$, 0.717 g/L $Na_2HPO_4.12H_2O$, 0.272 g/L KH_2PO_4, 0.2 g/L $CaCl_2.6H_2O$ and 0.005 g/L ferric ammonium EDTA	Dedysh and Dunfield (2011)[56]
Peat extract medium	500 ml of supernatant (400 g of wet peat mixed with 200 ml of distilled water) and 500 ml of base Medium M2	Dedysh *et al.* (2006)[54]
R2A	0.5 g/L yeast extract, 0.5 g/L proteose peptone, 0.5 g/L casamino acid, 0.5 g/L dextrose, 0.5 g/L soluble starch, 0.3 g/L sodium pyruvate, 0.3 g/L KH_2PO_4 and 0.05 g/L $MgCl_2$	Dedysh *et al.* (2006)[54]; Edenborn and Sexstone (2007)[57]; Taylor *et al.* (2002)[58]

Based on other studies on northern wetlands, several types of bacteria were isolated using the minimal media shown in Table 3. For example, an acidophilic methane-oxidizing bacterium was isolated using minimal mineral medium containing vitamin mixture with methane as sole carbon source. In another study conducted by Dedysh *et al.* (2006)[54], Alpha-proteobacteria, Beta-proteobacteria, Gamma-proteobacteria, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* were isolated using Medium M1, Medium M2 and diluted R2A media. However, acidobacteria and planctomycetes were not found in the same study which might due to the reason that some Acidobacteria such as *Granulicella* species are inhibited by the presence of phosphates found in most minimal media[59]. Therefore, agar selection is one of the main criteria leading to successful cultivation of peat-inhabiting bacteria.

Prolonged incubation time

Most peat inhabiting bacteria are slow growing even under optimal growth conditions. These bacteria are usually fastidious facultative anaerobes such as methanotrophs, acidobacteria and planctomycetes. In a study conducted by Kulichevskaya *et al.* (2012a)[60], colonies of *Telmatocola sphagniphila* (planctomycetes) were developed after 4 weeks of incubation using modified Medium M2 supplemented with trace element and vitamins under 5% CO2 (v/v) condition. *Telmatobacter bradus* which is a facultative anaerobe belonging to the phylum Acidobacteria requires 4 weeks to grow using Medium M2[59]. In another example, *Telmatospirillum sibiriense* which is an acidotolerant facultative anaerobic, only had observable colonies after 5 months of incubation on N-free minimal media supplemented with a reducing agent[61]. This also indicates that the presence of reducing agents might promote the growth of fastidious facultative anaerobes. However, there is no published result on the successful isolation of strict anaerobes from either the northern wetlands or TPSFs, which suggest the need for other reducing agents to be included in the culture media[51]. Furthermore, a more stringent method should be applied during sampling collection where peat samples are to be placed immediately in anaerobic conditions prior to sample transportation. This reduces the exposure of oxygen to the anaerobic bacteria at the same time simulating the actual anoxic conditions in the TPSF. To sum up, there is a need to incubate the peat culture for a prolonged duration with anoxic conditions, minimal nutrients, and with appropriate supplements in order to isolate anaerobes from TPSF.

GENOMIC APPROACH TO DISCOVER BIOACTIVE SECONDARY METABOLITES PRODUCTION

Whole genome sequencing of bacteria has become increasingly common for routine use in microbiological

laboratories. Subsequently, a large quantity of DNA sequence data from different microorganisms is currently available in public databases. As a result, this creates a path for uncovering novel natural products from microbes by utilizing new bioinformatics tools[62]. For instance, many recent studies have been performing whole genome sequencing of drug-prolific producers such as the *Streptomyces* spp.[63–69] for further investigation of their secondary metabolite production ability This could also be useful for the prediction of novel products of non-ribosomal peptide synthetases (NRPSs) and polyketides synthases (PKSs) through application of various sequence analysis tools[62]. Similarly, this strategy has been applied for *Burkholderia* and *Paenibacillus*[70,71,72]. By taking the genus *Burkholderia* as an example, the determination of genome sequences of these bacteria has essentially created a route for in silico structural prediction, wet lab experimental design, and execution. Genome-guided approaches, which are made possible through accessibility of extensive genome sequence data coupled with genome-mining technologies, have warrant the discovery of structurally and functionally diverse natural products from numerous *Burkholderia* strains[71, 73,74].

CONCLUSION

Tropical peat swamp forests are indeed a promising environment to source for secondary metabolites. However, culture-dependent methodologies should be scrutinized to ensure cultivation of rare bacterial species with important ecological and commercial roles which have never been captured before. Besides, whole genome sequencing of the bacteria in the near future may allow further understanding of the antimicrobial synthesizing capability of these bacteria. Nevertheless, efforts should be made to culture microbes from different genera with similar potential to discover new bioactive secondary metabolites.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

Author Contributions

K-SO performed the literature search, critical data analysis and performed the writing of this review. Technical support and proofreading were contributed by JW-FL and VL. K-SO, CMY and S-ML founded the review writing project.

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Review Article

Progress in Microbes and Molecular Biology

HH PUBLISHER

Dietary Glycaemic Index and Type 2 Diabetes Mellitus: Potential Modulation of Gut Microbiota

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Abstract: Diet therapy is often the first-line approach in prevention and management of type 2 diabetes mellitus (T2DM). Adoption of low glycaemic index (GI) diet one of the recent dietary strategies to modulate glycaemic response in individuals with T2DM. Generally, diet has strong influence on the gut microbiota, which recently have been found to be associated with insulin resistance and the inflammatory response in diabetes. The possible modulation of the gut microbiota with dietary intervention is a topic of emerging interest, with limited evidence among T2DM population. In this review, we have narrated the available evidence and discussed the current knowledge about diet manipulation associated with dietary GI in order to shape the gut microbiota. As a conclusion, we have pointed out several key research directions that may have helpful impact on diet interventions with modulation of gut microbiota on the pathogenesis and therapeutic implications in T2DM.

Keywords: microbiome; type 2 diabetes mellitus; carbohydrate-glycaemic index; nutrition

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INTRODUCTION

Diabetes mellitus is defined as a "metabolic disorder of multiple aetiology characterised by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both"[1]. Type 2 diabetes mellitus (T2DM) is the most common form of diabetes, accounting for about 80% of all diabetes cases mostly after the age of 30[2]. T2DM is characterised by three basic abnormalities — insulin resistance, impaired insulin secretion and increased hepatic glucose production, either of which may be the predominant feature[3,4]. This results in a disorder of carbohydrate metabolism, and fat, protein and mineral metabolism also can be affected.

The World Health Organization has defined the diagnostic criteria for diabetes through a single raised glucose reading with symptoms, otherwise raised values on two occasions, of either fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or with a glucose tolerance test, two hours after the oral dose a plasma glucose ≥ 11.1 mmol/l (200 mg/dl)[1]. In diabetes management, nearly all T2DM related research outcomes are looking at reduction in glycosylated haemoglobin (HbA1c) and report from the UK Prospective Diabetes Study (UKPDS) has recommended HbA1c to be maintained below 7.1% to minimise T2DM-related complications[5].

It has been shown that individuals from primitive societies, where the incidence of diabetes is low who then move to societies where food is too readily available, often progress to develop diabetes[6]. Although many people with T2DM could be managed by dietary modification alone, eventually they may require insulin therapy due to its progressive nature.

Although T2DM could be inherited, modifiable factors such as body composition and nutrition play important roles in the etiology of T2DM[7]. The goals of Medical Nutrition Therapy (MNT) in people with diabetes are to achieve and maintain normal blood glucose, lipid and lipoprotein and blood pressure levels by addressing the nutritional needs and maintaining the pleasure of eating which eventually, prevent or slow the development of complication[8,9].

One of such dietary modifications that many MNTs recommend will be adoption of low glycaemic index (GI) diet. GI ranks carbohydrate-containing foods according to their effect on postprandial glycaemia[10,11]. GI is a measure of the increase in blood glucose two hours after consumption of the food of interest, with reference to glucose or white bread. Glycaemic load (GL), a product of GI and amount of carbohydrate in the food, is another measure of glycaemic response to carbohydrate-containing foods and could improve the

evaluation of glycaemic response to a diet[12]. Foods with low GI such as parboiled rice, barley, oats and legumes lower the postprandial hyperglycaemia, while high GI foods such as white bread, potatoes, white rice and commercial breakfast cereals will show the opposite effect[8,13].

Additionally, increasing evidence has highlighted that the role of gut microbiota in a multitude of human illnesses, both inside and outside of gut, including irritable bowel syndrome[14], colorectal cancer[15,16], skin diseases[17,18], neurological-related diseases[19,20]. Similarly, there are also emerging findings on the strong association between development of diabetes and gut microbiota[21,22]. Gut microbiota is a dynamic identity that can result in various pathophysiological changes in human's internal environment, though the composition changes gradually over the lifetime[23,24]. Gut microbiota plays a critical role in several metabolic functions namely amino-acid synthesis, absorption of dietary fats and fat-soluble vitamins, production of short-chain fatty acids (SCFAs), activation of glucose homeostasis, lipid energy metabolism, calorie removal and regulating bile acid transformation among others[25]. This may predispose or protect an individual against diabetes.

As gut microbiota can be easily influenced by environmental factors especially one's dietary intake, there is a potential for gut microbiota to act as a modulator of dietary GI and T2DM relationship. In this review we will be summarizing the evidence associating dietary GI and T2DM, and subsequently discuss the potential role of gut microbiota in modulating this relationship.

DIETARY GLYCAEMIC INDEX AND DIABETES

A cross-sectional study in South Italy found a dose-dependent relationship between dietary GI and GL with HbA1c, where patients with diabetes with highest GI and GL had the highest HbA1c levels[26]. GI-based dietary education has been suggested to be a useful tool in diabetes management[27,28]. Miller and Gutschall[29] reported a nine-week nutrition education regarding GI and GL which improved dietary intake, knowledge, outcome and efficacy expectations and empowerment for diabetes management. Although GI could lead to a better dietary intake in people with diabetes, only few organisations recommended the use of low GI diets[30–32].

Retrospective study by Burani *et al.*[26] found a reduction in HbA1c of 19% and body mass index (BMI) of 8% following inclusion of low GI diet in lifestyle intervention, which was well accepted by the participants. Several trials have supported the role of GI in glycaemic control in patients with T2DM[34]. Significant improvements have been seen in HbA1c and/or FPG[34–37], insulin sensitivity[34] and serum fructosamine[36] with low GI diets. The role of GI diets in glycaemic control has been confirmed by several meta-analyses and reviews[13,38,39]. However, there is a lack of studies on the technology assisted low GI interventions, and only one feasibility nutritionist-delivered, PDA-assisted low-GI dietary intervention by Ma *et al*.[40] has been discovered.

Low GI diets also have been shown to have favourable impact on lipid profile and reduction in cardiovascular risk of patients with T2DM[35,41–44]. Studies have reported significant decrease in total cholesterol and LDL-C with prescribed low GI diets[34]. Longer educational programmes to improve diet quality by emphasizing low GI diets also have shown to lower the LDL-C[45,46] and increase the HDL-C[37]. Besides the cholesterol levels, a low GI or GL diet may be preferred for the dietary management of T2DM because of sustained reductions in postprandial glucose and c-reactive protein[47] and the increase in the plasma adiponection concentrations[48].

There is also evidence showing the positive impact of low GI diet on other health outcomes in people with diabetes. Low GI diet accompanied with exercise programme was found to improve cardiovascular health[49] and protect against-induced hypoglycaemia in T2DM patients[50]. Low GI diet could assist with the weight management programme in patients with diabetes[43,46]. Yusoff and colleagues[36] reported a significant reduction in waist circumference in Asian patients after 4 months of following a low GI diet. Low GI diet has also generally results in better cognitive performance in the postprandial period in adults with T2DM and reduce their dependency on diabetes medication[45,51].

Interestingly, there were studies which did not support the role of GI in diabetes management[52]. In one of such studies, low GI diet with calorie restriction in overweight patients with T2DM did not find any significant reduction in HbA1c[46]. Data derived from the Atherosclerosis Risk in Communities study suggest high GL intake to be a CHD risk factor only among Whites without diabetes and not in individuals with diabetes[53]. Cheong *et al.*[54] concluded addition of a low-GI component to a walking did not improve anthropometric or metabolic outcomes in diabetic patients. A review by Barojek and Morello[55] has identified short coming in terms of power of the study and confounders, which could have affected otherwise positive findings in these studies.

As low GI foods are generally rich in fiber and other nutrients, the consumption of this diet has been shown beneficial to diabetic patients[56]. Incorporation of such foods in every day diet may be an effective approach for weight management, glycaemic control and favourable lipid profile. However, the concept of GI should not be used in isolation, but to be used as an adjunct treatment to existing lifestyle management of T2DM in fine-tuning the glycaemic control[45,57].

DIETARY GLYCAEMIC INDEX AND GUT MICROBIOTA

Exploration of a potential association between dietary GI and changes in the proportion of certain gut microbiota is relatively a newer research area, with limited evidence among T2DM population.

An experimental study among individuals at risk of metabolic syndrome showed largest increase in the *Bifidobacterium* spp. (an established gut health biomarker) level in high carbohydrate/high glycaemic index (HC/HGI) group compared to control group[58]. This change was

associated with reduced fasting glucose, fasted insulin and cholesterol levels compared to baseline. Furthermore, HC/HGI group was also associated with increased *Bacteroides* numbers as well as reduction in body weight, BMI and waist circumference. Both *Bacteroides* and *Bifidobacterium* spp. have been independently associated with reduction in risk factors for metabolic syndrome and improved body energy regulation in the past[59]. Interestingly, the study found an increased abundance of *Faecalibacterium prausnitzii* with both high saturated fat (HS) diet and high carbohydrate/low glycaemic index (HC/LGI) diets. HS group also experienced an increase in faecal SCFA concentration. However, faecal acetate percentage may be inversely correlated with absorbed acetate percentage (after rectal infusion), as demonstrated by Vogt and Wolever[60]. In that case, higher SCFA noted in the HS group may be due to decreased absorption, instead of higher colonic fermentation.

A study exploring specific foods with lower GI responses *in vitro*, found minimally processed wholegrain cereals such as wholegrain oats and granola resulted in significant growth in the friendly bacteria namely *Bifidobacterium* genus and *Lactobacillus-Enterococcus* group[61]. Wholegrains with minimal processing also resulted in increase in *Atopobium* cluster and *Bacteroides-Prevotella* group after 10 hours. Increase in *Clostridium histolyticum* after instant porridge fermentation (highly processed wholegrains) is also noted to be significantly higher compared to the decrease observed in minimally processed wholegrains.

Another low GI grains that were investigated in the past were barley grains. An *in vivo* study found barley intake lead to increased abundance of *Prevotella* and *Lactobacillus*, as well as *Candidatus homeothermaceae* in obese and lean mice[62]. Barley intake was also linked with lower levels of plasma insulin and resistin, a cysteine-rich peptide secreted by adipocytes, immune cells, and epithelial cells which are found in higher levels in metabolic syndrome cases[63].

Another study utilised models such as static *in vitro* digestion and dynamic gastric model to simulate the normal digestion process in investigating relationship between different types of barley grains and microbiota[64]. During early stage of digestion in the study, it noted that the digesta of wild-type barley *Hordeum vulgare* cv Golden Promise (Hv) and Amylose-only (AO) breads portrayed higher abundancy of Firmicutes, whereas wheat and wild barley *Hordeum vulgare* subsp. Spontaneum (Hs) grain bread digesta contained lower levels of it. Hs grain bread digesta demonstrated increased abundance of actinobacteria (30-fold higher than control) to the detriment of Bacteroidetes and Firmicutes. However, samples representing later stages of digestion behaved differently as Bacteroidetes and Actinobacteria were found to be abundant in all digesta. Specifically, increased Actinobacteria was noted in AO fermentation (compared to early digestion stage samples) while Bacteroidetes was more abundant in fermentation of Hs grain digesta. Aside from that, Proteobacteria levels in wheat bread digesta fluctuated from early digestion to late digestion stage. Therefore, these results portray the potential of low GI barley-based bread in regulating gut microbiota.

ROLE OF GUT MICROBIOTA IN TYPE 2 DIABETES

Several studies have investigated and showed some forms of association between gut microbiota and development of T2DM. The role of gut microbiota may be evaluated in several aspects.

Insulin resistance in T2DM patients may have resulted from increased production of hepatic triglyceride facilitated by Firmicutes and Bacteroidetes, as they enhance the monosaccharide uptake from the host gut[65]. Aside from the impact on carbohydrate metabolism, high ratios of Firmicutes to Bacteroidetes is also found to alter the production of SCFAs with an increase in acetate production and decrease in butyrate production[66]. The increased levels of acetate in the blood is found to result in insulin resistance and heightened production of ghrelin (an-appetite stimulating hormone) in the stomach, as illustrated by a recent study among individuals with metabolic syndrome[67]. On the other hand, decrease in butyrate levels also encourage insulin resistance through promotion of low-level inflammation[68].

Aside from the diversity of gut microbiota, another aspect that could be looked into is its role in facilitating immune response which lead towards development of T2DM. Abundance of *Prevotella* bacterial species was found particularly in obese T2DM individuals, and known to increase the levels of pro-inflammatory cytokines, besides encouraging low-grade inflammation and insulin resistance[69]. *Verrucomicrobia*, on the other hand, which are known to contribute towards the maintenance of anti-inflammatory state of gut and improve insulin sensitivity were found less in T2DM individuals in a study conducted in Pakistan[70]. The same study also found an increase in the levels of gram-negative bacteria such as *Dialister* and *Allisonella*, which may have contributed to the rise in levels of lipopolysaccharide (LPS), which eventually binds with CD14 and mediates inflammatory response. Another class of bacteria which is found to be abundant among people with diabetes is *Fusobacteria*, which plays critical role in inflammatory responses, mounting adhesiveness to host epithelial cells and energy generation[71,72].

Furthermore, the link between gut microbiota and T2DM can also be explored by looking into their association with bile acids. It is well-known that one of the important symbiotic roles played by gut microbiota is in terms of bile acid transformation, as their composition has been found to affect the concentration and composition of circulating bile acids[73]. This in turn affects the individual's risk in developing obesity and related disorders. Most of the bile acid biotransformation occurs in the large intestine which is extremely rich in microbiota, through a complex process[74]. This is one process in which the role of gut bacteria can be directly observed, as it is catalysed by bile salt hydrolase (BSH), an enzyme which is present in several gut bacteria such as *Clostridium*, *Bacteroides*, *Lactobacillus*, *Bifidobacterium* and *Enterococcus*[75]. Bile acids in the blood circulation play a role in homeostasis of carbohydrates and lipids, further reinforcing their importance as regulatory molecules in that aspect[73]. Bile acids also pose some direct

antimicrobial action which subsequently impacts the survival and colonisation of certain gut microbiota[76].

As an individual's food consumptions affect both gut microbiota and development of diabetes, further exploration can be made to investigate the changes in gut microbiota according to the type of prescribed dietary pattern, and its impact on diabetes development and/or severity.

CONCLUSION

The search for the optimal nutritional strategy in T2DM patients remains an unresolved issue. It is important that the potential benefits of suitable GI diet and the T2DM patient's microbiota, which in turn will impact on the progression of disease complication, are taken into consideration. In this review, we have provided evidence that different dietary patterns have different impacts on gut microbial composition. These findings suggest that the gut microbiota contribute to the pathophysiological regulation of glucose tolerance, insulin secretion and in inflammation.

Hence, future studies should define the features of the gut microbiome in diet consumption that contribute to the T2DM in defined populations. Furthermore, with the emerging advances in technology, the relationship between important biomarkers to changes in gut microbiota and microbiota metabolites modulated by recommended diets are worth investigating in future studies to aid prevent or treat diabetes-related disorders in a strategic manner. In addition, it is also important to focus on bile stress and its effects on the gut microbiota in future direction in finding therapeutic strategies to reduce the aggregate metabolic burden in human populations. Some bacteria can use bile as their host to regulate virulence determinants and produce secondary bile acids that regulate the normal homeostasis of tissues in human body which leads to unwanted complications. Thus, further studies should investigate how bacteria sense bile and regulate it response it induces and reveal the effect of different bile acid profiles in host tissues by virulence factor production. Lastly, approaches to modulate the microbiome-bile acid formation through diet may likely reduce the risk and/or treat metabolic diseases and this need further investigation.

Author Contributions

The review and manuscript writing were performed by HD, TK and AR.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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Review Article

Progress in Microbes and Molecular Biology

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Insight of microRNA role in Colorectal Cancer

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Abstract: The colorectal cancer is among the most predominant cancer in the world including Malaysia. Numerous factors could contribute towards colorectal carcinogenesis and one of the factors is genetic predisposition. Mutations in the V-Ki-Ras2 (Kras) oncogene have been implicated in 30-50% of the colorectal cancer patients and usually lead to poorer prognosis. The challenging ability for the early detection of colorectal cancer still poses an enormous challenge to oncologist as there are limited or no signs or symptoms in the early stage of colorectal cancer. Many studies were conducted hoping to further understand colorectal cancer for a better diagnosis and prognosis. As early detection of colorectal cancer frequently leads to good prognosis. The gold standard for prognosis depends on the stage of the tumor at the time of diagnosis. Lately a group of small, non-coding RNAs termed microRNAs (miRNAs) exhibited capable outcomes in cancer research. Numerous miRNAs were discovered to play a key role in regulatory mechanism in numerous cancers. Differential miRNAs expression among tumors and non-tumor controls are highly valuable in recognizing miRNAs that could have vital role in carcinogenesis. Recently some miRNAs were discovered to play a vital role in colorectal carcinogenesis. Thus, miRNAs have emerged as highly useful tool for scientists to comprehend carcinogenesis better. For example, miR-21 and miR-106a were highly expressed in colorectal cancer. While miRNAs including miR-17-92 cluster, miR-21, miR-34, miR-135 and miR-196a also exhibited high association with colorectal cancer. Therefore, this article aims to provide insight of miRNAs role in colorectal cancer for a better understanding of this disease.

Keywords: microRNA; miRNA; colorectal cancer; Malaysia; cancer

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INTRODUCTION

Colorectal Cancer

Colorectal cancer is a kind of cancer that forms in the tissues of the colon and rectum. Classically, most colorectal cancers are adenocarcinomas or cancer arises from cells making and releasing fluids such as mucus. Alike other cancers[1], colorectal cancer is a multifactorial disease that exhibited complex interactions among inherited susceptibility and environmental factors leading towards the development of the disease[2,3].

Prevalence of colorectal cancer

A total of 115,238 new cancer cases were diagnosed in Malaysia for the period of 2012-2016. The Malaysian National Cancer Registry Report 2012–2016 indicated that age-standardized incidence rates (ASR) for all cancers were 86.1 for males and 101.6 for females per 100,000 populations[3]. The 5 most common cancers among Malaysian were reported as breast, colorectal, trachea, bronchus and lung (TBL), lymphoma and nasopharynx cancer. The statistics indicated that colorectal cancer ranked 2nd most common cancer in Malaysia. Total ASR was at 13.5 per 100000 population. Colorectal cancer was somewhat higher in males (14.8 per 100000) as compared to females (11.1 per 100000)[3]. Looking at different ethnicities, Chinese documented the highest ASR (21.4 per 100000), followed by Indian (11.3 per 100000) and Malay (9.5 per 100000)[4]. Data obtained from the National Cancer Registry 2007 indicated that colorectal cancer ranked 2nd as one of the most common cancers in Malaysia, with ASR for male at 85.1 per 100000 and female at 94.4 per 100000 population[5].

Diagnosis of colorectal cancer

Diagnosis is extremely important in the management of colorectal cancer. Currently there are few types of tests and procedures used to diagnose and detect colorectal cancer as listed here:

- Fecal occult blood test — detecting occult blood in the feces
- Double contrast barium enema — liquid containing barium is placed into the rectum and coats the lower gastrointestinal tract. X-rays are undertaken to detect abnormal regions of the colorectal
- Colonoscopy — a colonoscope is introduced via the rectum into the colon to investigate polyps, abnormal areas or cancer
- Sigmoidoscopy — a sigmoidoscope is introduced via the rectum into the sigmoid colon to view for abnormal areas, polyps or cancer.
- Virtual colonoscopy — using computed tomography to generate series of photos of the colon and allows the view of any abnormal areas such as polyps in the colon.

Staging of colorectal cancer

Staging is the important procedure of investigating how extensive a cancer has spread and is one of the most important factors in defining the prognosis and treatment choices for cancer patients. Several staging systems are employed for colorectal cancer. The most common staging system is the TNM system established by American Joint Committee on Cancer (AJCC). TNM system illustrates three key evidence:

- **T** illustrates how extensive the main tumor has spread into the wall of the intestine.
- **N** explains the extent of spread to nearby (regional) lymph nodes.
- **M** specifies whether the cancer has metastases or spread to the other organs of the body.

The wider staging of a cancer is usually represented by roman numbering for instance I, II, III and IV originated from the TNM values. Specifics of this staging system are demonstrated in Table 1.

Table 1. Colorectal cancer staging system.

AJCC stage	TNM stage	Details
0	Tis N0 M0	Tumor has not expanded beyond the inner layer (mucosa)
I	T1 N0 M0	Tumor has invaded submucosa
I	T2 N0 M0	Tumor has invaded muscularis propria
II A	T3 N0 M0	Tumor has invaded subserosa
II B	T4 N0 M0	Tumor has invaded adjacent organs or perforated the visceral peritoneum
III A	T1-2 N1 M0	Tumor has extended to 1-3 regional lymph nodes. T1 or T2.
III B	T3-4 N1 M0	Tumor has extended to 1-3 regional lymph nodes. T3 or T4.
III C	T1-4 N2 M0	Tumor has extended to 4 or more regional lymph nodes.
IV	T1-4 N0-2 M1	Tumor has extended to 1 or more distant organ(s) or set of lymph nodes.

Another staging system utilized is the Dukes system, it is a less complex staging system. Itemized here are the descriptions of the stages in the Dukes system:

- **A** denotes the tumor that is confined to the intestinal wall.
- **B** denotes the tumor that begins invading over the intestinal wall.
- **C** denotes the involvement of lymph node(s)
- **D** denotes distant metastases

Present treatment for colorectal cancer

Different kinds of treatments are available for colorectal cancer and are administered according to the diagnosis of the colorectal cancer by oncologist. The available treatments for colorectal cancer are discussed below:

- Surgery — as one of the most frequent treatment for colorectal cancer. A clinician could do local excision if the cancer is still at an early stage. If the cancer is larger, the clinician could do colectomy to remove the cancer and nearby normal tissues. Additional options consist of radiofrequency ablation and cryotherapy.
- Chemotherapy — to use of medications that destroys cancer cells or inhibiting cancer cells division. Numerous medications could be given concurrently to increase treatment effectiveness. One of the regular medicines for treating colorectal cancer is 5-Fluorouracil (5-FU), commonly apply concurrently with oxaliplatin and leucovorin in a combination recognized as FOLFOX.
- Radiotherapy — the use of high doses of radiation to kill cancer cells via destructing the targeted cells genetic materials. There are 2 types of radiotherapy, the external radiation therapy that uses a beam of radiation to target on the cancer area and repeated over a few days. The other would be the internal radiation therapy that uses radioactive materials inserted into or near the tumor via small thin tubes or needle.
- Immunotherapy — the use of individual body's immune system to combat cancer. This therapy primarily promotes the immune system to response and combat more effectively against cancer. Monoclonal antibodies therapy is one of the immunotherapies using generated monoclonal antibodies to target the tumor in the body and to deliver drug or radioactive materials directly to tumor cells. Other examples of immunotherapy are colony-stimulating factors, tumor vaccines and biological response modifiers.

Other than existing treatment options, scientists conducted many experiments in hope to search for useful metabolites

or compounds that could potentially inhibit the growth of cancer cells[6–12] especially colorectal cancer[13–19]. Researches have gained some good findings, but more tests need to be done before these potential compounds could be ready for clinical application.

THE RAS ONCOGENE

The *Ras* is a family of related proteins known as small GTPase. They are vital for signal transmission between cells. The naming of "Ras" derived from "Rat sarcoma", indicating the source from which the first member of the protein family was discovered. The ras family comprises of 3 members, Nras, Hras and Kras[20]. Members of the *ras* family are triggered after a nearby transmembrane receptor is bound by its corresponding ligand. The ras protein is activated by guanine nucleotide exchange factors (GEFs) that leads to the development of GTP-bound state, followed by inactivation by GTPase activating proteins (GAPs) forming the GDP-bound state by GTP hydrolysis. Consequently, these will be activated and regulating other genes participating in cell differentiation, growth and survival. Hence, mutation in *ras* genes could lead to overexcited *ras* signaling, therefore triggering uncontrolled cell division and cell growth that would eventually become cancerous[21].

Kras mutation in colorectal cancer

Kras gene is a vital gene in colorectal cancer, this gene expresses a protein involved in the epidermal growth factor receptor (EGFR) signaling pathway. The Kirsten rat sarcoma viral oncogene homolog or Kras gene belongs to the *Ras* family of oncogenes, and mutations are common notably in colorectal cancer, pancreatic cancer and lung cancer. *Kras* gene mutation is among the earlier events in colorectal carcinogenesis, and the ability to detect *Kras* gene mutation is very important for diagnosis. Kras mutation was reported in the earlier stages of molecular alteration contributing to the development of colorectal adenoma to carcinoma. The Kras protein has vital role in tumor growth via the regulation of downstream proteins involved in survival, proliferation, and metastasis[21,22].

The prevalence rate of Kras mutation was reported to be 20% to 50%[23,24]. Reported here are countries and their respective rate of Kras mutation amongst colorectal cancer patients; Italy (46.3%), USA (40%), Iran (37.4%), Turkey (34.2%), Jordan (33.3%), Taiwan (26.5%) and Egypt (18.4%)[25,26,27]. One of the most common Kras mutation occurs at codon 12 and codon 13 [28]. It was reported that codon 12 has higher mutation rate as compared to codon 13 [21,22], with mutation of codon 12 reported at ~35% based by Bazan *et al.*[29]. Researchers suggested the high mutation rate of codon 12 is because of the vulnerability of codon 12 to carcinogen binding, furthermore along with the poor repair mechanism of the resulting adduct[21,22]. Also, other studies indicated that Kras mutation normally implicated codon 12, 13, 59 or 61[30,31].

Reports indicated that Kras mutations contributed to higher probability of death and lower progression-free survival[22]. Reinacher-Schick *et al.*[32] also showed that Kras mutations associated to lower progression-free survival in patients with advanced colorectal cancer treated with oxaliplatin chemotherapy. The increase of response rate associates with increased progression free survival in colorectal cancer. Nevertheless, Kras mutations have been connected to decreased response degree towards chemotherapeutic agents. Lievre *et al.*[33] reported that Kras mutation was decreasing the reaction of anti-epidermal growth factor receptor (EGFR), therefore patients with mutant Kras demonstrated lower survival rate as compared to patients with wild-type Kras. Hence, the prognosis of patients could be improved by verifying the mutation status of the *Kras* gene.

THE MicroRNAs

MicroRNAs (miRNAs) are small, non-coding RNA found in the genomes of vertebrates, invertebrates and plants[34]. The typical size of mature miRNAs is 21–25 nucleotides. MiRNAs demonstrated important role in many vital processes for instance cell proliferation, differentiation and apoptosis[35]. MiRNAs controls gene expression by various ways for example mRNA cleavage, deadenylation and translational repression. The exciting element is that miRNA is able to control the expression level by partial complementary binding to the target mRNA. This allows a single miRNA to control and regulate more than one target mRNA and perform several roles in our biological processes.

Discovery of miRNAs

The first miRNA, the *lin-4* was discovered in 1993 by the Ambros's and Ruvkun's research team[36,37]. The gene *lin-4* was detected by isolation of null mutation that triggers a breakdown in temporal growth in *Caenorhabditis elegans*[36]. Ambros *et al.*[34] reported a 700bp fragment that may well have *lin-4* gene but unable to locate the conventional start and stop codons, hence indicated that *lin-4* is not encoding protein. Researchers also discovered two small transcripts of *lin-4* of 61nt and 22nt in length that correspond to the common precursor miRNA and mature miRNA length[37].

The second miRNA discovered was *let-7*, that is likewise a heterochronic gene of C. *elegans*. Reinhart *et al.*[39] discovered that *let-7* was a 21nt RNA regulating the transition stage from L4 to adult in the larval development. Dissimilar to the *lin-4*, *let-7* sequence is conserved across species from invertebrates to complex organisms for instance humans. Nevertheless, *let-7* was not found in unicellular organisms and plants. Curiously, the expression level of *let-7* is dissimilar in different types of human tissue[40]. The finding that *let-7* was conserved across numerous species initiated the surge of research in the small RNA called microRNA (miRNA). Till date, more than 38 thousand miRNAs is listed in the miRBase database (http:// http://www.mirbase.org/) and keeps rising.

miRNA Synthesis

As depicted in Figure 1, miRNAs synthesis starts in the nucleus, with the primary transcripts (pri-miRNAs) processed into miRNA precursor (pre-miRNA) facilitated by Drosha and Dicer, which are RNase III enzymes. The pre-miRNAs are next exported from the nucleus into the cytoplasm by Exportin-5 and cut by Dicer into a 22-nucleotide mature double stranded miRNA[41]. This strand is then fused into the Argonaute protein to create the effector RNA-induced

silencing complex (RISC) after which the miRNA and its mRNA target interact. The miRNA will only interact with mRNAs containing anti-sense sequences. Nevertheless, this interaction could occur even if they are partially complementary to each other[42].

miRNA Role in Human Diseases

The discovery of miRNAs had quickly led to key research conducted to investigate their roles in humans especially in disease progression. Cancers were extensively studied, as one of the most common chronic disease affecting human. Furthermore, miRNAs were as well correlated to a multitude of other diseases. Van Rooij *et al.*[43] firstly reported the association of miRNAs with cardiac hypertrophy and heart failure. The study utilized miRNA microarray analysis demonstrated 12 miRNAs were deregulated during cardiac hypertrophy and heart failure[43]. Furthermore Tijsen *et al.*[44] reported another miRNA, miR-423-5p was elevated in heart failure patients. While for acute myocardial infarction patients, miR-208b and miR-499 was discovered to be greatly elevated and associated with the plasma troponin level[45].

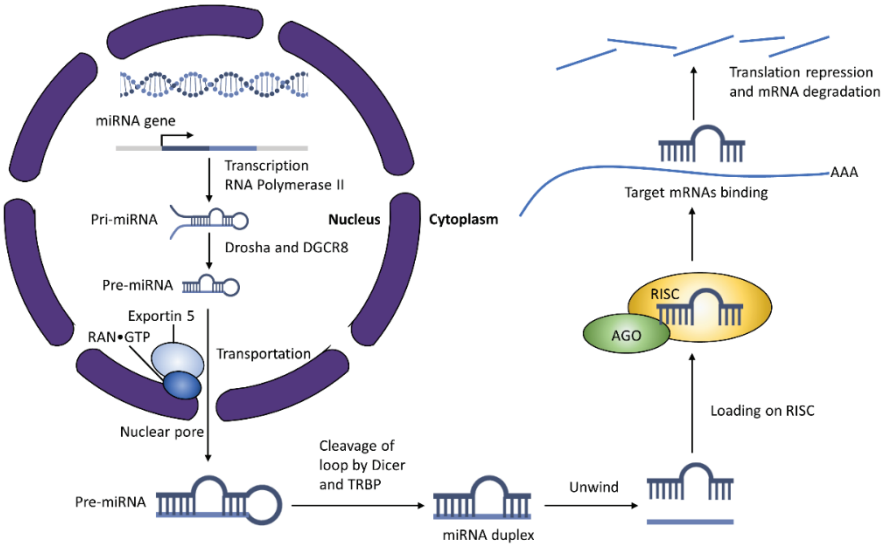

Figure 1. The miRNA synthesis pathway.

`Moreover, miRNAs have demonstrated important role in autoimmune diseases. Stanczyk *et al.*[46] demonstrated miRNAs role in autoimmune diseases with the discovery of miR-146 and miR-155 overexpressed in rheumatoid arthritis synovial fibroblast and synovial tissue. The finding agreed with another study by Nakasa *et al.*[47] that validated the expression of miR-146 in rheumatoid arthritis synovial tissue. In 2007, Dai *et al.*[48] described a total of 16 miRNAs were differentially expressed in patients of systemic lupus erythematosus (SLE). Both the miR-21 and miR-148a were overexpressed in CD4+ T cells of SLE patients and contributing to down-regulation of the *DNMT1* gene, triggering DNA hypomethylation[49].

A couple of miRNAs were correlated to neurodegenerative diseases for instance Parkinson, Alzheimer and Huntington's disease. Lukiw (2007)[50] reported that in Alzheimer's patients, miR-9, miR-25b and miR-128 were up-regulated, whereas miR-124a was down-regulated. An increase in miR-9, miR-128 and miR-125b were observed on cultured human fetal brain-derived primary neural cells, that was treated with metal salts to create reactive oxygen species (ROS)[51]. While miR-133b was absent in brain tissue of Parkinson's disease patients, indicating miR-133b vital roles for maturation and function of dopamigernic neurons[52].

miRNA in cancer

In 2002, miRNAs were first reported to be involved in cancer, with miR-15 and miR-16 discovered to be down-regulated or deleted in chronic lymphocytic leukemia (CLL)[53]. Cimmino *et al.*[54] demonstrated that miR-15 and miR-16 have a role in apoptosis by targeting Bcl2 mRNA. Various studies then reported different miRNA expression in almost all types of cancer. MiRNAs can behave as tumor suppressors or oncomiRs in carcinogenesis[55,56,57,58]. MiRNAs that were up-regulated act as oncomiRs, while down-regulated miRNAs in cancer usually operate as tumor suppressor. The overexpressed miRNAs leading to cancerous growth operate as oncogenes, whereas under-expressed miRNAs leading to cancerous growth act as tumor suppressor. There were numerous miRNAs identified as tumor suppressor. Johnson *et al.*[59] demonstrated that *let-7* expression was down-regulated in lung cancer tissue as compared to normal tissue. Furthermore, it associates with the increased of Ras protein in the lung cancer samples. While study by Garzon *et al.*[60] indicated that miR-29 was down-regulated in acute myeloid leukemia. While miR-34 was reported to be down-regulated in colon, pancreatic and breast cancers[61]. The miR-155 was among the first miRNA to be linked as oncomiRs. It was up-regulated in various cancers such as acute myeloid leukemia[60], Hodgkin disease[62], Burkitt lymphoma[63], and lung cancer[64].

miRNAs in colorectal cancer

MicroRNAs (miRNAs) have exhibited vital role in colorectal cancer genesis, progression and response to

treatments. Researchers demonstrated that in colorectal cancer samples, more miRNAs were up-regulated than down-regulated[65,66,67]. The miR-21 is one of the properly studied miRNA and discovered to be associated in many types of cancers. It was reported among the first miRNA discovered as oncomiR and was associated to a multitude of tumor suppressor genes for instance *PDCD4*, *PTEN* and *BCL-2*. Faltejskova *et al.*[68] indicated higher expression of miR-21 was associated with shorter general survival of colorectal cancer patients. They performed silencing of miR-21 expression in DLD1 cell lines and observed 30% suppression of the cancer cells migration ability, thus leading to lower cancer cells viability. This finding demonstrated the role of miR-21 in cancer cells migration in tumorigenesis. Likewise, Link *et al.*[69] demonstrated that miR-21 was highly expressed in patients with adenomas and colorectal cancer as compared to healthy individuals. Another study of stage II colon cancer patient exhibited increased miR-21 expression levels were associated to decrease recurrence-free cancer-specific survival[70]. Furthermore, the plasma miR-21 was able to differentiate colorectal cancer patients from normal controls with 90% sensitivity and specificity[71]. These findings demonstrated that miR-21 could be a reliable and non-invasive marker for colorectal cancer.

Link *et al.*[69] reported that miR-106a was up-regulated in colorectal cancer patients when compared with normal patients. Furthermore miR-106a was overexpressed in colorectal cancer and regulates the retinoblastoma 1 (*RB1*) gene in sporadic colorectal cancer patients[72]. Lately, Feng *et al.*[73] demonstrated that miR-106a was highly expressed in metastatic colorectal cancer cells and regulating the migration and invasion both *in vitro* and *in vivo*. The miR-106a prevents the expression of transforming growth factor-b receptor 2 (TGFBR2), leading to increase tumor cells migration and invasion. While Diaz *et al.*[74] stated the downregulation of miR-106a contributes to lower disease-free survival and overall survival of colon cancer patients, regardless of the tumor stage.

The miR-135b was regularly found up-regulated in cancer samples. Faltejskova *et al.*[75], reported the increased of miR-135b expression in CRC tumor tissues. Furthermore, miR-135b was correlated with higher serum levels of CEA and CA19-9. Moreover, Xu *et al.*[76] indicated the elevated expression levels of miR-135b in CRC tissues compared to normal tissues, and the positive association of miR-135b with the clinical stage. The miR-135b targets the adenomatous polyposis coli (APC) gene, an important gene in colorectal carcinogenesis[77]. They discovered that miR-135b was up-regulated in colorectal carcinomas and associates with the low APC mRNA levels leading to colorectal cancer.

The high-throughput sequencing was used to compare between paired tumor and normal tissue, and results identified that 37 miRNAs were dysregulated, with miR-1 among the down-regulated miRNAs[77,78]. Furthermore, miR-1 down-regulation was correlated to colorectal cancer progression, hence attributes that miR-1 can be a potential tumor suppressor via down-regulating MET oncogene at RNA and protein level[79].

For miR-504, researchers have reported its association in cancer in various studies[80,81,82]. The miR-504 was up-regulated in oral cancer, by increasing the invasion and migration capabilities of oral cancer cells[80]. While Hu *et al.*[81] indicated that miR-504 down-regulates the p53 protein via binding to the 3'-UTR of *p53* gene, hence stimulating tumorigenesis. In 2011, another study also demonstrated that miR-504 down-regulates p53 protein levels and damages its function particularly in p53-mediated apoptosis and G1 cell cycle arrest[82].

Bauer and Hummon (2012)[83] indicated that miR-145 was down-regulated in colon cancer and created distinctive molecular alterations. Earlier studies have indicated that these miRNAs were also down-regulated in more types of cancers. Kent *et al.*[84] established that Ras activation leads to the down-regulation of miR-145 that propels tumorigenesis. The down-regulation of miR-145 was reported to increase risk of esophageal cancer[85]. Whereas in bladder cancer, both miR-133a and miR-145 were discovered to be down-regulated and targeted the oncogenic FSCN1 mRNA[86]. Hence, miR-133a and miR-145 could act as a probable tumor suppressor by regulating the *FSCN1* gene.

Three miRNAs from the miR-182/183 cluster, namely miR-182, miR-183 and miR-96 were up-regulated various studies[78,87,88,89]. Sarver *et al.*[78] indicated that these miRNAs (miR-182, miR-183 and miR-96) were up-regulated in colon cancer tissues. The miR-183 was overexpressed in rhabdomyosarcoma and colon cancer[90], and the miR-183 demonstrated a function as oncomiR via controlling the tumor suppressor EGR1 and PTEN and advocating tumor cell migration[90]. Cekaite *et al.*[87] demonstrated that miR-182 was overexpressed by more than 2 fold in colon cancers throughout all clinical stages. Likewise, miR-96 was among the miRNAs reported to be up-regulated in colorectal cancer sample as compared to adjacent normal tissue[78,86]. Intriguingly, Yu *et al.*[89] reported differing finding of miR-96 down-regulated in pancreatic cancer and act as a tumor suppressor gene via inhibiting Kras oncogenic gene.

The miR-224 expression was elevated in colorectal cancer cell lines and in wild type Kras and BRAF colorectal tumors. Arndt *et al.*[91] indicated that miR-224 was overexpressed and correlated to colorectal cancer tumor progression. Yet, Mencia *et al.*[92] suggested that miR-224 was underexpressed in colorectal cancer cell lines and leading to the cells exhibiting rise in resistance towards methotrexate. Likewise, previous studies have suggested that miR-203 was up-regulated in colorectal cancer, but down-regulated in another study[93]. Chiang *et al.*[94] reported that miR-203 has really low expression in colorectal cancer tissue and cell lines.

MiR-31, a miRNA discovered to be highly expressed in colorectal cancer tissues and correlated with advance tumor stage and poor differentiation[95]. Furthermore Wang *et al.*[96] also reported miR-31 to be up-regulated in CRC samples and positively related to advanced TNM stage. While reports indicated that miR-17 was up-regulated in colorectal cancer and promotes tumor cell proliferation, growth and cell cycle progression[97].

miRNAs in cancer pathways

MiRNAs participation in cancer pathways have been

highlighted in many studies. Many proteins in key signaling pathways of colorectal cancer are changed and controlled by miRNAs. The Wnt pathway is one of the important pathways in early colorectal cancer development. In the Wnt pathway, inactivation of the APC gene is one of the key beginning steps for colorectal carcinogenesis[98]. Nagel *et al.*[77] suggested that miR-135a and miR-135b reduce the translation of APC gene *in vitro*. Also, the expression of these miRNAs increases in colorectal cancer and associated with low expression of APC gene.

EGFR and Kras signaling pathways lead to the initiation of numerous signal transduction molecules which started a cascade of downstream effectors regulating tumor growth, angiogenesis and metastasis[99]. The up-regulation of Kras will start a cascade of downstream activation of MEK gene, RAF gene and MAPK gene, therefore increasing the proliferation of tumor cells[100]. The miR-1 and miR-106a were reported for presumed targets of the MAPK gene family, therefore play a part in the Kras signaling pathway. The PI3K pathway is an important signaling pathway downstream of the EGFR pathway. Researchers reported that miR-135b and miR-21 targets the genes involved in PI3K pathway. With miR-21 clearly repressing the tumor suppressor, *PTEN* gene leading to lower survival rate of cancer patients[101].

Another renowned tumor suppressor gene, *p53* was mutated in estimated 50–75% of all colorectal cancer and other tumors. The p53 protein responds to DNA damage and deregulation of oncogenes through the initiation of cell cycle checkpoints, cellular senescence or apoptosis[102,103]. The miR-504 exhibited putative target of *BCL-2* gene, one of the gene that regulates the p53 pathway. Researchers indicated that miR-504 down-regulates the p53 protein, thus promoting cancer progression[81]. These results was also in agreement by Feng *et al.*[82] that indicated that miR-504 down-regulates p53 protein and damages its function in p53-mediated apoptosis and G1 cell cycle arrest.

CONCLUSION

This article provided vital insight into the roles of miRNAs in colorectal cancer. In colorectal cancer, studies indicated that many miRNAs are involved in the pathogenesis of the disease. They control the known oncogenes or tumor suppressor pathways by targeting proteins for instance p53, Kras and phophatidylinositol-3-kinase (PI3K). Nagel *et al.*[77] demonstrated that miR-135a and miR-135b reduce the translation of the APC transcript *in vitro*. The inactivation of the APC gene an important stage in colorectal carcinogenesis. Furthermore, *let-7* and miR-143 were described to target Kras oncogene. The Kras signaling leads to the initiation of numerous signal transduction molecules that starts a cascade of downstream effectors regulating angiogenesis, tumorigenesis and metastasis. Some miRNAs were discovered to be correlated with colorectal cancer for instance the miR-17-92 cluster, miR-21, miR-34, miR-135 and miR-196a[104,105,106,107]. In conclusion, this article shed light of miRNAs role in colorectal cancer that enabled a much better understanding of the disease.

Author Contributions

The literature review and manuscript writing were performed by K-LL, LT-HT and H-MY.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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Review Article

Progress in Microbes and Molecular Biology

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Bioprospecting of Microbes for Valuable Compounds to Mankind

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Abstract: The most biological multiplicity on this planet is almost certainly concealed in soils. Many valuable bacteria had been extensively dispersed in soils worldwide, with soils from terrestrial, desserts and Antarctic. Hence, soils become an intensively utilized ecological niche for the inhabitants to generate various useful biologically active natural products such as antibiotics, antifungal, antiviral, antioxidant, neuroprotection, anticancer and other important compounds. Bacteria including Actinobacteria have been exceptionally valuable for the pharmaceutical industry due to their limitless capability to generate secondary metabolites with various biological activities and chemical structure. Therefore, this article aims to provide critical insight of bioprospecting of microbes for valuable compounds to mankind.

Keywords: Bioprospecting; microbes; compounds; metabolites; actinobacteria; soil

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INTRODUCTION

Biotechnology is an illustration of biodiversity as new products via the utilization of living organisms and bioprocesses in medicine, engineering, technology, and other fields that required bioproducts. The greater the biodiversity offered, the greater probabilities of discoveries that could be transformed into vital technologies[1]. The estimation of the environmental and economic gains that are a direct or indirect result of microbial diversity were approximate to be in the range of 16-54 trillion US dollars per year, with an average of 33 trillion US dollars per year[2].

The primary and secondary metabolism of prokaryotes has been utilized by industrial for the creation of diverse products such as antibiotics[3–6], amino acids[7,8], nucleotides[7], organic acids[9] and vitamins[10]. Bacteria like Actinobacteria are a particularly rich source of compounds with activities such as antimicrobial[6,11–22], anticancer[23–29], antioxidants[30–35], neuroprotective[36,37], enzymes[38–41] and immunosuppressive[29] as illustrated by Figure 1. Bérdy (2005)[42] reported that in 2002, over 10,000 bioactive compounds (45% of all microbial metabolites) were obtained from filamentous Actinobacteria, out of which 7600 (75%) were obtained from *Streptomyces* and 2500 (25%) from rare Actinomycetes for instance *Actinomadura*, *Streptoverticillium* and *Micromonospora*.

Despite the tremendous success of the past in obtaining

useful secondary metabolite, the probabilities of discovery novel biologically active molecules from bacteria such as Actinobacteria was reduced and appears to be reaching a saturation curve. Recently, isolating well known Actinobacteria such as *Streptomyces* from diverse environments were reported to obtain similar compound, potentially due to regular genetic exchange between species[43]. These challenges had led to intensely amplified in serious demand for new structures in pharmacology, hence propelled the investigation of new habitats with poorly explored areas and uncommon environments to become vital for the discovery of novel bacteria (e.g. Actinobacteria) and useful metabolites[44–55]. Reports from poorly explored areas from these regions (e.g. Antarctic, Australia, China, Malaysia and Jordan) suggested that the investigation of new habitats remain to be valuable in discovering novel microorganisms and useful metabolites[47,56–61]. Moreover, the progression of new selective methods allows the screening and isolation of 'rare' Actinobacteria that can lead to finding useful bioactive compounds[62–64]. The finding of "rare" Actinobacteria has increased the array and diversity of genetic resources available for biotechnological utilization[62–66]. It is apparent that the findings of novel bacteria such as Actinobacteria could increase the discovery novel bioactive metabolites[62,66–68].

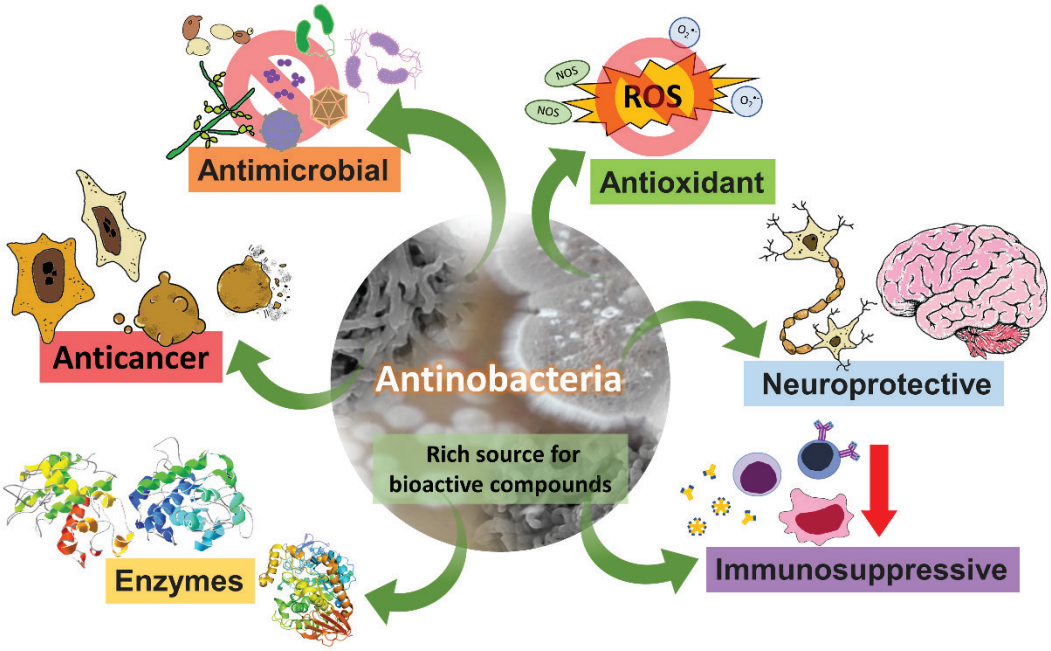

Figure 1. Actinobacteria are prolific producers for metabolites with diverse activities.

The genome sequencing of *Streptomyces coelicolor* A(3)2^{T}[69] and *Streptomyces avermitilis* MA-4689^{T}[70,71] discovered that these bacteria comprise more than 20 natural product gene clusters. This number of gene clusters is much more as compared to genomes of bacteria from another phylum[72,73]. For instance, *Bacillus subtilis* strain 168^{T} with three, *Ralstonia solanacearum* strain GMI 1000^{T} with two[74], and *Pseudomonas aeruginosa strain* PA01^{T} with four[75] *Pseudomonas aeruginosa* strain. While most other bacteria genomes lacking any detected natural product gene clusters[69]. These reports indicated the capability to produce secondary metabolites are not evenly distributed among microbes. Moreover, multiple gene clusters encoding for alike classes of secondary metabolites have been discovered in the genomes of other Actinobacteria[76,77]. Thus, explaining Actinobacteria are highly prolific sources of bioactive metabolites[78] with high capacity to utilize a extensive range of compounds and create secondary metabolites with diverse chemical structures and biological activities[79,80].

Unexplored environment — The Antarctic

The Antarctic is the area at the Earth's South Pole, contrary the Arctic region at the North Pole. The Antarctic includes the continent of Antarctica and the ice shelves, waters and island territories in the Southern Ocean situated south of the Antarctic Convergence. The area covers approximately 20% of the Southern Hemisphere, of which 5.5% (14 million km^2) is the surface area of the continent itself. The Antarctic is the coldest and windiest continent, it is a hostile, remote, and uninhabited area with its surrounding marine sites, provides an appropriate chance to investigate a still unexplored microbial biodiversity[81–87]. The uneven mixture of selection pressures has led to the evolution of novel biochemical adaptations and the likelihood of native species[88,89]. The production of metabolites such as antibiotics and toxins could confer a competitive survival benefit in this environment. Therefore, the investigation of poorly explored areas such as the Antarctic seemed as important region for discovering of potential novel bacteria and useful biological active metabolites[59,82,85,90,91].

Bacteria from Antarctic territories

The information of prokaryotic biodiversity remains very sparse across Antarctica[82,92,93]. Nevertheless, in recent decades, the improvement in both culture dependent and culture-independent methodologies allow some studies focused on Signy Island were done[94,95,96,97,98,99]. This area

act as a benchmark site within the maritime Antarctic, whose terrestrial ecosystems are demonstrative of the region[100]. Furthermore, more studies are also emerging from other sites along the Antarctic Peninsula, such as the study of the prokaryotic communities of a series of Antarctic terrestrial habitats along a latitudinal gradient as part of a larger regional microbial diversity study covering between the Falkland Islands (~50^0S) and Mars Oasis, Alexander Island (~72^0S)[101–103]. Based on the restricted habitats studied, a fairly large bacterial diversity has been reported[96–99,104,105].

There is an agreement that spatial distinction between soil organisms is not random but displays expectable patterns over dissimilar spatial scales. The small-scale difference is found to exhibit superior diversity than large scale difference[106–108]. Small-scale difference might be more vulnerable to local environmental effects such as areas of increased substrate availability[109]. Scientists indicated that water content, organic content (loss on ignition) and total N showed substantial direct correlations with microbial counts from soil at 6 different sites on Signy Island, whereas pH exhibited an inverse association[94]. Some recent culture-independent reports have demonstrated that soil prokaryote biodiversity on Signy Island have high association with elements such as conductivity, pH, lead and copper content. Moreover, significant overlap was reported across sites evidently affected by penguins, seals, and the existence of vegetation[99]. The direct effect of soil properties for instance soil pH, nutrients and moisture on bacterial diversity were demonstrated[110–113], and remarkably these parameters also exhibited close connection to specific functional genes for instance glutamate dehydrogenase and nitrate reductase[102].

Studies of the bacterial ecology of Antarctic soils by means of culturing dependent methods demonstrated that bacterial abundance and diversity can differ with soil factors for instance moisture, pH, available nutrients, salinity, elevation, slope, solar radiation, and drainage[114]. Suzuki et al. (1997)[115] isolated an obligate psychrophilic Actinobacteria, *Cryobacterium psychrophilum* from the Antarctica soil. This bacterium grew best at 9–12°C and did not grow at temperatures higher than 18°C. While psychrophilic strains of *Modestobacter multiseptatus* with optimum growth temperatures of 11-13°C have also been isolated from transantarctic mountain soils[116].

Normally, the early studies on the bacterial diversity of Antarctic soils were disadvantaged by the readiness of appropriate approaches. With the accessibility of DNA-based culture-independent assays, analysis of mineral soils of the Antarctic area has discovered that the soil bacterial communities have low diversity compared with temperate soils and may be dominated by a few bacterial phylotypes. Bacteria reported from the soils typically group with the phyla *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes*, *Cyanobacteria* and *Proteobacteria*[117-119]. Apart from *Deinococcus* and *Cyanobacteria*, they are among the phyla normally described from non-Antarctic soils[120]. The phyla *Actinobacteria* and *Bacteroidetes* appear to be prevalent in Antarctica while other phyla less broadly spread (e.g. *Acidobacteria)*. Remarkably some bacteria have no close relatives demonstrating soils of the Antarctic (e.g. Ross Sea Region) are extremely potential as a natural reserve of novel and cold-adapted bacteria[118]. The closest relatives include members of the genera *Arthrobacter*, *Brevundimonas*, *Leptolyngby*a, *Hymenobacter*, *Nocardioides*, *Sphingomonas* and *Sporosarcina*[117–119] all of which have been isolated from Antarctic soil.

The Barrientos Island of Antarctic is situated at 62˚24'S, 59˚47'W, north entrance to English Strait between Robert and Greenwich Islands. The north coast of the 1.5km island is dominated by steep cliffs, reaching a height of nearly 70 metres, with a gentle slope down to the south coast. The eastern and western ends of the island are black sand and cobbled beaches. The western end has columnar basalt outcrops as a notable feature. The whole center of the island is covered by widespread moss carpet. Lichens *Xanthoria* spp., *Caloplaca* spp. and other crustose lichen species are present. Moreover, the green alga *Prasiola crispa* is prevalent. Soil samples were collected from this island and molecular identification, which was based on 16S rDNA sequences analysis, discovered eight genera of Actinobacteria namely *Actinomyces*, *Actinobacterium*, an uncultured *Actinomycete*, *Streptomyces*, *Leifsonia*, *Frankinea*, *Rhodococcus* and *Mycobacterium*. The uncultured *Actinomyces* sp. and *Rhodococcus* sp. appear to be the prominent genera of Actinobacteria in Barrientos Island soil[121]. Molecular methods were applied to investigate correlations between actinobacteria abundance and environmental features, for instance vegetation and type of rookery. There was a substantial positive association between type of rookery and the abundance of actinobacteria; soil samples collected from active chinstrap penguin rookeries had the highest actinobacteria abundance. Vegetation type, for instance moss, which could provide a microhabitat for bacteria did not associate significantly with actinobacteria abundance[121].

In Barrientos Island, the selective isolation of culturable bacteria using 12 different isolation media were performed and total 96 bacteria isolates were isolated with 39 and 57 isolates belonged to phylum *Actinobacteria* and *Proteobacteria*, respectively. Through 16S rRNA gene analysis, 13 (*Arthrobacter*, *Brevibacterium*, *Demetria*, *Gordonia*, *Rhodococcus*, *Janibacter*, *Leifsonia*, *Dermacoccus*, *Kocuria*, *Lapillicoccus*, *Micromonospora*, *Microbacterium*, *Nocardioides*) and 8 (*Bradyrhizobium*, *Caulobacter*, *Sphingomonas*, *Methylobacterium*, *Paracoccus*, *Ralstonia*, *Rhizobium*, *Staphylococcus*) different genera of *Actinobacteria* and *Proteobacteria*, respectively were discovered[122,123]. Comparatively *Actinobacteria* (13 genera) had substantial higher diversity than *Proteobacteria* (8 genera)[122,123], hence showed that *Actinobacteria* are proficient to prosper in an extensive range of diverse soil environments, and they could resist the pressure of harsh environment as they could persist in the viable but inactive state for a extended time with form of spore[124]. Their extensive disseminations in Antarctic suggest that their dispersals are extremely endemic,

predominantly in soil and sediment[112,125]. Therefore, allowing the bio-prospecting of bacteria from sampling soil from widespread array of geographic sites, such as the Antarctic areas to be benefitted. Results showed that *Streptomyces* agar (SA) was the most suitable medium for isolating actinobacteria from soil of Barrientos Island with 54% isolation rate, while starch casein agar (SCA) was the most suitable medium to isolate proteobacteria with 19% isolation rate[122,123].

Furthermore, researchers studied actinobacteria and proteobacteria isolates from Barrientos Island for ability of producing antibacterial and antifungal secondary metabolites[122,123]. By means of high-throughput screening models, about 23%, 9%, 6% and 1% of isolates inhibited growth of *Candida albicans* ATCC 10231^T, *Staphyloccoccus aurues* ATCC 51650^T, methicillin-resistant *S. aurues* (MRSA) ATCC BAA-44^T and *Pseudomonas aeruginosa* ATCC 10145^T, respectively. A total 34 bioactive isolates were isolated and categorized into 13 genera, particularly 9 genera were actinobacteria. The high bioactivities of actinobacteria isolates (38%) as compared to proteobacteria isolates (25%) in this study[122,123] showed that *Actinobacteria* still remain as the better source for bioprospecting of novel bioactive metabolites owing to their tremendous capability to produce secondary metabolites with varied chemical structure and biological activities[79,80,126]. These findings provided vital baseline data that Barrientos Island is a good source of isolation for bioactive actinobacteria and proteobacteria with good antibacterial and antifungal metabolites[122,123].

In Barrientos Island, the application of the polyphasic taxonomic such as on the basis of phylogenetic, chemotaxonomic, phenotypic and signature nucleotide pattern of the 16S rRNA gene, these results indicated that strain 39^T is unlike all the genera in the family *Dermacoccaceae*. Hence, it is recommended that strain 39^T to be categorized in a novel genus in the family *Dermacoccaceae*, as *Barrientosiimonas* gen. nov., the type species of which is *Barrientosiimonas humi* gen. nov., sp. nov. The strain was named after Barrientos Island, the origin of the sampling site[127].

Bacteria as source of new natural products

The natural products have been demonstrated to be the richest source for discovery of novel bioactive compounds[128]. Previously, the majority bioactive products of microbial origin obtained from few taxonomic groups and mainly terrestrial environments[42,48]. In these decades, microbial natural products research inspired the progress of integrated methods merging specific isolation methods and the access to geographically diverse sources and to different ecological niches[128]. Lately the advancement of technologies enables other initiatives like targeting the exploitation of the metabolic potential of environmental gene libraries without undertaking the need of culturing microbes[129–131].

The microbial secondary metabolites comprise of antitumor agents, antibiotics, pesticides, enzyme inhibitors, toxins, and pigments. The biosynthesis of these metabolites is usually coded by genes clusters on chromosomal DNA and irregularly on plasmid DNA[132]. The discovery of new classes of antibiotics are vital to fight the increased occurrence of multiple resistances among pathogens to the available drugs presently in clinical use[133]. The utmost producers of natural product antibiotics are Actinobacteria as nearly two thirds of natural products have been derived from Actinobacteria[20], with streptomycetes accountable for more than 80% of them.

The phylum *Actinobacteria* signify a significant constituent of the microbial population in most soils[134–138]; such as the Antarctic region[117-119,139]. Also, *Actinobacteria* present in rhizosphere soil were reported for discovery of antimicrobial agents and other useful metabolites[140–151]. The genus *Streptomyces* exhibited potential as bio-control agent of commercial crops against fungal pathogens[17,152]. Moreover, *Streptomyces* spp. derived from grapes exhibited antifungal activity that is pathogenic to fungi and yeast from the same habitat[153]. While the genus *Arthrobacter,* a pervasive genus repeatedly discovered in Antarctic and Arctic areas is recognized for secondary bioactive metabolite production and for bioconversions[154,155]. Rojas et al. (2009)[128] examined Antarctic bacteria for creation of novel metabolites discovered a novel molecules associated to cyclic thiazolyl peptides active on gram positive pathogens produced by *Arthrobacter agilis* derived from Lake Hoare and Lake Fryxell from the McMurdo Dry Valley area in Antartic[128].

The Antarctic γ- and β-Proteobacteria strains R-12535 and R-7687 derived from Lake Reid in the Larsemann Hills and Lake Hoare in the McMurdo Dry Valleys produced bioactive metabolites that inhibited the growth of gram positive and negative pathogens such as *E. coli* and *S. aureus*[128]. Moreover, the MS spectra of bioactive metabolites obtained from the γ- and β-Proteobacteria strains R-12535 and R-7687 indicated no relatedness with any known compounds, suggesting a chemical novelty related to the bioactivity of these Antarctic bacteria. These studies demonstrated the high occurrences of antimicrobial activities discovered from Antarctic bacteria, which exhibited them as a prolific source of antimicrobial agents[42,62,156]. These findings support the notion that bacteria from Antarctic habitats comprise a rich metabolic diversity and the production of antimicrobial agents could provide a competitive benefit in this situation[157].

Other than antimicrobial agents, bacteria such as Actinobacteria produced enzymes that are vital and extensively used in medical therapy, bio-organic chemistry, molecular biology, detergent manufacturing, food processing, the textile and pharmaceutical industries[158]. For instance, Thermophilic *ThermoActinomyces candidus* could yield extracellular enzyme keratinase that could degrade wool[159]. The antimicrobial agents and keratin-degrading producing *Actinobacteria* (*Streptomyces, Nocardioides, Saccharomonospora, Nonomuraea* and *Nocardiopsis*) have been utilized to transformed poultry farm feather waste by composting into pathogen-free

and odourless bio-fertilizer with complete biological degradation[160].

Crawford (1978)[161] reported that streptomycetes can decay lignin by producing the enzyme lignin peroxidase. The extracellular lignin peroxidase derived from *Streptomyces viridosporus* has been studied[162] and it was the first report of a lignin peroxidase from a bacterium. In nature, lignin physically covers cellulose to form lignocellulose (65% cellulose, 25% lignin, and small quantities of hemicellulose glucans), and is resilient to degradation by most microorganisms. *Streptomyces viridosporus* T7A could depolymerizes lignin while degrading cellulose[161] and generates a modified water-soluble, acid-precipitable polymeric lignin (APPL) as a key lignin degradation product[163]. Pasti et al. (1990)[164] revealed novel *Streptomyces* strains, the *S. rochei* and *S. chromofuscus* that were discovered to be superior or equivalent in lignocellulose-degrading capability to *Streptomyces viridosporus* T7A.

The enzyme chitinase were discovered from the culture filtrate of *Streptomyces cinereorube*[165]. The enzyme was inhibited by Ag^{+}, Hg^{+}, Hg^{2+} and ρ-chloromercuribenzoate. This enzyme is stable in pH range 4.0-10.0 and the optimum pH and temperature for chitinase activity were 4.5 and 50°C, respectively. Gomes et al. (2000)[166] reported that *Streptomyces* spp. obtained from a Brazilian forest soil exhibited exceptional endochitinase activity and very active against three phytopathogenic fungi, namely *Fusarium solani*, *Magnaphorte grisea* and *Aspergillus parasiticus*.

Streptomyces ipomoea CECT3341 and *S. scabies* CECT3340 in liquid culture produces great levels of enzyme mannanase[167]. The potential of mannanase enzyme in refining the bleachability of pine kraft pulp was demonstrated. With bio-bleaching examinations by means of treatment of the enzyme to result in the release of chromophoric and color material from the pine kraft pulp, together with an increase in pulp brightness and an absence of differences in the viscosity values.

Berens et al. (1996)[168] effectively obtained the enzyme endoxylanases from the thermophilic actinobacteria *Microtetraspora flexuosa* SIIX. These thermostable enzymes reported to have optimal activities at pH 6.0 and 80°C. The hydrolysis of hemicellulose generated mostly xylobiose and xylotriose, the latter will be hydrolysed to xylobiose and xylose. Researchers demonstrated the production of endoxylanase from *Streptomyces noboritoensis*[169]. Moreover, a cellulase-free and endoxylanase-producing streptomycete, *Streptomyces thermocoprophilus* sp. nov. was discovered by Kim et al. (2000)[170].

Busch and Stutzenberger (1997)[171] discovered the *Thermomonospora fusca*, a facultative thermoalkalophilic Actinobacteria that produces an extracellular α-amylase which generates maltotriose as the key product. The optimum pH and temperature for the amylase activity were 6.0 and 65°C, respectively. The enzyme activity was not blocked by the addition of glucose due to the preference of the Actinobacteria for maltotriose.

Pasti and Belli (1985)[172] reported isolation of *Streptomyces* sp. and *Micromonospora* sp. from termite gut whereby these strains produce enzyme cellulose that contributed to their cellulolytic activity. A total of 4 different termites were reported for the isolation of cellulolytic Actinobacteria, namely *Armitermes, Macrotermes*, *Odontotermes* and *Microcerotermes* spp. All Actinobacteria strains effectively degraded both soluble and insoluble cellulose with some shown persistent activity up to a week. Waldron et al. (1986)[173] reported the isolation of *Microbispora bispora* from soil samples of hot springs, geysers and composts was found to grow at 55°C and create thermo-stable extracellular endoglucanase in good concentration with broad pH range of 5.5–7.2.

All these reports indicated the practicality of various enzymes produced by various bacteria such as Actinobacteria. The value of bacteria in the production of enzymes is heightened by their comparatively high produces, cost efficiency and susceptibility to genetic manipulation. These enzymes enabled bacteria to have a key role in numerous areas for instance the biodegradation of plant litter especially the recalcitrant lignocellulose component [174] and the decomposition of soil organic matter[175].

CONCLUSION

As a conclusion, the research of microbial diversity and the isolation of novel microorganisms signify a key chance for developments in biology[67,176–181]. The search and discovery of novel microbes that produce new useful secondary metabolites remains important in the fight against antibiotic resistant pathogens[182], and new emerging diseases[183–185].

Author Contributions

N-SAM, SHW, H-LS, LT-HT and JW-FL performed the literature search, critical review and performed the writing of this review. Guidance, support, and proofreading were contributed by AD, SR and VL. N-SAM and VL founded the review writing project.

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Review Article

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Cancer, Natural Products and Nanodrug Delivery Systems

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Abstract: As an offshoot of nanotechnology, nanomedicine has made great impact in the field of pharmaceutical and biomedical sciences by achieving breakthroughs in therapeutics and diagnostics of diseases in living organisms. One of the promising breakthroughs is the application of natural product-based nanoformulations for the treatment of various human diseases, such as cancer. Principally, the nanoparticle-based drug delivery system (NDDS) aims to overcome the limitations of conventional drug delivery system. NDDS improves the *in vivo* pharmacological and therapeutic properties of the poorly soluble drugs by dissolving, encapsulating, absorbing and/or attaching the drugs with the matrices of the nanoparticles. The nanoparticles that act as drug reservoirs also aim to control the drug release, enhance the drug uptake by targeted delivery and protect the drug against enzymatic degradation. This review presents a summary of the integration of nanotechnology and phytotherapy to achieve an improved pharmacological response and better clinical outcome in patients undergoing chemotherapy.

Keywords: nanomedicine; chemotherapy; natural product; drug delivery; nanoparticles

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INTRODUCTION

Cancer remains as one of the leading causes of mortality globally, irrespective of the advent of current available forms of cancer treatment. In 2018, cancer has accounted for an estimated of 9.6 million deaths worldwide[1]. The current therapeutic modalities, such as chemotherapy and radiotherapy, are associated with limitations, including damaging to proliferating healthy tissues, systemic toxicity, chronic side effects and emergence of drug resistance within tumor cells. Hence, there is always a continuous need of more effective strategies for the treatment of different human malignancies.

Throughout the history of mankind, natural products have played a pivotal role in the treatment of various diseases. Natural products derived from plants, animals and microorganisms represent the prolific bioresources for pharmacologically active compounds, including as chemotherapeutic agents[2–12]. These natural products have been extensively studied for the prevention and treatment of cancer, such as paclitaxel, vincristine, camptothecin and resveratrol[13]. Although they have been demonstrated to possess strong therapeutic value, the clinical application of these promising natural product derived compounds is severely hampered by their poor solubility and bioavailability[14].

As an offshoot of nanotechnology, nanomedicine has made great impact in the field of pharmaceutical and biomedical sciences by achieving breakthroughs in therapeutics and diagnostics of diseases in living organisms. One of the promising breakthroughs is the use of nanoparticles in the development of drug delivery system to improve the treatment of various diseases, including cancer[15]. Principally, the nanoparticle-based drug delivery system (NDDS) aims to improve the *in vivo* pharmacological and therapeutic properties of the poorly soluble drugs by dissolving, encapsulating, absorbing and/or attaching the drugs with the matrices of the nanoparticles. The nanoparticles that act as drug reservoirs also aim to control the drug release, enhance the drug uptake by targeted delivery and protect the drug against enzymatic degradation. In 2005, the National Cancer Institute (NCI) has launched the Alliance for Nanotechnology in Cancer due to the emerging of nanomedicine and their potential applications in

cancer research[16]. Under this approach, nanoparticle-based drug delivery system has been approved as one of the effective strategies to overcome the limitations of conventional chemotherapy with numerous nanoparticle-based drugs and delivery system that are in clinical use. In this review, we aim to critically consolidate the advent of nanotechnology in the context of chemotherapeutic drug delivery. This review also presents the examples of the extensively studied natural product-derived compounds using nanotechnology, including doxorubicin, paclitaxel and vincristine.

BRIEF OVERVIEW OF CANCER

Cancer is a collection of genetic diseases characterized by the uncontrolled cell growth as a result of dysregulated processes of cell division and cell death. Cancer cells are abnormal cells which lose their ability to undergo apoptosis (the programmed cell death) and uncontrollably proliferate, subsequently leading to formation of malignant tumour that invades to adjacent tissues[16]. The formation of tumour (tumourigenesis) involves a complex multistep process (tumour initiation, tumour promotion and tumour progression) that transforms a normal cell into a malignant one due to DNA mutation. In 2011, "the hallmarks of cancer" have been revised, explaining the defect mechanisms of malignant cells that deviate from normal cellular functions (**Figure 1**)[17].

Figure 1. The ten hallmarks of cancer[17].

These cancer hallmarks are the common traits of tumour cells with their capabilities to alternate the cell growth (sustaining proliferative signalling and evading growth suppressors), to evade apoptosis and cell cycle (resisting cell death and enabling replicative immortality), to modify cellular metabolism (deregulating cellular energetic), to destruct immunological function, to induce angiogenesis, invasion and metastasis. Additionally, the genomic instability[18-22] and inflammation by immune cells facilitate the tumourigenesis[17].

APOPTOSIS AND CANCER

One of the prominent hallmarks of cancer is the ability to resist cell death[17]. Cell death is a fundamental programme that happens for two purposes; under defence mechanism when the cells received stressful stimuli or under homeostasis mechanism to remove damaged or aged cells for maintaining cell population[23, 24]. The cell death could occur by either necrosis or apoptosis, depending on the response of the cell to the stimuli[25].

Necrosis is an "accident" or premature cell death that occurs when the cells encountered irreversible stimuli such as infectious agents, hypoxia, heat and radiation[26]. The necrotic cells experience rupture in the plasma membrane and organelles due to the swelling of cytoplasm. The cytoplasmic contents are then released to the extracellular space and triggered an inflammatory response[27].

In contrast, apoptosis is a "suicide" or programmed cell death that occurs under some physiological conditions to maintain homeostasis[28]. Aged and damaged cells that may interfere with body function are removed from the system[29]. Apoptotic cells experience the blebbing of plasma membrane, cell shrinkage, DNA fragmentation and activation of specific proteases (caspases). As different from necrosis, the apoptotic process does not trigger the inflammatory response as the apoptotic cell externalises the phosphatidylserine at the outer leaflet of plasma membrane to be recognised and engulfed by .phagocytes[30,31].

Tumour initiation (hyperproliferation of cell) happens when apoptosis fails to eliminate the genetic mutated cells[32]Ryia-Illani Mohd. These mutated cells proliferate uncontrollably by evading apoptosis via several mechanisms. One of the common mechanisms is the loss of function of *TP53* tumour suppressor gene[17]. *TP53* tumour suppressor gene encodes the p53 protein which is responsible to transcript more than 125 genes coordinating the repairing of DNA, cell cycle and apoptosis. The mutation of *TP53* gene has been identified as a common molecular characteristic in human cancer[33]. Therefore, *TP53* has become a potential target in cancer treatment with the aim to restore the transcription function[34].

Apoptosis is executed via two downstream signalling pathways: intrinsic and extrinsic pathways, depending on the source of the stimuli (**Figure 2**)[35]. The intrinsic pathway receives the intracellular death signals from non-receptor-mediated stimuli and initiates the events in mitochondria[24]. The up-regulation of pro-apoptotic proteins (Bax, Bad and Bim) or/and down-regulation of anti-apoptotic proteins (Bcl-2, Bcl-xL and Bag) stimulate the release of cytochrome c from mitochondria, leading to the formation of apoptosome in the cytosol. The apoptosome then initiates a cascade of proteolysis with the effector caspases (caspase-3 and -7) that lead to the execution of apoptosis[36].

On the other hand, extrinsic pathway receives extracellular death signals from transmembrane death receptors such as tumour necrosis factor (TNF) receptor and Fas receptors after binding with their homologous ligands (TNF-α and FasL). Activation of these death receptors results in the activation of Fas-associated death domain protein (FADD) and caspase-8 that further initiates the effector caspases (caspase-3 and -7) and triggers apoptosis[37, 38].

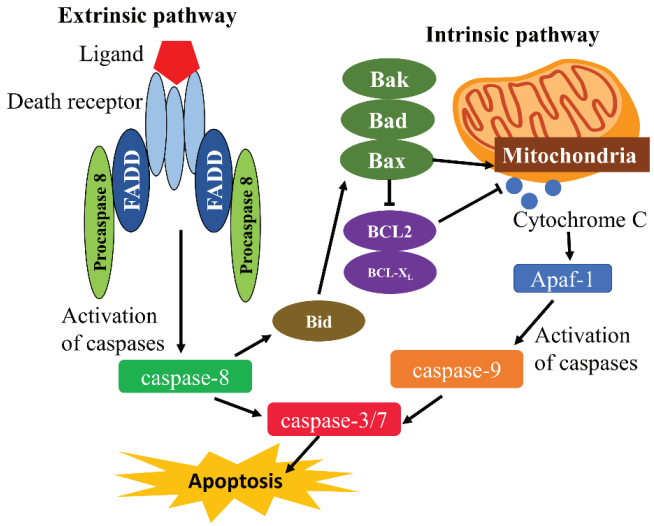

Figure 2. The intrinsic and extrinsic pathways of apoptosis[35].

Angiogenesis and Cancer

Another hallmark of cancer is the induction of angiogenesis[17]. Angiogenesis is the formation of new blood capillaries from existing blood vessels[39]. Formation of new capillaries in the tumour enables the tumour cells to obtain nutrients and oxygen and to remove the metabolic waste and carbon dioxide. This has promoted the tumour growth and metastasis of tumour cells to other organs[17,40]. Vascular endothelial growth factor (VEGF) family members such as VEGF A and B proteins are the key components to induce angiogenesis. In response to hypoxia, the level of hypoxia inducible factor-1 α (HIF-1α) promotes the expression of these pro-angiogenic VEGFs in tumour[41]. The VEGFs then bind to their respective receptors on the endothelial cells in the extracellular matrix, causing the differentiation of endothelial cells to form new capillaries[42]. Therefore, VEGF has become the therapeutic target to control the tumour growth, angiogenesis and metastasis[41,43].

Metastasis and Cancer

Activation of invasion and metastasis is one of the characteristics of malignant cancer[17]. Metastasis is defined as the spread or development of cancer in other distinct organs from the primary tumour through blood and/or lymphatic vessels[44]. It is always related to poor prognosis as it is the leading cause of cancer death[45]. For tumour cells to pass through the tissue barriers, they have to develop the ability to break through the extracellular matrix (ECM)[46]. One of the proteolytic enzymes that are responsible to degrade ECM is the matrix metalloproteinases (MMPs). ECM metalloprotease inducers (EMMPRIN) are produced on the membrane of the tumour cells to activate MMPs including the MMP-2 (72-kDa Gelatinase A) and MMP-9 (92-kDa Gelatinase A)[47].

NATURAL PRODUCTS AS A SOURCE OF CHEMOTHERAPEUTIC AGENTS

Natural products are defined as the compounds that are produced from nature such as plants, microorganisms and animals as a result of nutritional needs and evolution to adapt the environmental challenges[48–62]. Historically, plants have been extensively documented in traditional medical systems such as traditional Chinese medicine, Indian Ayurvedic system and Egyptian "Ebers Papyrus" to promote health and cure diseases[63]. Nowadays, plants have become an important source for discovery of bioactive compounds (phytochemicals) with promising therapeutic activities such as anti-cancer, anti-inflammatory and anti-bacterial effects[13,64–75].

Majority of the marketed chemotherapeutic agents were isolated and derived from medicinal plants due to the advantages of readily available and cost effective[76]. The well-known examples of plant-derived chemotherapeutic compounds are paclitaxel from *Taxus brevifolia*[77], camptothecin from *Camptotheca acuminata*[78], vinblastin and vincristine from *Catharanthus roseus*[79]. These phytochemicals can be further classified into alkaloids, flavonoids, taxanes, lignans, stilbenes and more[76]. **Table 1** summarises some of the potential and clinically approved plant-derived chemotherapeutic compounds with their mechanisms of action.

Table 1. Potential and clinically approved plant-derived chemotherapeutic compounds with their mechanisms of action

Phytochemical	Plant name and part	Mechanism of action and type of cancer	Reference
Camptothecin - Synthetic derivatives: topotecan and irinotecan	*Camptotheca acuminata* (bark and stem)	- DNA topoisomerase I inhibitor in glioblastoma, ovarian, lung and colorectal cancers	[78]
Vinca alkaloids (vincristine and vinblastine) - Synthetic derivatives: vinorelbine, vindesine, and vinovelbine	*Catharanthus roseus* (leaves and bark)	- Microtubules acting agent that arrests cell cycle in leukemia, breast, lung and liver cancers	[79,80]

Colchicine - Synthetic derivatives: colchicinamide, deacetylcolchicine	*Colchicum autumnale* (leaves)	- Arrest cell cycle by binding to tubulin in melanoma, colorectal and breast cancers	[81,82]
Pomiferin	*Maclura pomifera* (fruits and flowers)	- Inhibit histone deacetylase and cause oxidative DNA damage in colorectal cancer	[83]
Paclitaxel - Synthetic derivative: docetaxel	*Taxus brevifolia* (barks and leaves)	- Microtubules acting agent that binds to β-tubulin and arrests cell cycle in breast, ovarian and prostate cancers	[77,84]
Podophyllotoxin - Synthetic derivatives: etoposide and teniposide	*Podophyllum peltatum* (leaves)	- Inhibit microtubule polymerization and arrest cell cycle at G2/M phase - Induce apoptosis via modulation of intrinsic and extrinsic pathways in non-small cell lung carcinoma	[85,86]
Resveratrol	*Veratrum grandiflorum* (leaves)	- Inhibit angiogenesis and induce apoptosis via modulation of VEGF, p53, Bax, Bcl-2, cytokines, and COX-2 proteins in breast, liver, prostate, lung and colorectal cancers	[87,88]
Thymoquinone	*Nigella sativa* (seed oil)	- Induce apoptosis via modulation of intrinsic pathway in breast cancer	[89]
Geniposide	*Gardenia jasminoides* (Fruit)	- Antioxidant and anti-inflammatory via induction of Nrf2 and GPx	[90]

NANOTECHNOLOGY IN DRUG DELIVERY: NANOMEDICINE

The word "Nano" comes from the Greek "*nannos*" (dwarf or a very short man) that refers to the prefix of one-billionth of a meter (a factor of 10^{-9})[91]. According to the National Nanotechnology Initiative (NNI), nanotechnology is defined as "the knowledge and manipulation of matter within nanometer scale (1–100 nm) that comprises multidisciplinary fields of nanoscale science, engineering and technology". Owing to the abilities to shape, process and create things at nanoscale, nanotechnology has been addressed as the next "Industrial Revolution" that offers tremendous advances to human being in the application of communications, chemistry, engineering, medicine and robotics[91,92].

One of the most active applications of nanotechnology is nanomedicine[93]. Nanomedicine is defined as "the application of nanotechnology in the field of medicine"[94]. With the utilisation of nanoparticles, nanotechnology has brought positive impact to human health in diagnosis, prevention and treatment of diseases[95]. It involves the development of nanomaterials and nanodevices for the applications of drug delivery, *in vivo* diagnosis, implants and nanotheranostics[96]. Nowadays, various types of material have emerged as useful nanomaterials for the development of drug delivery system. The nanoparticles can take various shapes and sizes with distinct properties depending on the types of nanomaterials used. Generally, nanoparticle-based drug delivery systems can be categorized based on biological-origin materials (such as phospholipids, dextran, lipids, chitosan, and lactic acid) or inorganic materials (such as polymers, carbon, silica and metals)[97,98]. The family of nanoparticles, including polymeric nanoparticles, lipid nanoparticles and inorganic nanoparticles, is illustrated in Figure 3[99].

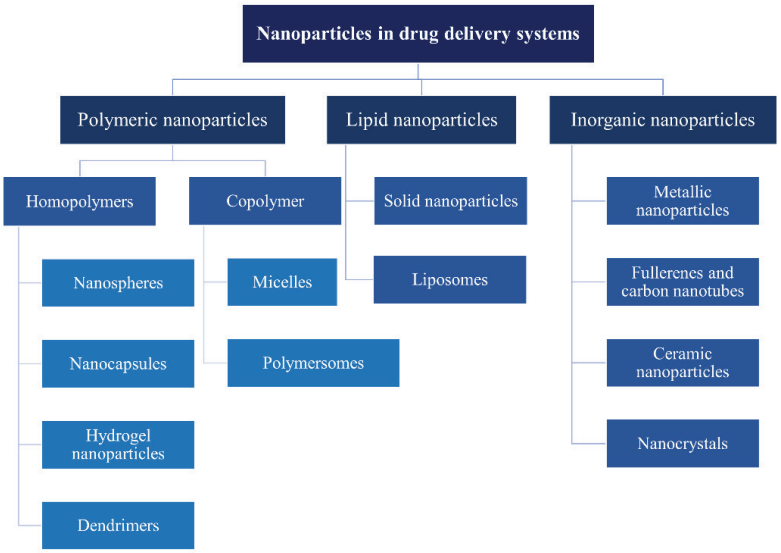

Figure 3. Family of nanoparticles in drug delivery systems.

Drug delivery is defined as "a process of delivering a pharmaceutical agent for pharmacological reactions"[100]. An ideal drug carrier used in the drug delivery system should possess the ability to transport the optimum dose of pharmaceutical agent to the desired site without causing adverse side effects in other tissues due to unwanted accumulation[101]. Besides, the intentions of drug carriers are to protect the therapeutic agents from degradation by gastrointestinal enzymes, to improve the bioavailability of hydrophobic or lipophilic therapeutic agents and to facilitate controlled release of drug[102–106].On top of these criteria, nanoparticles have been developed as the novel drug delivery system that provide extra advantages of low possibility of rapid clearance from the body through extravasation or prevention from phagocytosis by macrophage due to their nanometer size[107,108].

Limitations of Conventional Delivery of Chemotherapeutic Agents

Chemotherapy is the most common therapeutic approach for breast cancer. The highly-cytotoxic chemotherapeutic agents are directly administered into the blood circulating system by intravenous or oral route to kill rapidly-dividing cells[110]. However, this conventional drug delivery system encounters some drawbacks as follows:

A **Non-specificity of chemotherapeutic agents:** The random distribution and non-specific targeting of chemotherapeutic agents in the body system have caused unwanted accumulation and toxicity to other normal cells that divide rapidly such as bone marrow, hair follicle and digestive tract cells[111]. The most common adverse effects of chemotherapy are anaemia, alopecia, nausea, vomiting and acute cholinergic gastrointestinal effects[112].

B **Poor pharmacokinetics via oral administration:** Cancer is considered as chronic disease that requires long term frequent treatment. Therefore, oral route of drug administration has been the most preferred choice due to the reasons of patient's convenience, compliance, lower cost and painless[113]. Nevertheless, the desired therapeutic dose needed for maximum therapeutic effect is hard to achieve via oral administration of active drugs due to the enzymatic and hydrolytic degradation in the gastro-intestinal fluids, low cell uptake in the gastro-intestinal tract, first-pass hepatic metabolism, susceptibility to efflux transport and short biological half-life[114, 115].

C **Poor aqueous solubility:** Conventional delivery of chemotherapeutic agents remains a challenge as more than 40% of the potential anti-cancer compounds are hydrophobic or lipophilic. The insolubility of these drugs in water becomes an issue as they could not achieve the desired concentration in the systemic circulation. Therefore, various methods have been applied to improve the drug solubility such as drug carrier, chemical/physical modification of drugs, use of surfactant and salt formation[14].

APPLICATION OF NOVEL DRUG DELIVERY SYSTEM IN CANCER THERAPY

Nanotechnology has appeared as an attractive approach for solving the drawbacks of conventional drug delivery system of chemotherapy. In principle, the potential or existing chemotherapeutic agents are conjugated or encapsulated in the nanoparticles in order to improve their pharmacokinetics and bioavailability[116,117]. As compared to discovery of new chemotherapeutic agents, the development of this novel drug delivery system only utilized half of the time (6–8 years) and 20% of the cost ($50–$60 million) to be clinically approved and marketed[111].

In 2005, the National Cancer Institute (NCI) has launched the Alliance for Nanotechnology in Cancer due to the emerging of nanomedicine and their potential applications in cancer research. Under this approach, nanoparticle-based drug delivery system has been approved as one of the promising strategies to overcome the limitations of conventional chemotherapy with numerous nanoparticle-based drugs and delivery system that are in clinical use.

An effective chemotherapeutic drug delivery to a solid tumour involves five steps which are circulation in blood, accumulation and penetration in the tumour, internalization by cancerous cell and drug release (CAPIR)[118] (Figure 4).

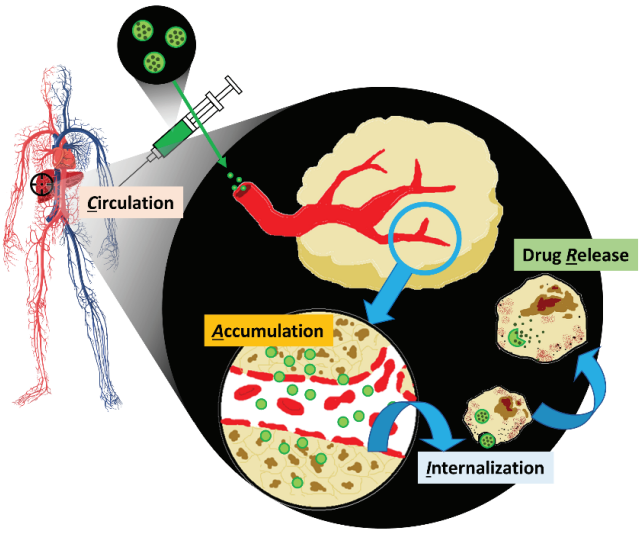

Figure 4. The CAPIR cascade of chemotherapeutic drug delivery[118].

The overall therapeutic efficiency of chemotherapeutic drugs is improved by nanoparticle-based drug delivery system by its abilities of accumulation and penetration in tumour, which could be achieved by either passive or active targeting (Figure 5)[119].

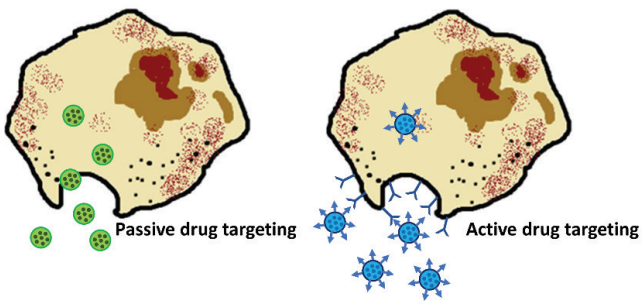

Figure 5. Targeted delivery system by nanoparticles[15].

In passive targeting, the circulating nanoparticles could passively extravasate from blood vessels and accumulate in the tumour via enhanced permeability and retention (EPR) effect. This EPR effect could only be achieved in tumour due to its unique characteristics of leaky blood vasculature and poor lymphatic drainage[120]. The normal blood vessels are endowed with tight junctions that could allow molecules less than 10 nm to permeate; meanwhile, the fast growing blood vessels that surround the tumour are highly disorganized and dilated due to the defective smooth muscle and enlarged gap junctions, allowing molecules less than 600 nm to permeate[121,122]. Furthermore, the tumour possesses poor lymphatic system that further enhances the entrapment of nanoparticles in the tumour[123].

On the other hand, active targeting can be achieved by attaching the ligands (proteins or antibodies) on the surface of nanoparticles that can interact with specific over-expressed receptors on the tumour[123,124]. In principle, this mechanism allows the nanoparticles to identify and recognise cancerous cells, thus minimising the unwanted systemic exposure of chemotherapeutics to other tissue[123]. Table 2 shows some examples of clinically approved nanomedicine for cancer chemotherapy[109,125].

CONCLUSION

Natural products have been showing impressive potentials as chemotherapeutic agents for cancer treatment, but their success in clinical trial has been limited as they have low solubility and bioavailability. The era of nanomedicine in cancer therapeutics has promisingly overcome the challenges hampering conventional therapy regimes hurdled by barriers of low solubility and physiological stability, poor bioavailability and specificity and high toxicity. Nanotechnology has revolutionized current cancer therapeutic modalities with the introduction of nano-drug delivery systems which are more efficient and less toxic as well as enhanced specificity to target tumor cells. The present *in vitro* and *in vivo* results pose a promising picture, but much more efforts are required from basic molecular aspects to preclinical and clinical trials before advancing more clinical use of nanoformulations in cancer chemotherapy.

Table 2. Clinically approved nanomedicine for cancer treatment[109,125].

Nanoparticle	Trade name	Description	Type of cancer	Manufacturer
Liposome	Doxil®	- Doxorubicin encapsulated in PEGylated liposomes - Reduced toxicity of doxorubicin	Ovarian and breast cancer	Orthobiotech, Schering-Plough
	Myocet®	- Doxorubicin citrate encapsulated in liposomes - Lower clearance and higher half life of drug	Breast cancer	Elan/Sopherion therapeutics
	Marqibo®	- Vincristine sulfate encapsulated in liposomes - Lower clearance	Acute lymphoblastic leukemia	Talon Therapeutics, Inc.
	DaunoXome®	- Doxorubicin encapsulated in non-PEGylated liposomes - Prolonged circulation time and higher accumulation in tumour site	HIV-associated Kaposi's sarcoma	Galen Pharmaceutics
	Marqibo®	- Vincristine encapsulated in non-PEGylated liposomes - Reduced toxicity and higher accumulation in tumour site	Philadelphia chromosome-negative acute lymphoblastic leukemia	Spectrum Pharmaceuticals
	Onivyde®	- Irinotecan encapsulated in non-PEGylated liposomes - Prolonged circulation time, and higher accumulation in tumour site	Pancreatic cancer	Merrimack Pharmaceutics
Polymer-based nanoparticle	Genexol®	- Paclitaxel in PEG-PLA copolymer micelles - Increased solubility of paclitaxel	Metastatic breast cancer and pancreatic cancer	Samyang Biopharmaceuticals
	Opaxio®	- Soluble ester conjugate of paclitaxel and α-poly(L)-glutamic acid - Prolonged tumour exposure of paclitaxel - Reduced neutropenia and alopecia	Ovarian cancer	Cell therapeutics
	Eligard®	- Lueprolide acetate and polymer - Prolonged drug circulation time and controlled drug release	Prostate cancer	Tolmar Pharmaceutics

Protein-drug conjugates	Abraxane®	- Paclitaxel conjugated with albumin - Enhanced endothelial transcytosis via albumin-receptor mediated pathway	Breast cancer and non-small cell lung cancer	Abraxis Bioscience, Astra Zenica
	Ontak®	- Denileukin diftitox - Specifically targeted to T-cell	Cutaneous T-cell lymphoma	Eisai
Metal nanoparticle	Nano-Therm®	- Aminosilane-coated with superparamagnetic iron oxide - Thermal ablation by generating heat directly within the tumour tissue	Glioblastoma and prostate cancer	MagForce AG

Abbreviation: PEG-PLA, poly(ethylene glycol)-poly(D,L-lactide).

Author Contributions

Y-SO, LT-HT participated in the writing of the manuscript. Y-SO conceptualized the project.

Conflict of interest

The authors hereby declare no competing interest.

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Review Article

Progress in Microbes and Molecular Biology

HH PUBLISHER

Resveratrol as a potential broad-spectrum compound for cancer treatment

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Abstract: There is a need to shift the paradigm of cancer therapeutic approach. The severe adverse side effects, drug resistance and unaffordable price plagued with chemotherapeutic drugs has spurred the development of "dirty drug". Natural products, specifically phytochemicals, have gained much attention due to their ability to target multiple interconnected pathways. Resveratrol (RSV), the stilbenes found in red wine, is one of the phytochemicals that exhibits various pharmacological therapeutic effects including cancer. In this review, we highlighted RSV as a potential "broad-spectrum" anticancer compound, by summarising its targeting mechanisms in the pathways relevant to the cancer hallmarks.

Keywords: stilbene; hallmarks; dirty drug; natural product; metabolites

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INTRODUCTION

Cancer, the most commonly diagnosed non-communicable disease, has imparted significant mortality and morbidity worldwide[1]. In 2018, the World Health Organisation ranked cancers as the leading cause of premature death with 9.6 million cases reported worldwide[2]. Despite the advancement in cancer therapy, the overall survival rate and quality of life of cancer patients have not been improved. The development of personalised drug on the other hand, is slow, costly and may not guarantee good clinical outcomes. Particular attention has been devoted to explore the potential of pleiotropic, "broad-spectrum" compounds or "dirty drugs" which could simultaneously target multiple mechanisms to overcome the aforementioned issues in achieving effective cancer treatment[3].

The ten cancer hallmarks proposed by Hanahan and Weinberg in 2011, has become a general a guide to evaluate the potential of specific compound as a promising "dirty drug"[4]. This means compounds that could disrupt any of the processes responsible for the cancer hallmarks would almost certainly hinder cancer progression. In an ideal circumstance, a compound that could disrupt multiple pathways not only would produce superior effect, it would also minimise the risk of side effects that is otherwise introduced by multiple drug administration. Natural products have been a rich and excellent source to search for multi-target bioactive compounds with improved therapeutic efficacy and safety[5,6]. These multi-target bioactive compounds are derived from natural sources such as plants[7,8], microorganisms[9–16] and animals[17]. In a work published by Block *et al.* (2015)[3], they found that most of the compounds that targets all cancer hallmarks were the phytochemicals. Phytochemicals are naturally produced as a result of evolution against pathogens with evident role playing in human health. These natural compounds have attracted much attention from community for their promising effects in treating diseases[18–22]. Stilbenes are one of the examples of secondary metabolites that was produced in stressful condition to fight against fungal infection and UV radiation[23]. It is one of the nonflavonoids which consists of two aromatic rings linked by an ethylene

or ethane bridge (C6-C2-C6 carbon skeleton) and usually found in plants. Among the 400 natural stilbenes discovered, RSV is the most widely investigated compound due to its vast pharmacological activities[24].

RESVERATROL

Resveratrol (3,4',5-trihydroxy-trans-stillbene) (RSV) **(Figure 1)** can be found in the skin of grapes, red wines, peanuts, pineapple and mulberries. This compound could be synthesised from phenylalanine pathway through several enzyme reactions into para-coumaroyl-CoA, which is condensed with malonyl CoA to form RSV[25]. Since the discovery of RSV by Siemann and Creasy in red wine in 1992[26], extensive studies have proven that RSV exhibits its biological functions such as antimicrobial, cardiovascular disease, anticancer, anti-inflammatory, antidiabetic and neurodegenerative diseases[27,28]. It is believed that most of the pharmacological activities are attributed to its anti-oxidant mechanisms involving the competition with coenzyme Q, free radical scavenging and inhibition of lipid peroxidase in the mitochondria[29].

RSV has demonstrated anti-cancer properties in both *in vitro* and *in vivo* settings. The type of cancers tested are ovarian[30], lung[31], colon[32], prostate[33], breast[34] and cervical cancers[35]. As a pleiotropic compound which acts on different mechanisms, it also showed to play a possible role to counteract multidrug resistance[36]. As of June 2020, the keywords of "resveratrol and cancer" brings up 3660 results in PubMed database. Correspondingly, RSV possesses great commercial value as evidence by a surge in patent filed in *The Lens* patent database (http://lens.org) related to the application of RSV in cancer therapy in recent decades **(Figure 2)**.

MODULATION OF CANCER HALLMARKS

The surge of publications and patents undeniable reflect the increased interest of the scientific community toward the therapeutic effect of RSV. In an attempt to investigate the potential of RSV as a "broad spectrum" chemotherapeutic agent, this review summarised the cancer hallmarks and the target molecules that have been claimed to be modulated by RSV in various cancer cell lines and animal models **(Figure 3)**.

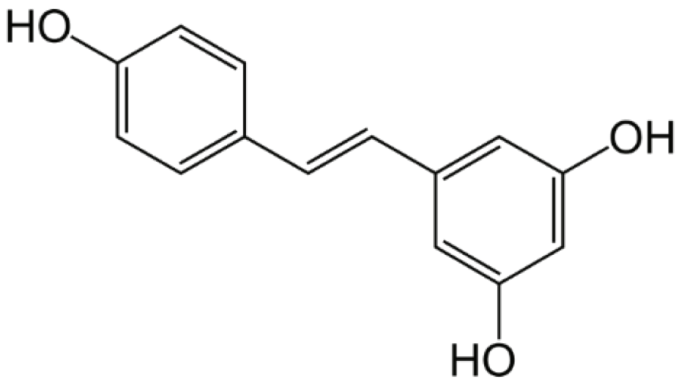

Figure 1. Chemical structure of resveratrol.

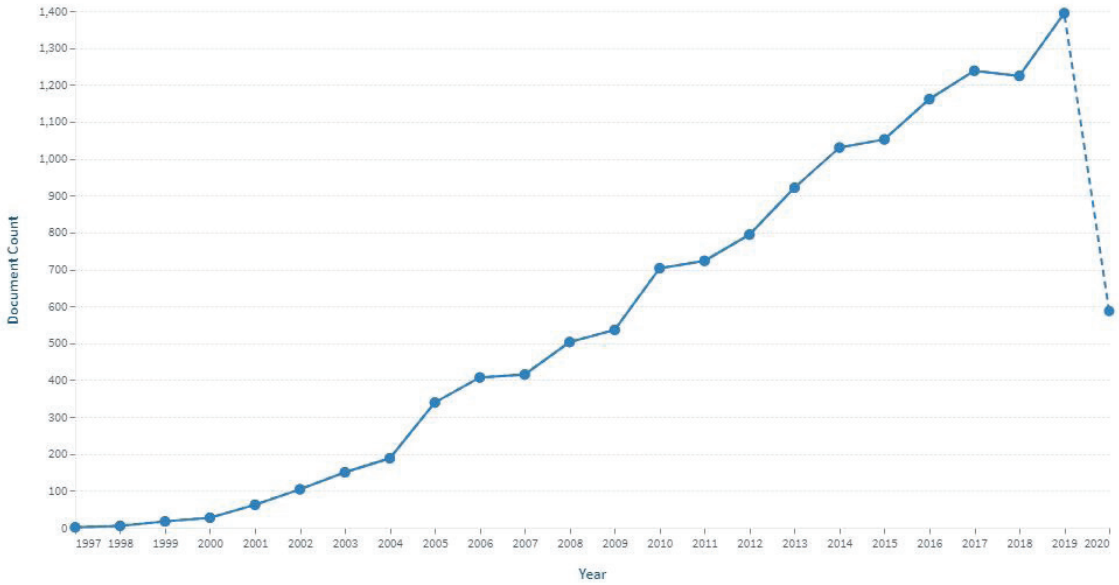

Figure 2. Number of patents filed with keywords "resveratrol" and "cancer" from 1997 to 2020. Data was obtained from *The Lens* patent search engine.

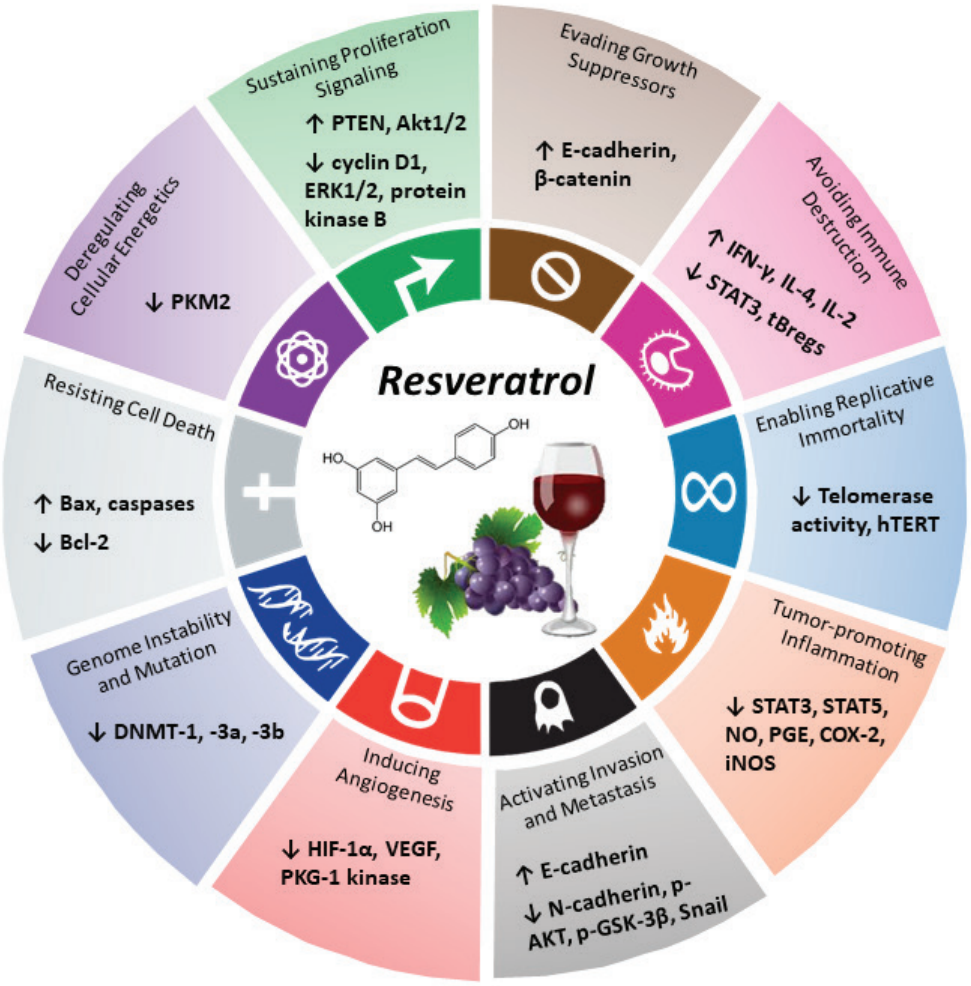

Figure 3. Cancer hallmarks and molecular targets modulated by resveratrol.

Prevention of Proliferative Signalling

Most cancer cells have upregulated proliferative signalling pathways in order to maintain their survival. The signalling pathways are often associated to the alteration in the expression of proteins related to cell cycle, metabolism and cell proliferation[37–39]. RSV promoted the growth of ovarian cancer cell (OVCAR-3) by blocking PI3K and Akt signalling pathways, through reduction of phosphorylated extracellular signal-regulated kinase (ERK)1/2, downregulation of cyclin D1 and protein kinase B[40]. Cancer cells have prevented the proliferation via activation of autophagy-suppressing pathway such as phosphatidylinositol 3-kinase (PI3K)/Mammalian Target of Rapamycin (mTOR)[30]. Autophagy has become a target that may play an important role in cancer treatment. Studies have shown that RSV could induce autophagy cell death. Zhang and the research team proved that the treatment with RSV caused accumulation of calcium ions which then led to the activation of phosphorylated AMPK and phosphorylated Raptor via Ca^{2+}/AMPK-mTOR signalling pathway in the A549 human lung adenocarcinoma cells[31]. The anti-proliferative effect of RSV has been determined in xenograft animal model (HCT116 colon cancer cells in athymic nude mice). The RSV induced the gene expression of PTEN, the tumour suppressor gene, which then led to the downregulation PI3K/Akt signalling evidenced by the phosphorylation of Akt1/2[32].

Activation of Growth Suppressors

On top of sustaining the proliferation, the cancerous cells are also overpowered the growth suppression pathway. There are two commonly known mechanisms of tumour suppressors, the retinoblastoma-associated (RB) and TP53 proteins. These proteins prevent the progression of cancerous cells growth through cell cycle arrest and cell death via induction of apoptosis. Evidence suggests that RSV induces cell death through the activation of p53 pathway, which further triggered apoptosis via mitogen-activated protein (MAP) kinases in T98G glioblastoma cells[41]. The results were in congruent to with another study in which the p53-dependent apoptosis was activated due to the binding of RSV to integrin αVβ3, more specifically the β3 monomer[42].

Research found that the abruption and suppression in the transforming growth factor β (TGF-β) and "contact inhibition" pathway which involve E-cadherin and epidermal growth factor (EGF) receptors has led to the proliferation of cancerous cells[36]. Zhong *et al.* (2015)[43] postulated that RSV suppressed the proliferation of OVAR3 ovarian cancer

cells by upregulated the protein expression of E-cadherin which subsequently increased the β-catenin on the cell membrane, leading to the inhibition of proliferation.

Promotion of Cancerous Cell Death

Normal cells maintain the homeostasis by programmed cell death through apoptosis. However, this normal cellular homeostasis is abrogated during tumorigenesis. Cancerous cells modulate the regulatory and effector components in the cell via increase the anti-apoptotic proteins such as Bcl-2 and downregulate the pro-apoptotic proteins such as Bax, Puma, Bim and caspases. Induction of apoptosis is considered as one of the strategies in cancer treatment due to the minimal inflammatory response[18]. Convincing evidences showed that RSV induced apoptosis in various cancer cells such as T acute lymphoblastic leukaemia cell line[44], HCT116 colorectal carcinoma cell[45], Caco-2 colorectal cancer cells[46], TRAMP murine prostate cancer cells[33] and MCF-7 breast cancer cell[34] through upregulation of Bax and downregulation of Bcl-2 expression. Some studies have suggested that the changes in the expression on both Bax and Bcl-2 were due to the disruption in the mitochondria membrane potential ($\Delta\psi m$) after treatment with RSV[33].

Apart from apoptosis, autophagy plays an important role in maintaining the cellular homeostasis by clearing up the aggregated proteins, damaged organelles or exogenous components via a lysosomal degradation process[30]. Studies suggested that RSV could be a natural autophagy agent. Luyten and the research team found out that RSV induced autophagy via mTOR-dependent pathway with the presence of IP_3Rs and extracellular Ca^{2+} in HeLa human cervix carcinoma cells[35]. The results is in compliance with Park *et al.* (2016)[47] in which they suggested that RSV inhibited mTOR by directly docking onto the ATP-biding site of mTOR[47].

Disabling Replicative Immortality

Telomeres have long been regarded as the guardian of genome stability. The telomere theory of aging and longevity of cells is well-recognised through its ability to elongate the DNA[48]. Notwithstanding, the association of telomeres with cancer has been suggested in several studies that consistently demonstrated that the tumour cells contains higher percentage of telomerase activity as compared to normal cells. These observations strengthened the coherency that explains the immortality of cancerous cells. The understanding of the mechanisms underlying telomerase activity and telomere structures have offered another option for cancer therapy[49]. Wang *et al.* (2011)[50] reported that RSV delayed the senescence of endothelial progenitor cells isolated from human peripheral blood via the downregulation of telomerase activity. The study also suggested that the compound has regulated the expression of human telomerase reverse transcriptase (hTERT), the rate limiting component of telomerase activity, via the PI3K/Akt pathway[50]. The results were in accordance to Mirzazadeh *et al.* (2017)[49] in which they pointed out the role of RSV in regulating the replication immortality by inhibition of the hTERT gene expression in a dose-dependent manner in human glioblastoma cell line U-87MG[49].

Inhibition of Angiogenesis

One of the vital components for cancer development and progression is the formation of blood vessels (angiogenesis) in the tumour area. Cancerous cells regulate the pro-angiogenic factors such as vascular endothelial growth factor (VEGF) to stimulate vascular endothelial cells to enter the tumour hypoxic areas and anti-angiogenic factors such as thrombospondin-1 (TSP1) to inhibit the effect of VEGF[51]. Studies showed that treatment with RSV has decreased the stabilisation of hypoxia-inducible factor- 1 (HIF-1α) which in turn downregulated the VEGF protein expression and upregulated the TSP1 protein expression in spheroid A375 melanoma cells[52]. Another study by Mikuła-Pietrasik *et al.* (2012)[53] suggested that RSV suppressed the angiogenesis by downregulating IL-8/CXCL-8, the proangiogenic chemokine which regulated the expression of VEGF, in human peritoneal mesothelial cells (HUVEC, HMVEC and HMEC-1)[53] . A combination of RSV with 5-fluorouracil (5FU) has enhanced the *in vitro* anti-angiogenic effect in B16 melanoma cells, as compared to treatment with either drug alone. The results demonstrated that the combined treatment has synergistically decreased the VEGF protein expression. As a proof of concept, further study was performed with B16 tumour-bearing BALB/c nude mice in the effort to assess the *in vivo* anti-angiogenic effect. After subcutaneous injection for 10 days, the combined treatment of RSV and 5FU has significantly decreased the VEGF protein expression with reduced microvessel density as compared to the negative control group[54]. RSV has shown its anti-angiogenic effect by inhibiting the tube formation and cell migration in primary human vascular endothelial cells (HUVECs). The authors reported that the process was achieved by the suppression of PKG-1 kinase and four inhibitors of apoptosis proteins (c-IAP1, c-IAP2, livin and XIAP)[55].

Deactivation of Invasion and Metastasis

Epithelial-mesenchymal transition (EMT) is a process where the epithelial cells lose the cell-cell contact by exhibiting mesenchymal phenotype. This process potentially worsen the prognosis in cancer patients by enhancing the invasiveness and migration of cancerous cells[56]. Several studies have confirmed that RSV inhibited metastasis and invasion in various cancer cells through EMT signalling pathway[57–59]. Yuan and team proved that RSV reversed EMT via regulation of AKT/GSK-3β/Snail signalling pathway both *in vitro* colon cancer cells (SW480 and SW620) and *in vivo* lung metastasis animal model (SW480 tumor bearing nude mice)[58]. The effect was verified using proteomic experiments, indicating that the treatment with RSV has upregulated the expression of E-Cadherin and downregulated the expression of N-cadherin, p-AKT, p-GSK-3β and Snail in both models[58]. Another key regulator, the metastasis-associated protein 1 (MTA1), is also known to correlate with the aggressiveness of the tumour metastasis. Li *et al.* (2013)[60] has first demonstrated that RSV modulated the expression of MTA1-mediated proteins such as Ac-p53 in

prostate cancer xenografts animal model, consolidating the ability of RSV in deactivation of metastasis.

Epigenetic Modification

Epigenetic modification such as DNA methylation and histone modification are the important key regulators in affecting tumorigenesis[61]. A number of research has been done to investigate the interaction of RSV with epigenetic targets such as DNA methyltransferase (DNMT), histone deacetylase (HDAC) and lysine-specific demethylase-1 (LSD-1) in regulating the histone proteins and DNA molecules[62]. RSV showed its ability to reverse carcinogenesis by significantly reducing the DNMT mRNA expression (DNMT1, DNMT3a and DNMT3b) in human breast cancer cell lines (MCF-7 and MDA-MB-231), evidenced by Mirza *et al.* (2013)[63]. Similarly, in combination of another plant-based pterostilbene, RSV has significantly decreased the activation of DNMT enzyme via restoration of oestrogen receptor-α (ERα) expression in ERα-negative breast cancer MDA-MB-157 cell line[64].

Ameliorating the Tumour-Promoting Inflammation

Studies showed that 25% of the cancer occurred due to the chronic inflammation as a result of simultaneous destruction and healing of body tissues. Tumour-associated inflammation is always linked with poor prognosis[65–67]. Signal transducer and activators of transcription (STAT) and NF-κB signalling pathways are the two promising targets for cancer therapy as both collaboratively play role in inflammatory response via inducing the expression of pro-tumorigenenic genes such as *IL-1β, COX-2 and cyclin D1*. Many studies have been focusing on the effect of polyphenols including RSV in tumour-associated inflammation. RSV has shown its efficacy by suppressing both STAT3 and STAT5 phosphorylation in 786-O renal cell carcinoma cells as evidenced by Kim *et al.* (2016)[68]. It also co-regulated both STAT3 and NF-κB pathways in medulloblastoma UW228-2 and UW228-3 cell lines, which subsequently lead to the up-regulation of Bcl-2 protein expression[69]. Interestingly, RSV also regulated JAK-STAT pathway in colon cancer HT-29 cell line with significantly reduced expression level of nitric oxide, prostaglandin E_2, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), consolidating the effect of RSV in inflammatory process in various cancers.

Programming Energy Metabolism

Warburg effect was observed in cancer where the cells altered energy metabolisms, including increased glycolysis and lactate production, to cope with their growth requirements. Therefore, the proteins that are involved in the metabolism become the key elements in tumour progression and therapeutic targets for cancer treatment[70–72]. Saunier *et al.* (2017)[70] proved that RSV managed to reverse Warburg effect via activation of pyruvate dehydrogenase in colon cancer Caco2 cells. Interestingly, the reversal effect could be observed even with low doses of RSV that mimicked the drug concentration in human patients[70]. Another study proposed that RSV modulated the Warburg effect via mTOR signalling pathway by down-regulation of pyruvate kinase M2 (PKM2), a catalyst in converting the phosphoenol-pyruvate to pyruvate[73], in cervical cancer HeLa, liver carcinoma HepG2 and breast cancer MCF-7 cell lines[74].

Promotion of Immune Destruction

In normal cells, immune system acts as a surveillance to protect our body from against foreign microorganisms. In events where infection occurs, the immune system will initiate a series of mechanism to eliminate the source of infection. Principally, the immune cells could mount a response in eliminating the cancer cells which are recognised as "non-self"[75]. However, the cancerous cells gain ability to alter the immune system through several mechanisms such as modulating the expression of major histocompatibility class (MHC1) or immunosuppressive products[76](Print. Studies have suggested that RSV could modulate the molecular modulators of the inflammatory response *in vitro* and *in vivo*, via the activation of macrophage, T cells and natural killer[77]. RSV suppressed the proliferation of cancerous cells via the secretion of IFN-γ, IL-4, IL-2, CD4+ in lymphocytes and elevated the secretion IL-10 via downregulation of the CD80 on macrophages[78]. The compound was proven to enhance the expression of FcγRIIB, a receptor for IgG that blocks the activation of B cells in mice[79]. As a result of the regulation of immune system, Lee-Chang *et al.* (2013)[80] showed that RSV could inhibit the lung metastasis in 4T1-tumor bearing BALB/c mice via inhibition of phosphorylation of STAT3 and hence inactivation of tBregs[80].

MOVING TOWARDS CLINICAL TRIALS

In order to realise the clinical translation for cancer therapy, the toxicity of RSV have been investigated to gauge its safety and effectiveness in both the animals and human. A commercialised RSV (ResVida) was found to have no-observed-adverse effect levels (NOAELs) of 750 mg/kg/bw in rats via oral administration, which then converted to human equivalent dose of 450 mg/kg[81]. At higher dose of 3,000 mg/kg, oral administration of RSV does seem to caused renal and bladder toxicity after a daily administration for one month in rats[82]. In human, the oral consumption of 5 g of SRT501, a proprietary micronised formulation of RSV by GlaxoSmithKline (GSK) for 14 days are well-tolerated in colorectal cancer patients[83]. However, the toxicity seems to be largely affected by the pre-existing conditions of patients. For instance, one clinical trial that recruited subjects with multiple myeloma was prematurely terminated due to high incidence of renal toxicity after given a dose of RSV similar to SRT501. The patients had experienced an elevated serum creatinine level with more than 500 μmol/L with minority developed crystal nephropathy and acute tubular damage[84]. The toxic effect is believed to happen specifically to multiple myeloma patients which had developed pre-existing renal damage prior to the treatment.

A number of clinical trials have been conducted in recent decades to investigate the anti-cancer effect of RSV. Table 1 summarizes the clinical trials involved the use of resveratrol as a single therapeutic agent or combination therapy in cancer treatment:

Table 1. Clinical trials of resveratrol in cancer treatment, data retrieved from https://clinicaltrials.gov/.

Type of cancer	Phase and status	Treatment	Outcome	Ref.
Colon	Phase I (Completed)	- Oral administration of 20, 80, 160 mg/day of RSV - Two weeks treatment	Significant inhibition of the gene expression of Wnt target in colonic mucosa	[85]
Colon and Rectal	Phase I (Completed)	- Treatment is given after surgical resection - Oral administration of RSV for 9 days continuously (dose is not mentioned)	Outcome is not provided	-
Multiple Myeloma	Phase II (Terminated)	- Oral administration of 5.0 g SRT501 (RSV) for 20 days	The trial was terminated due to reported renal toxicity	[84]
Lymphangioleiomyomatosis	Phase II (Recruiting)	- Escalating doses of RSV from 250 mg daily (for 8 weeks, 500 mg daily (8 weeks) and 500 mg twice a day (for 8 weeks) - (Route of administration is not reported)	Outcome is not yet reported	-
Polycystic ovary syndrome	Phase 4 (Recruiting)	- A combination treatment of 500 mg of RSV and 20 mg simvastatin daily - (Route of administration is not reported)	Outcome is not yet reported	-
Unspecific solid tumour	Phase I (Completed)	- Oral administration of 0.5, 1.0, 2.5, or 5.0 g RSV daily for 29 days	A decrease in level of IGF-I and IGFBP-3, suggesting RSV exhibits the chemo- preventive property	[86]

CONCLUSION

Currently, the search and development of a more effective cancer therapeutic regimen with low toxicity and "broad spectrum" that could simultaneously target several mechanisms has been encouraged in response to the intolerable conventional chemotherapy. The cancer hallmarks have been adopted to provide a clear insight in understanding the potential of specific compounds as "dirty drugs" on the molecular basis. As can be seen, RSV was found to be interfering with all the cancer hallmarks and modulating the key signalling pathways in tumour development, hence suggesting that the compound hold a great promise for future anticancer drug development. On top of that, several studies have found that RSV exhibit synergistic anticancer effect when combined with other therapeutic agents. Even though the preclinical studies showed promising results, the compound has been disappointing with its toxicity and poor pharmacokinetics in human. We believe that RSV should be considered favourably as a broad-spectrum chemotherapeutic agent that might overcome the limitations of conventional treatment. The drug design and delivery formulation of this compound should be further improved for clinical translation.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Review Article

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Molecular profiling and detection methods of microRNA in cancer research

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Abstract: A large portion of human genome was believed to be "useless" and termed as "junk DNA" in the past, given that these sequences did not have any protein coding role. However, with more researchers dwelling into the world of these mysterious genetic codes, a group of non-protein coding RNA (ncRNA) known as microRNAs (miRNAs) is now being recognized to play important roles than they were thought to be. In truth, the first discovery of miRNA was in a simple organism - nematode (scientific name: *Caenorhabditis elegans)*, whereby a mutant displayed aberrant morphological changes. Years after that, researchers then realized that these miRNAs are actually important regulatory molecules — controlling cell division signaling, apoptosis and so on. In fact, the unusual expression of miRNAs has also been associated in etiology of various cancers. Acting like a "double-edge" sword, miRNAs can control and/or act as tumor suppressor genes and oncogenes, thus any unwanted alterations in their expression would bring upon disastrous effects on the host. Therefore, the current review aims to summarize the molecular detection tools that are available for miRNA profiling in cancer research.

Keywords: miRNA; noncoding RNA; detection; profiling; cancer

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Introduction

In the 1972, a geneticist Susumu Ohno coined the concept of "junk DNA", which was used to describe all the non-protein coding regions within the human genome[1]. Since then, scientists around the world have been trying to decode the human genetic code or DNA, particularly to illuminate and understand the genetics of gene regulation and function. Less than twenty years later, the Human Genome Project (HGP) commenced in the early 1990s as an international, collaborative research which is known to be an important milestone in understanding the human genome[2,3]. More importantly, apart from identifying the genes, one of the eight major goals proposed by HGP in 1998 was to elucidate functions of these genes, including non-protein coding sequences. One of the most game-changing discovery was a type of small RNA called microRNAs (miRNAs) in 1993 by Lee and colleagues, in which they cloned the *lin-4* locus almost two decades after its first description and this locus was proven to have exceptional characteristics compared with other normal coding genes[4]. Rather than coding a protein, the *lin-4* gene presents as a small RNA molecule. Two small *lin-4* transcripts with approximately length of 22 and 61 nucleotides were discovered in *Caenorhabditis elegans*; these were significantly smaller in size compared with other genes. Also, *lin-4* mRNA transcripts displayed antisense complementarity to multiple sites in the 3' untranslated region (UTR) of another gene known as *lin-14*, implying that *lin-4* regulates the translation of latter gene via an antisense RNA-RNA interaction. These remarkable breakthroughs subsequently instigated the concept of a unique class of small ncRNA regulatory molecules acting via an antisense-like interaction[5,6]. Accumulating evidence is pointing directly at the role of miRNA in human chronic diseases, including diabetes,

Alzheimer's disease, cardiovascular diseases as well as cancer[7,8]. Carcinogenesis is a very complex, multi-step process which involves at least three stages: initiation, promotion and progression/metastasis[9]. The mutated cells somewhat managed to overcome fate of cell death or apoptosis and subsequently divide and expand itself; some scenarios would bring about life threating events particularly when these mutated cells gain the ability to promote blood vessel formation in order to gain access to nutrients and oxygen supply as well as ability to spread via moving or metastasize to nearby local tissue[9,10]. With the increasing interest in human genome, it was then noted that miRNA expression patterns were certainly tissue-specific, with cancer cells demonstrating significantly different profile than their normal counterpart[11,12]. Furthermore, additional studies also demonstrated that miRNA expression in peripheral blood could be used as an alternative way to "gauge" miRNA expression in the tumor biopsy. Therefore, the current study is to summarize and discuss conventional molecular detection tools as well as some newer, innovative methods that are available currently for miRNA profiling in cancer research.

What are miRNAs and their role in cancer?

miRNAs are recognized as a group of small non-protein coding RNA (ncRNA) sequences with regulatory potential. The biogenesis of miRNA begins with the processing of RNA polymerase II/III transcripts post- or co-transcriptionally, with most of the miRNA being intragenic and processed largely from introns[13]. Two main pathways have been described for miRNA biogenesis: (a) canonical pathway which involves processing by a microprocessor complex an RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and a ribonuclease III enzyme, Drosha and (b) non-canonical pathway that can be either Drosha/DGCR8-independent and Dicer-independent pathways[13,14]. Nonetheless, it has been shown that animals cannot survive or reproduce normally without miRNAs expression[15]. Using *C. elegans* as an *in vivo* model in genetic study, Sulston and Brenner were able to screen for 300 mutants that gave rise to *C. elegans* displaying diverse developmental defects and behavioral changes in the early 1970s[16]. One of the mutants, designated as lin-4, exhibited elongated and flaccid body morphology with diverse reiterations for particular cell lineages. Little did the scientists noticed that this discovery became the founding member of the miRNA family, whereby it was shown to be involved in the early development of *C. elegans* larval by influencing timing of developmental events across various cell types[17,18]. Nonetheless, the atypical expression of miRNAs has also been associated in etiology of various cancers, given that it can regulate and/or act as tumor suppressor genes and oncogenes[19–21]. For example, Calin and team failed to notice any protein coding genes within chromosome 13q14 and later found that the loss of two miRNAs, miR-15a and miR-16-1 in more than 65 % of B cell chronic lymphocytic leukemia patients[22]. Later on, another study by Bandi and team also revealed that these miR-15a and miR-16 are often deleted or downregulated in non-small cell lung cancer (NSCLC) tissues[23]. Even though the target for these two miRNA seems to be unknown, some studies showed that these miRNA may possess a negative regulatory role for anti-apoptotic B cell lymphoma 2 (Bcl2) protein and eventually induces apoptosis in leukemic cell model[20,24,25]. As such, alterations in miRNA may indeed sound like "total chaos" for the human body, contributing to the hallmark features of human cancer including supporting cell division signaling, dodging growth suppressors, avoiding cell death, activating replicative immortality, increasing invasion and metastasis ability as well as promoting angiogenesis[26–29]. Rather than a loss of function, oncogenic miRNAs or "oncomiR" grant cancer cells survival/growth advantages by evading apoptosis and tend to cleave target mRNAs more frequently than those miRNAs with tumor suppressors function[30–32]. One of the classic example for oncogenic miRNAs would be miR-17-92 which exists as a miRNA polycistron at chromosome 13q31 and is highly expressed in a range of human cancers including lymphoma, lung cancer, breast cancer, colon cancer as well as head and neck cancers[33–38]. All in all, extensive dysregulation of miRNAs can have serious impact on the development of various human cancers, which in turn explains for its importance as diagnostic and prognostic markers as well as targets for new therapeutic agents[39].

Detection and profiling of miRNA

Currently, there are several methods to capture and quantify expression of these small non-coding miRNA. In terms of miRNA detection, there are three main techniques: (a) hybridization-based techniques (e.g. Northern blots, microarrays), (b) amplification-based (e.g. RT-qPCR) and (c) cloning-based (e.g. miRAGE) (Figure 1)[40]. The decision on which techniques to be used for detection is greatly dependent on number of specimens, running time and cost. For examples, Norther blots developed by Alwine and team in the late 1970s remained as the gold standard for gene expression changes and miRNA studies[41]. However, in terms of expression throughput, multiplex RT-PCR and miRAGE can produce results in shorter time, but the newer techniques like microarrays are generally better choices if the study involves large number samples or requires in-depth analysis[40]. Having said that, the quantitative power of these techniques varies; RT-qPCR produces quantitative data, whereas results from Northern blots are semi-quantitative. Hybridization-based technique like *in-situ* hybridization is normally non-quantitative. Each of these techniques have got their advantages as well as limitations, therefore researchers may opt to combine these techniques in a study to improve and/or strengthen their findings.

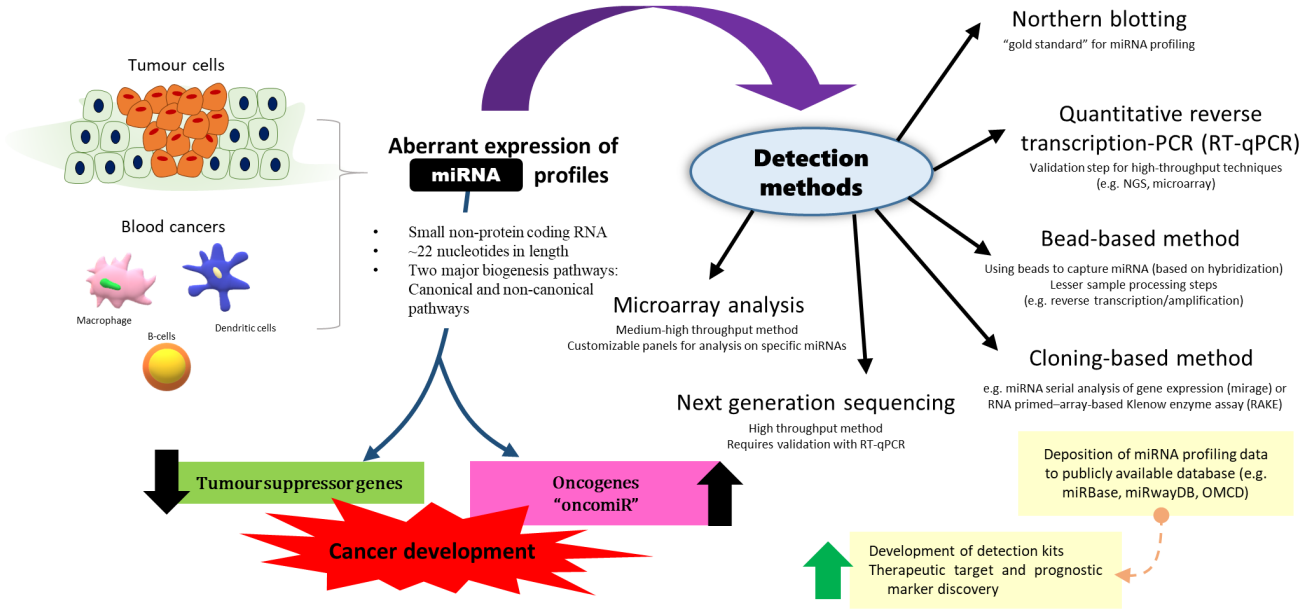

Figure 1. Examples of miRNA profiling detection methods in cancer research.

Northern blotting

Even though close of half a decade has passed since the first introduction of Northern blots, this technique is still the gold standard for miRNA expression profiling. In spite of that, there are several technical considerations which have been raised up by researchers when using Northern blot as a routine miRNA expression profiling tool. As much as the technique stands as a high sensitivity assay, Northern blotting requires huge running time and large amounts (5–25 mg) of total RNA from each sample, on top of potentially dangerous radioactive probes[40].

As a result, researchers have come up with alternatives in probe design, specifically to allow the detection of miRNA without compromising its sensitivity and accuracy. One of which was a technique designed based on the Northern-blot principle by Kim and team, known as LED which consists of three "key players" – digoxigenin-labelled oligonucleotide probes containing locked nucleic acids (LNA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for cross-linkage with membrane[42]. The authors claimed that LED is able to generate clearly visible signals for RNA amounts as low as 0.05 fmol and requires only few seconds of membrane exposure, equivalent to ~1000-fold improvement in exposure-time (Kim et al., 2010). As a non-radioactive label, DIG assay is safer than radioactive methods, while offering the same/similar sensitivity as isotope labeling-based methods[43]. Along with this, locked nucleic acid (LNA)-modified oligonucleotides greatly improve the sensitivity of Northern blotting technique, as high as ten-fold compared to conventional DNA probes[44,45]. LNAs were described as a new class of bicyclic high-affinity RNA analogues, containing a furanose ring in the sugar-phosphate backbone that is chemically locked in an N-type (C3'-*endo*) conformation by the introduction of a 2'-*O*,4'-*C* methylene bridge[45]. Furthermore, Válóczi and team explained that the oligonucleotide probes with LNA was designed in a way that every third nucleotide position was substituted by a LNA monomer. This design subsequently resulted in an increase in its sensitivity yet retained its high specificity, which enables detection of both mature and precursor miRNAs[46]. Similarly, EDC chemically mediates cross linking of RNA to (nylon) membrane which has been shown to improve miRNA detection by up to 25 to 50-fold, depending on plants or mammalian samples[47,48]. Therefore, by combining the goodness of these three components, Kim and team highlighted that LED probes offer multiple advantages; it's more environmentally friendly compared to conventional probes which can be stored for at least six months [42]. Having said that, these "upgrades" seem to incur higher cost compared with the conventional old method, especially when LNA probes can be more expensive than DNA probe and DIG-labeling which will also add up to the final cost.

Microarray-based method

Microarrays are amazing tools in cancer research. Based on nucleic acid hybridizations between target molecules and corresponding complementary probes, it allows genotyping at multiple loci or targets at once. In reality, majority of the published studies reporting miRNA profiling used a variety of microarray technologies. Its high utilization in miRNA profiling studies is fairly self-explanatory, as most of the experimental (and analysis) steps can be done at any molecular biology laboratory and it also allows customization including probes design, chemistry of probe immobilization, labeling of samples and detection of signal[49,50]. At some point, some researchers have also implicated its potential to be used as a standard tool in the near future[51].

With the availability of miRNA databases (e.g. miRBASE, miRwayDB, OMCD), these efforts in turn speeds up the

development of commercially available miRNA microarrays.

In general, a ready-to-use miRNA microarray contains miRNA oligonucleotide probes that have a amine-modified 5'-end which are immobilized onto glass slides by covalent crosslinking[52]. The detection of miRNA type is based on the binding location of fluorescent-dye labelled miRNA on the slides, while its expression level can be quantitated based on the levels of fluorescence emission. Along with this, LNA can also be used in miRNA microarray due to its exceptional affinity and specificity to the complementary RNA. On top of the efficient visualization fostered by the extraordinary thermal stability between LNAs and their target RNA molecules, LNA seems to improve the mismatch discrimination which then lead to multiple attempts to incorporate LNA-based probes in miRNA microarrays design for profiling purposes[53–55]. Exiqon (now under Qiagen) used to manufacture microarray platform with probes containing LNA bases, providing higher annealing affinities[49]. As a result, several studies attempted to compare the performance of commercial miRNA expression array platforms[49,56]. Git and team included six commercially miRNA expression array platforms which consist of both single- and dual-channel fluorescence technologies in their studies[49]. Based on their studies, all six platforms tested were equally applicable to cell line and tissue, but factors such as input sample amount may need to be considered when selecting a platform to be used. On the other side, another study compared four platforms including Agilent, Illumina (platform withdrawn since 2010), Exiqon and Miltenyi[56]. It was noted that higher modulated miRNAs were identified via Agilent and Illumina platforms for class comparison analysis between tumor and normal samples. Even though Exiqon did not produce the same number of modulated miRNAs as the two platforms, most of miRNAs modulated in Agilent and Illumina were detectable, although they did not reach statistical significance. On the contrary, the same miRNAs were mostly undetected on the Miltenyi platform which can be explained by its sensitivity to GC content. Sah and colleagues found similar results with Ambion and Agilent which exhibite better accuracy while Illumina and Exiqon displayed higher specificity[51]. Another company, Invitrogen also developed a series of microarray for miRNA profiling, known as NCode™ Multi-Species miRNA Microarray. Tchernitsa and team used the Invitrogen NCode(™) Multi-Species miRNA Microarray Probe Set containing 857 mammalian probes to study six primary gastric cancers (compared with normal/ non-cancerous tissue); out of which three of them presented lymph node metastases, while the other three did not[57]. Comparing gastric carcinoma with non-cancerous tissue, twenty miRNAs were differentially regulated and six of these miRNAs showed distinct expression which separated node-positive from node-negative gastric cancers, including miR-103, miR-21, miR-145, miR-106b, miR-146a, and miR-148a. These results indeed emphasized the importance of microarray as high throughput method in miRNA studies. Despite of that, researchers have also pointed out that the periodic changes to miRNA database like miRBase then imposes a reannotation of microarray and qPCR probes prior to analysis. Nevertheless, this would also provide researchers the flexibility in selecting types of microarrays to be used, be it pre-designed microarrays or customized microarrays.

PCR-based technique: reverse transcription quantitative PCR (RT-qPCR)

Even though there are several options which allow measurement of multiple target miRNAs simultaneously, RT-qPCR remains one of the popular choice for miRNA profiling as it's an important technique for validating expression data obtained from high-throughput screening (e.g. with microarray).The first step of RT-qPCR begins from precise and absolute conversion of RNA into complementary DNA (cDNA) via reverse transcription. However, given its nature of miRNA which has a limited length (~22 nucleotides), reverse transcription can be difficult to perform. Apart from that, mature miRNA sequence can exist in two forms – pre- and the pri-miRNAs, along with little or no common sequence feature to be used for their enrichment and amplification[58]. Before quantification, there are two approaches for reverse transcription of miRNAs: (a) by using miRNAs-specific reverse transcription primers or (b) using a universal primer that targets the miRNAs that are tailed with a common sequence (e.g. poly-A tail). While the use of miRNA-specific primers (MSPs) reduces background "noise", using a universal reverse transcription method is useful when there are several different miRNAs needed to be studied from a small amount of input. Additionally, there is another alternative which enables multiple miRNAs to be reverse transcribed by pooling stem-loop primers[59,60]. Step-loop primers consist of a short single stranded sequence at their 3'-ends that anneals to the 3'-end of the miRNA of interest, a double-stranded segment (the stem) and a loop. Due to its structure, the primer will not be able to bind to pri- and pre-miRNAs and to any dsDNA that may be present.

RT-qPCR possesses numerous benefits compared to microarray as it presents higher speed and sensitivity as well as larger dynamic range[40,61]. Furthermore, RT-qPCR also requires low amounts of starting specimen which makes it more user-friendly. At the time of writing, there is currently over 1,900 human miRNA curated in miRBase (http://www.mirbase.org/). As single target RT-qPCR can be both time and reagent consuming, several strategies can be used to perform parallel reverse transcription when there is a need to detect a large number of miRNAs in a single sample. A study by Tang and colleagues incorporated 220 individual stem-loop primers in their study to develop a multiplex reverse transcription assay[62]. A pre-PCR process was included to reduce significant loss of detection sensitivity using low amounts of RT-qPCR primers (pre-PCR) and subsequently the cDNA product was diluted before multi-well plates containing MSPs, universal primers and TaqMan probes for RT-qPCR.

Apart from that, there are also miRNA arrays available for

the use of miRNA profiling. Zhang and team measured differential expression of 95 miRNAs in pancreatic cancer tissues and cell lines by RT-qPCR using the QuantiMir System (SBI System Biosciences)[63]. As a consequence, unique miRNA profiles were seen in pancreatic cancer tissues and cell lines which reflected individual diversity, compared to adjacent normal pancreatic tissue or cells. After another validation using RT-qPCR, a total of eight miRNA were upregulated in most of pancreatic cancer tissues and cell types. On the other hand, as a medium-throughput method for RT-qPCR, Taqman array microRNA cards (by Applied Biosystems) combine microfluidics technique and classic/advance chemistries which can measure up to 384 miRNAs. Based on project's needs and directions, user can also select to use either pre-designed (standard) or custom-made cards. Mees and colleagues used TaqMan Low density microRNA Arrays (TLDA) to investigate miRNA expression in ductal adenocarcinomas of the pancreas[64]. The main advantage is that this system allows as little as 1 ng of RNA in circumstances whereby pre-amplification PCR step is performed[65]. For this approach, each TLDA card contains pre-loaded primers and TaqMan probes to amplify a single miRNA[64,65]. After cDNA synthesis using predefined pools of reverse transcription primers, cDNAs are loaded into the micro fluidic card where amplification of individual miRNA occurs[64]. This method provides a simple and convenient way for parallel monitoring of a large number of miRNAs by using RT-qPCR.

Conversely, there are several innovative methods for quantification of miRNA such as LNA-enhanced primers with droplet digital PCR (ddPCR) and the Two-tailed RT-aPCR method which uses primers composed of two hemiprobes (and connected by a hairpin structure)[66-69]. The inclusion of LNA in the forward PCR primers keeps its sequence to be short but highly specific and detects most of the miRNA sequences, while in the reverse primer binds to the 3′ end of the miRNA sequences[66]. Andreasen and team concluded that this unique combination confers specificity plus extreme sensitivity thus making it useful especially for quantification of miRNAs in difficult samples like FFPE tissues. Complementing to the strategy of using LNA-enhanced primer, a relatively new technology that serves the same purpose as qPCR machines comes into the picture, known as ddPCR[69]. This technique eludes several problems with conventional qPCR such as the need for reference gene or replicate samples as the system detects fluorescence signals in the nanoliter-sized water-in-oil droplets containing target molecule[70,71]. Using FFPE specimens, Laprovitera and team used a customized, pre-spotted 96-well plates to study the expression of 92 miRNA with different miRCURY LNA miRNA primers (Qiagen, former Exiqon)[69]. The customized plate was designed to cover most cancer-specific miRNAs (including 89 cancer-specific miRNA and 3 reference genes). In their study, the team managed to detect miRNAs expression in 14 FFPE specimens representing different type of tumors comprising of liver, skin, breast, gastric, colon, ovary, prostate, gastrointestinal-neuroendocrine and so on. Even though there wasn't a direct comparison between this technique and microarray in the same study, the team observed a highly significant correlation ($p < 0.0001$, Spearman $r > 0.7$) between results generated from this technique and their previous microarray experiment (after data normalization).

In 2017, Androvic and team introduced a novel and cost-effective strategy to quantify miRNA expression known as the Two-tailed RT-qPCR[67]. As the primers are made up of two hemiprobes (which are complementary to different regions of target miRNA) with a oligonucleotide tether folded into a hairpin structure, this design then subsequently increase the binding strength to template, increasing its sensitivity while detecting all terminal variants of any miRNA (isomiRs). In another words, this assay enables a "true" reflection or measurement of total miRNA content (of interests) in a sample. Having the advantage of allowing multiplex during the reverse transcription step, the miRNA profiles generated by Two-tailed RT-qPCR displayed excellent correlation with the standard TaqMan miRNA assays ($r^2 = 0.985$). Following the success of developing the rapid technique for miRNA expression with a total analysis time of less than 2.5 hours, the team led by Androvic subsequently published another work last year, highlighting the use of this technique as a quality control test for circulating miRNA studies[68]. As a matter of fact, even though RT-qPCR is described as a "gold standard" for typical gene expression studies, its application in miRNA profiling may be limited by the constantly increase number of miRNA on a genomic scale, thus suggesting its utilization as a validation test for other high-throughput techniques such as microarray and next generation sequencing (NGS) which might be a more affordable plan (depending on factors such specimen and miRNA target numbers).

Next generation sequencing (NGS)

In biomedical science, Sanger sequencing has always been the principal approach and gold standard for DNA sequencing[40]. Two decades ago, a team from Lynx Therapeutics (USA) (which was later acquired by Illumina) launched the first NGS technologies known as Massively Parallel Signature Sequencing (MPSS)[72]. The technological feature of this method is that clonally amplified or single DNA molecules in MPSS are spatially separated in a flow cell, making it different from the Sanger sequencing which works based on the electrophoretic separation of chain-termination products produced in individual sequencing reactions[40]. As a general rule, NGS technologies involve repeated cycles of nucleotide extensions mediated by polymerase or by iterative cycles of oligonucleotide ligation in one format[73]. NGS platforms available today are Roche 454 GS FLX sequencing, Illumina/Solexa Genome Analyzer sequencing, Applied Biosystems/SOLiD as well as Helicos Biosciences and Single-Molecule, Real-Time (SMRT) Sequencing by Pacific Biosciences (PacBio).

In truth, quite a number of known miRNAs were discovered by conventional cloning and Sanger sequencing approach[74]. However, the sequencing power of NGS opens up a new window for researchers to discover novel miRNAs as it is not hindered by variability in melting temperatures, neither co-expression of almost identical miRNA family members nor post-transcriptional modifications as in other molecular techniques like microarray and RT-qPCR. At earlier times, several groups have attempted to use different NGS

technologies to uncover novel miRNA or even generate miRNAome. In 2010, Ramsingh and team used 454-based sequencing to study miRNAome in a patient suffering from acute myeloid leukemia[75]. A total of 472 miRNA (including 7 of them being novel) was identified from leukemic myeloblasts; some of them showed differential expression compared to normal (healthy) $CD34^+$ cells. Besides that, Creighton and team took advantage of another platform — Illumina/Solexa Genome Analyzer sequencing to discover of novel miRNAs in female reproductive tract[76]. In the same study, they have added nearly 100 putative novel miRNA (with mid-high confidence) derived from various organs of the female reproductive system (in both diseased and normal states), representing diseases such as ovarian cancer, endometriosis, and uterine tumors (benign and malignant). Comparing two different systems, the Illumina platform (e.g. HiSeq) which uses typically generates more reads at lower cost compared to Roche 454 pyrosequencing system[53,77]. Furthermore, one of the main problems with the latter system is that it has got relatively high error rate (for poly-bases longer than 6 bp), even though the system does offer an automated process for library construction. Even so, Applied Biosystems purchased SOLiD or Sequencing by Oligo Ligation Detection sequencing in 2006, which stands as a two-base sequencing technology based on ligation. Schulte and colleagues have adopted SOLiD NGS in their study analysing small RNA transcriptomes of neuroblastoma cases. The team subsequently successfully revealed the differential expression of miRNAs in favourable versus unfavourable neuroblastoma[78].

The third generation of NGS was firstly introduced in 2008 and defined as single molecule sequencing which was entirely different from the clonal based second-generation sequencing methods[79,80]. The short running time of SMRT sequencing by PacBio makes it particularly attractive for diagnostic use. A research team in India performed IsoSeq analysis on infratentorial ependymoma tumor tissue using SMRT technology, PacBio RSII[81]. A total of 2952 unique transcripts were identified to be involved in 307 KEGG pathways, with 22 transcripts coding 18 genes related with miRNA biosynthesis/processes (KO0520622). With the fast development in NGS technology, the fourth generation of NGS technology such as the MinION, a commercially available device from Oxford Nanopore Technologies (ONT) has also begun to gain attention as a potential tool for miRNA profiling work[82,83]. Even though it comes at a lower pricing compared to the third generation NGS, it is rather unfortunate to mention that more work/modification may need to be done to overcome the compatibility issue of the technology for short nucleic acid strands or miRNA due to their short length[83,84].

As much as NGS offers as somewhat powerful tool to identify and detect miRNA in various samples, some researchers pointed out that there are possibilities of bias resulting from RNA ligation and amplification steps[49,85]. Furthermore, the increasing amount of data generated from these NGS technologies require high computation power for analysis. Also, for high-throughput methods like NGS, additional data validation step is still required using other techniques like qRT-PCR[86,87].

Bead-based method

Apart from techniques mentioned above, some researchers developed another exciting method for the profiling of miRNA expression which works based on "beads" that capture different types of miRNA. Luminex Corporation developed the bead-based array known as xMAP™ system, which is a multiplexed microsphere-based suspension array (http://www.luminexcorp.com/technology/index.html). For this method, the oligonucleotide-capturing probes complementary to miRNAs of interest are linked to carboxilated 5-micron polystirene beads impregnated with mixture of two fluorescent dyes, each coded for a single miRNA[88]. In 2005, Lu and colleagues were able to differentiate tumors that were inaccurately classified by mRNA profiles[89], while another study in United Kingdom discovered new markers of human breast cancer subtype[90]. The bead-based miRNA arrays offers several benefits compared to glass-slide microarrays: (a) user-friendly and easy to use, (b) relatively low cost with advanced statistical performance, (c) faster hybridization kinetics and (d) higher flexibility in preparation of the array[40]. This technique remains as a popular tool in cancer research studying miRNA expression at present day. Wang and team studied the expression of miRNA in NSCLC tissues using Luminex xMAP bead-based suspension array and highlighted that the system requires as little as 2 μL of sample volume without the need of running reverse transcription or amplification step[91]. Though, this method demands for specialized equipment, thus limiting its usage.

Others: Cloning based assay, miRAGE and RNA primed–array-based Klenow enzyme assay (RAKE)

Before witnessing the major breakthrough in developing NGS techniques, researchers have heavily relied on traditional methods like amplification and cloning methods to increase the efficiency of small RNA species discovery including miRNA. miRNA serial analysis of gene expression or miRAGE was developed by Cummins and colleagues that merged the aspects of direct miRNA cloning and SAGE[92,93]. While allowing identification of new miRNA, miRAGE is similar with conventional cloning approaches: it starts with the isolation of 18-26 base RNA molecules and then ligation with specialized linkers and adapters before reverse-transcription step into cDNA[93,94]. The cDNA then will be subjected to another PCR reaction with the help of biotinylated primers before purification step with column of streptavidin-coated beads (to remove biotin tagged linkers). The eluded product will be purified miRNAs and these miRNAs are then concatenated, cloned, and sequenced for analysis. The main advantage of this approach is that it is able to generate large concatemers, enabling as many as 35 tags to be identified in a single sequencing reaction, whereas existing cloning protocols analyse approximately five miRNAs per reaction[92].

Aside from that, there is another method available for miRNA detection known as RNA-primed, array-based Klenow enzyme (RAKE) assay developed by Nelson and colleagues in 2004[95]. As it doesn't require any sample RNA manipulation prior to hybridization, the un-modified miRNA is hybridized to immobilized DNA probes. Biotinylated dATP is incorporated to DNA probe with hybridized miRNAs acting as primers by Klenow enzyme and subsequently a streptavidin-conjugated fluorophore is applied to visualize and analyze the expression of miRNAs. RAKE offers a high throughput protocol with unique advantages for specificity over northern blots or other microarray-based expression profiling platforms[96]. Moreover, the team demonstrated that miRNAs can be isolated and profiled from FFPE tissue, which provides another alternative for analyses of small RNAs from archival human tissue. On top of that, Berezikov and colleagues proved that novel mammalian miRNA candidates can be identified by extensive cloning and RAKE analysis[97].

Conclusion and future recommendations

The continuous expansion of miRNA knowledge has deemed the importance of these small non-coding nucleic acid in the area of cancer research, particularly as biomarkers in diagnostic and therapeutic targets in drug discovery field[98]. Even though newer methods are constantly being developed/introduced for miRNA profiling use, there is still much room for improvement, given that these tools are still unable to replace the conventional method that "sits on the throne". One of the bottlenecks would be experimental confirmation of miRNA targets behind these phenotypes computed via data generated from high-throughput technology. Upon obtaining a comprehensive understanding on their roles, selected miRNA could be used in RNA interference strategy to achieve therapeutic effects against different types of human cancers[99]. In fact, a quick search on clinical trials registry website (e.g. clinicaltrial.gov) revealed that there is a total of 327 studies related to miRNA, out of which 98 studies were interventional studies (as of 8th June 2020). However, no completed results reported results related to the use of miRNA as therapeutic agent(s) on the database yet. Just like any other drugs, it is impossible to observe success with every miRNA tested, but the "magic bullet" would pledge a promising future in the war against cancer.

Authors contribution

The literature review and manuscript writing were performed by NSAM and H-LS. NSAM and II provided vital guidance of the research and proof of the writing.

Conflict of interest

The authors declare that there is no conflict of interest in this work.

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Review Article

Progress in Microbes and Molecular Biology

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Updates on the development of vaccines and therapeutic options against rabies

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Abstract: Even though rabies has been claiming more than 50,000 deaths annually worldwide, it is considered as a vaccine-preventable viral disease. More than 95 % of the total human rabies cases are caused by dogs. During the initial stage of infection, affected individuals usually show weakess at the bitten extremities and the virus can ultimately travel to the brain causing neurological signs. In attenuated (inactivated) form, the currently in use vaccines have been recommended by WHO for the prevention (i.e. pre-exposure prophylaxis, PrEP) and treatment (i.e. post exposure prophylaxis, PEP) regime against the rabies virus (RABV). However, given that they normally require refrigeration and are costly, there have been discussions revolving around potential development of newer, safer and cheaper alternative that can perform better and more convenient than the ones that are currently in use. The current review aims to explore general characteristics of RABV before looking into potential candidates of vaccines that have been studied. Further studies on the pathogenic mechanism of RABV and therapeutic approaches are still required to prevent the deathly infection following clinical manifestation. In sum, integrated interventional strategy emphasizing human health and animal health is essential and requires collaboration between health authorities and the public.

Keywords: rabies; vaccines; treatment; development; therapeutic

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Introduction

Rabies is one of the dangerous zoonotic diseases, claiming more than 50,000 deaths per year worldwide[1]. The "culprit" behind rabies is known as rabies virus (RABV) which can be transmitted by animals including bats, raccoons and foxes[1–3]. Still, dog-mediated rabies infection accounts for more than 95% of the total human rabies cases as the virus can replicate in salivary glands of infected dogs. As a results, RABV can be easily transmitted from affected dogs through bite wounds, licking of damaged skin, or direct mucosal contact[1,4]. The virus attaches itself to its cellular targets by its surface protein (i.e. RABV-G), rapidly gaining access to peripheral nerves. Via retrograde axonal transport and trans-synaptic spread, RABV ultimately enters the brain[5]. If the disease is not treated in a prompt manner, death can occur within 5–7 days upon onset of symptoms[6]. The incubation time before clinical manifestation is influenced by several variables including distance of injection site from the central nervous system (CNS) and virus load at the wound site[7]. Shorter incubation period was observed in victims with a wound on the head/neck or category III exposure[8].Commonly, initial presentation of rabies-infected victims is the weakness at the bitten extremities, which subsequently progresses into acute neurological signs[6,9]. However, there are circumstances in which the victims presented unusual symptoms including severe abdominal pain and abnormal sexual behaviours[10,11]. Rabies infection can manifest as furious or paralytic form. Limbic signs are predominant in furious rabies while paralysis of lower motor neuron is hallmark for paralytic rabies[10,12]. Even after recovery, most rabies survivors suffer from neurological impairment[6,13]. As a consequence, an "ideal" effective immunological defense against rabies would be the interception of virus before productive neuronal infection, considering

there is still established, effective therapy for those who developed rabies encephalomyelitis[14].

Canine rabies remains endemic in most developing countries, it is a huge global burden with estimated 3.7 million disability-adjusted life years and 8.6 billion USD economic losses every year[15]. The World Health Organization (WHO) has issued a notice in the past to discontinue the usage of nerve tissue vaccine and replace the vaccination program with newer vaccine produced from cell-culture or embryonated eggs[1,16]. These newer vaccines typically consist of purified inactivated virus that can be used as prevention (i.e. pre-exposure prophylaxis, PrEP) and treatment (i.e. post exposure prophylaxis, PEP). Thus, the current review aims to provide an overview on the characteristics of RABV before exploring available vaccines and those which are in development. Indeed, rabies may not seem like a disease that can be eradicated completely worldwide, thus it is imperative to continuously seek for safer vaccines or drugs with higher efficacy to curb the spread of such harmful pathogens.

Discovery and characteristics of RABV

As one of the oldest communicable disease known to man, rabies was documented several times in historical records, as early as 4,000 years ago in the pre-Mosaic Eshnunna Code[17–19]. In the code, it was stated that the owner of a rabid dog that bit a person who later died due to rabies must pay a fine. Rabies is an acute, lethal disease marked by encephalomyelitis in which the causative agents are identified to be viruses belong to the genus *Lyssavirus*. RABV or taxonomically known as *Rabies Lyssavirus* (under family: Rhabdoviridae, genus: *Lyssavirus*) is a negative-strand RNA virus. In general, those within the genus *Lyssavirus* are enveloped RNA virus that are viewed as bullet-shaped when cut tangentially or "bulls-eye" in cross sectional view under transmission electron microscope[20,21].

The genome of Lyssaviruses is approximately 11–12kb in size, encoding five proteins including glycoprotein (G), phosphoprotein (P), nucleoprotein (N), matrix protein (M), RNA-dependent RNA polymerase (L) (Figure 1)[22,23]. Belonging to phylogroup I, RABV seems to be far more "adaptable" compared to other strain in the same phylogroup — circulates in both *Chiroptera* (i.e. bats) and *Carnivora* (i.e carnivores) including wolves, foxes, shunks and dogs[24]. Several strains of RABV have been previously described and it has been discussed that certain polymorphisms within the genome can alter virulence and transmission[25]. For RABV, its genome encodes viral proteins in the sequence of 3'-N-P-M-G-L-5'[26,27]. The viral structure consists of M protein encoded by gene M and transmembrane G protein encoded by gene G. G protein plays critical roles in the pathogenesis of rabies by binding to neural receptors and cellular entry via fusion with the cellular membrane[28–30]. As the only surface proteins, G protein is the only protein that is capable of inducing production of virus neutralizing antibodies (VNAs) by the host, therefore essential in determining the evasiveness of RABV against the host immune system[30,31]. A study in 2019 compared laboratory-adapted RABV strain (B2c) and a wild type (wt) RABV isolated from rabid dog in Mexico in 1990s (DRV); the team discovered that lesser G molecules were incorporated into mature virions by wt RABVs when compared to laboratory-adapted RABVs[30]. While recombinant virus with additional G protein (i.e. triple G expression) showed higher expression and incorporation of G protein, the virus activated more dendritic cells (DC) compared to its corresponding wild type form. Conversely, wild type RABVs that were treated with subtilisin or Dithiothreitol (DTT)/Nonidet P-40 (NP40) to remove G protein failed to activate any DC and/or VNAs expression. Without G protein, these G protein-depleted virus evaded the host immune response and caused lethal infection in mice. Furthermore, another study showed that single amino acid change(s) at position of 255 or 349 in G protein decreased the viral pathogenicity of RABV[31,32]. For instance, after introducing the amino acid change at position 349 nucleotide substituting glycine with glutamine ($Gly_{349} \rightarrow Glu_{349}$), the mutant strain (known as rGDSH-G349) exhibited decreased RABV pathogenicity without affecting its propagation rate[32]. On top of that, the same strain was able to induce higher immunogenicity in mice with higher level of VNA observed compared to its parent strain. Altogether, these important findings greatly benefited the scientific community by providing crucial insights into "behaviour changes" of the virus while at the same time enabling researchers to exploit these mutation points for development of therapeutic drugs and vaccines against rabies.

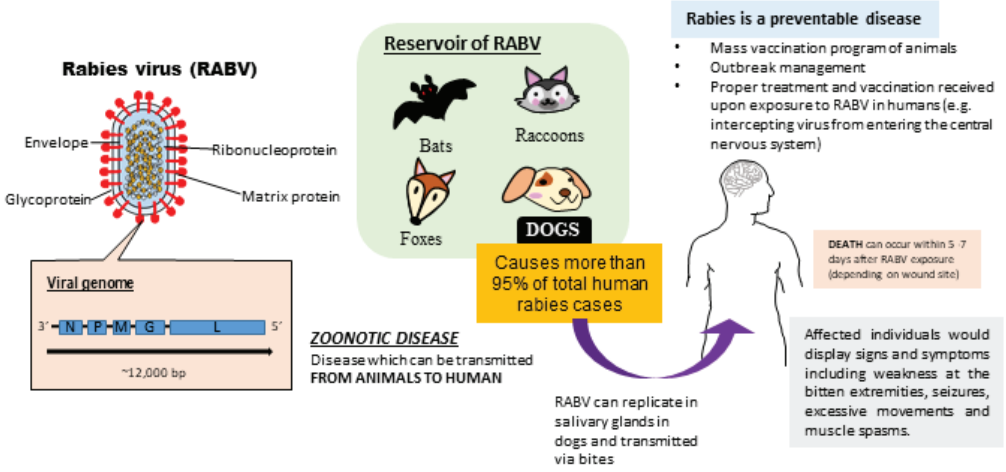

Figure 1. Illustration of rabies virus genome and its common animal reservoir.

On the other hand, the N protein is thought to be a preferred target for phylogenetic studies given that it's highly conserved and expressed while accountable for activating immunogenic response from the host[33–35]. As a matter of fact, N protein which forms the major component of helicoidal nucleocapsid that encapsidates the genomic RNA plays a determining role in viral replication; it facilitates the temporal transition between transcription and replication of the viral genome during the replicative cycle[35]. In contrast, the phosphoprotein encoded by gene P serves as a cofactor for L protein, connecting it to N protein and finally leading to the formation of ribonucleoprotein complex in viral RNA synthesis[36,37]. Besides polymorphism, the rearrangement of viral genes such as P and N has been shown to affect its pathogenicity and immunogenicity. A team led by Mei *et al.* in 2019 found that the rearrangement of gene P in RABV led to its low gene expression which then suppressed N gene and attenuated the pathogenicity of the virus [32,33]. Similar results were reported by Morimoto and team whereby the P-gene deficient (def-P) virus was apathogenic in adult and suckling mice. It was also described that even though the def-P virus can perform the primary RNA transcription, no further progeny virus was produced by the infected host (with def-P virus)[37].

Located on the third position in RABV genome, the M protein encoded by gene M is an important component during viral assembly and budding, covering the RNP coil and maintaining the viral bullet-shaped form [38,39]. On top of that, some studies have highlighted the role of M protein in viral transcription, whereby genetic manipulation on gene M via codon deoptimization led to inhibition of RABV replication at the initial stage of infection but increased viral titre at later stages[40,41]. Likewise, the codon deoptimization strain caused higher level of apoptosis in neuronal cell compared to its parental strain[42]. Besides shedding light on the transmission and replication mechanisms of RABV, the understanding on its genomic content allows researchers to identify and exploit these "weak points" in designing treatments or vaccines against this deadly virus, while monitoring RABV outbreaks and evolution.

Treatment and vaccines development against *Rabies lyssavirus* for human use

In order to fend off infections, the infected host needs to have sufficient and/or adequate immune response to first recognize the infectious agent(s) before eliminating it from the body. Before discussing in-depth about each vaccines that are in-use or in development (i.e. novel), it is important to note that currently in-use vaccines for rabies can be used as prevention (i.e. pre-exposure prophylaxis, PrEP) and treatment (i.e. post exposure prophylaxis, PEP); however, the only difference between these two lies in immunization schedule[43,44]. Moreover, there are two forms of immunizations: (a) passive immunization — by administration of monoclonal antibodies (e.g. human rabies immunoglobulins (HRIG), equine rabies immunoglobulin (ERIG)) and (b) active immunization which involves the use of cell culture- or embryonated egg-based inactivated virus[44,45]. In 1888, crude nerve tissue-based vaccines were developed and used as rabies vaccine but they are being phased out in most countries in the 21st century since the introduction of non-neural tissue-based vaccines. In addition, the crude neural vaccines made from sheep or mouse brains caused severe adverse effects and neurological sequalae including acute demyelinating encephalitis[46,47]. Even so, a few countries including Ethiopia are still using neural vaccines due to their affordability and high cost of newer vaccine[46,48]. As for preventive measures, WHO recommends rabies PrEP for individuals who are at high risk of exposure to rabies including veterinarians, laboratory workers, travellers or residents of rabies-endemic nations[1,49]. PrEP obviates rabies immunoglobulins administration and reduces the number of vaccine doses required when an individual is exposed to rabies[1,49-51]. According to WHO guidelines, a complete PrEP consists of single-dose intramuscular (IM) or two-site intradermal (ID) vaccination on both day 0 and day 7.

At the same time, WHO has also published a detailed guidelines and recommendation on PEP to assist physicians in making decision on treatment: (i) Category I involves touching of animals or licks on intact skin; (ii) Category II involves nibbling of uncovered skin or non-bleeding minor abrasions; and (iii) Category III involves transdermal bites, direct contact with bats, licks on broken skins or mucous membranes[1,51]. No PEP is indicated for category I exposure, but only active immunization (i.e. vaccine) will be given for those with category II exposure. For category III exposed individuals, they will be given both active and passive immunization (i.e. vaccine and monoclonal antibodies administration). The recommended dose of passive immunization is given at 20 IU/kg body weight for HRIG and 40 IU/kg body weight for ERIG and F(ab')2 products. Full dose of rabies immunoglobulins (RIG) can be given into or around wound site, but it can be diluted with physiological buffered saline to ensure better wound coverage in severe cases. Instead, purified cell-culture- or embryonated-egg-based rabies vaccines can be administered intramuscularly or intradermally[1,36]. PEP regimens recommended by WHO include two-sites ID rabies immunization (2-2-2-0-0) on day 0, 3 and 7; two-weeks IM rabies immunization (1-1-1-1-0) on day 0, 3, 7 and 14; three-weeks IM rabies immunization (2-0-1-0-1) on day 0, 7 and 21–28[1,50,51]. IM and ID immunization were commonly recommended because subcutaneous injections of rabies vaccines failed to induce sufficient antibody response after 1 month completing immunization protocol[52]. Receiving rabies vaccination and RIG within first 7 days and 48 hours respectively is considered as timely PEP response[53]. Though, people who received at least two doses of rabies pre-exposure vaccines do not require RIG infusion [1,51]. In events of re-exposure to animal bites, a previously immunised patient only require booster injections on day 0 and 3[49-51].

So the next important question would be — What are these vaccines make of? As discussed earlier, the host immune system must first recognize the pathogens before initiating "attacks" on the intruders. Having that said, it may seem to be unwise to inject someone with live virus to activate someone's immune system; nevertheless, looking at the history, one of the earlier version of "vaccination program" was done in small pox known as variolation, whereby they

inoculate a boy with materials from cowpox pustule and observed protective effect against matter from smallpox lesion[54]. For RABV, there have been many studies looking into potentially more effective vaccines over the years, apart from the attenuated RABV vaccines that are currently in use.

Nucleic acid-based vaccines are getting more popular these days, as researchers are working around the clock to develop them given that this approach combines the positive attributes of both live-attenuated and subunit vaccines[55]. A research team in Germany successfully developed a synthetic messenger RNA (mRNA) based vaccine which consists of an optimized non-replicating rabies virus glycoprotein (RABV-G) mRNA sequence in 2016. When compared with licensed rabies vaccines, this mRNA vaccine managed to induce comparable $CD4^+$ T cells and $CD8^+$ T cells responses upon two injections[56]. Subsequently in 2017, Stitz and team described another attractive feature for their mRNA vaccine — thermostability; they showed that the mRNA vaccine that retained its immunogenicity and protective effects against RABV even after exposure to temperatures as high as 70°C[57]. The development of thermostable vaccines provides extended shelf life in challenging conditions especially in tropical countries and economical vaccine stockpiling in preparation for epidemic threats. In fact, a Phase I clinical trial carried out in 2016 using the same mRNA vaccine technology (RNActive®), studying the safety of and immunogenicity of this vaccine in healthy volunteers (NCT02241135)[58]. A total of 101 participants were enrolled and vaccinated with 306 doses of mRNA (80–640 μg) by needle-syringe or needle-free devices (via intradermal or intramuscular route). As the first drug substance of mRNA vaccine against RABV, CV7201 or nadorameran (as named by WHO) was described as generally safe with a reasonable tolerability profile. The same study reported the observation on VNA titres of 0·5 IU/mL or more across dose levels and schedules in 71% of participants given 80 μg or 160 μg CV7201 doses intradermally and 46% of participants given 200 μg or 400 μg CV7201 doses intramuscularly. Nonetheless, 57% of them (i.e. 8 out of 14 participants) achieved titres of 0·5 IU/mL or more after receiving needle-free booster shot of CV7201 at 80 μg intradermally, while those underwent intradermal or intramuscular needle-syringe injection failed to respond (i.e. no immune response) except one participant who received 320 μg of CV7201 intradermally. Additionally, another recent study highlighted that the co-administration of RNA-based adjuvant CV8102 with licensed vaccine for rabies, Rabipur® in Phase I clinical trial (EudraCT No. 2013-004514-18, NCT02238756) indicated that CV8102 was safe up to 50 μg and enhanced immunogenicity of the licensed rabies vaccine significantly[59]. Even so, there is still much to do to determine the appropriate vaccination dose and schedule of mRNA vaccine alone or as adjuvant for PrEP and/or PEP regime.

Besides that, there are several groups discussing the use of viral vector-based vaccines against RABV, such as via the incorporation of RABV glycoprotein genes into West Nile virus backbone to induce protective effect [7,60–62]. In the study by Giel-Moloney and team, the RABV G protein expression remained stable after multiple *in vitro* passages and the vaccine exhibited durable protective immunity with high titres of complementing T helper cells[60]. The vaccine which uses RepliVax® technology is a highly promising vector delivery system, given that immunized dogs displayed durable protective immunity when tested at one- and two-year post immunization. In addition to that, there are other recombinant rabies vaccines generated using different viral vector systems, such as poxvirus[7,63,64], Newcastle disease virus[65], parainfluenza virus[66], adenovirus[67,68], or baculovirus[69,70]. Despite of that, these vaccines may not be available for clinical use at the moment, particularly regarding efficacy restricted to certain species but not human, safety considerations, and similar to the concern with mRNA vaccines—usage as PEP and/or PrEP vaccination. Looking on the bright side, there are two ongoing Phase I clinical trial studying the safety and immunogenicity of novel recombinant rabies vaccines, ChAd155-RG (NCT04019444) and ChAdOx2 RabG (NCT04162600)[71,72].

In reality, another critical point to consider in designing recombinant vaccine is that the viral vector used must not be pathogenic while being able to trigger protection against certain pathogens (including RABV)[73,74]. Decades have passed since the first vaccine for smallpox and an increasing number of researchers are considering the possibility of immunization against multiple pathogens with the use of single viral vector carrying fragments of another virus (e.g. multivalent vaccine)[74,75]. For instance, a novel vaccine consisting of inactivated RABV that expressed protein fragments of Middle East respiratory syndrome coronavirus (MERS-CoV) was proven to be effective in producing antibodies against rabies and MERS-CoV infection[74]. Developed by Wirblich and team, BNSP333-S1 is an inactivated RABV-MERS S-based vaccine and the team observed increased antigen-specific IgG responses over time after each immunization. Besides that, there is another genetically modified RABV vector-based Rift Valley fever virus (RVFV) vaccine which induced significant rabies VNA level but it is still unsure whether it can protect against RVFV as it failed to induce RVFV VNA (despite high titres of anti-RVFV IgG antibodies)[75]. Therefore, multivalent vaccines against rabies and other infectious diseases can be developed, but further validation tests should be conducted thoroughly in clinical studies to confirm its efficacy and safety.

Current measures in place to control the spread of RABV from animals to humans

Animal mass vaccination

While the development of RABV vaccine for human use is essential to combat against RABV, the preventive measures and management of wild life including carrier of RABV are equally important. WHO recommended that mass vaccination of at least 70% rabies-susceptible dog population is essential to achieve herd immunity and contain the virus as 95% of human rabies cases were caused by dog bites[1]. Although mass vaccination of dogs is the most cost-effective method for significant decrease

in human rabies cases and mortality, local government particularly in endemic countries often neglect these preventive efforts [76,77]. According to World Organization for Animal Health (OIE), animals are considered to have protective immunity against rabies infection if they have minimum post-vaccination rabies VNA of 0.5 IU/mL[78]. Several canine rabies control strategies including immunization, movement restriction and culling of stray dogs were carried out in several Asian and African countries over many decades but were not effective in eliminating rabies from the population[79,80]. Introduction of a simple centralised canine rabies vaccination campaign to a rural area in Africa increased vaccination coverage from initial estimated 9.5% to between 60 and 70%[79]. There was a significant decline in incidence of dog rabies by 97% after the second vaccination programme with more than 60% coverage of the dog population. However, in Korea, canine rabies was successfully controlled with low vaccination coverage, which ranged between 30% and 50%[81]. Genetic, temporal and spatial heterogeneities that influence contact and transmission rate can have significant impact on the design of immunization program[82]. Besides vaccination coverage, relative success of large-scale vaccination program is also determined by frequency of vaccination campaign and dog density in the area[83]. Satisfactory rabies knowledge and awareness in the population will increase rabies immunization coverage[84]. In countries with high birth and death rate of dogs, there is substantial risk of outbreaks occurrence between vaccination campaigns due to rapid decline in overall population coverage following a campaign[79].

Oral rabies vaccination (ORV) is a cost-effective and socially acceptable technique that can be incorporated into large scale rabies control programmes for canine or wildlife reservoirs[85]. International researchers have generated several effective vaccines over the years. In the late 20th century, a mass vaccination programme using live attenuated RABV vaccine (ERA-BHK21) successfully eliminated Arctic rabies virus variant from red fox population in eastern Ontario[86]. Similar result was observed in Europe where the spread of rabies infection was prevented by vaccinating approximately 60% of fox population with a different live vaccine (SAD)[2]. Despite the successful results and cost-effectiveness, the use of live-attenuated vaccines in ORV programmes remains controversial due to residual pathogenicity, vaccine-induced rabies infection, thermal instability and ineffectiveness of oral immunization in rabies reservoirs including skunks and raccoons[2,85–91]. Alternatively, recombinant vaccines were constructed from heterologous virus vectors expressing RABV glycoprotein and were proven to have improved safety profile and thermal stability[92,93]. ONRAB® is a recombinant oral RABV vaccine generated using human adenovirus vector that expresses RABV glycoprotein and often distributed as bait to animals[92]. ONRAB® induced sufficient immune response in wildlife reservoirs including red foxes, raccoons and skunks with high survival rate after rabies challenge test 1 year post-vaccination[92,94,95]. During oral vaccination campaign, muscle extracts and thoracic liquid are considered potential samples for virus neutralization tests when other samples collected are of low quality[96]. Therefore, bi-annual and annual bait distribution schedules are sufficient for rabies control in wildlife reservoirs[92].

Outbreak prevention

Appropriate health promotion measures, coordinated rabies surveillance and mass vaccination program can prevent rabies outbreaks[97]. Introduction of rabies-infected subject to a community could be an imminent threat and trigger an outbreak, especially for a previously rabies-free region[98]. In developing countries, significant stray dog population and inevitable dog movement are recognized as public health risk and could result in rabies outbreak with subsequent bites to other animals by an infected animal[99,100]. While stray dog population in rural regions is correlated with carcass availability, economic implications of dog bites and rabies infections are significant following decline in vultures population. Local government should implement strategies for carcass disposal including incinerations. As significant stray dog population and rabies infection are major concerns, Bhutan had implemented catch-neuter-vaccinate-release (CNVR) program[99]. Moreover, rabies outbreak in wildlife is of huge concern as animals like fox and wild dogs are highly mobile and travel over a long distance between habitats in different countries, further enhancing the spread of rabies infection[101]. Health promotion measures including domestic dog control regulation and mass vaccination program had been implemented in the early 19th century during the Japanese colonial period[102]. However, the Japanese colonial government was widely criticized in Korea for brutality and poor understanding of traditional dog-human relationship. In the 21st century, cultural obligations to dog population remains significant in the rural communities, especially Indigenous community, as harming dogs will result in sickness[98]. During an outbreak in India, most people only received rabies preventive measures from friends and consumed traditional herbal medicines[100]. Poor knowledge and practices of preventive measures reflected on the health-seeking behaviour of rural communities following an outbreak. As a previously rabies-free region, rabies remains endemic in Bali since the introduction of the virus by a sub-clinically infected dog in 2008[103,104]. Poor surveillances, diagnostic facilities and treatment policy in 2008 had resulted in circulation of rabies virus across the island following the outbreak[104]. However, local authority in Bali has implemented Program Dharma to improve dog care practices and facilitate mass vaccination program, in addition to reducing roaming dog density. Hence, public health officials should organise education awareness campaign to emphasize the significance of dog ownerships and public cooperation as preventive strategies of outbreak[103]. Poor handling of outbreak in wildlife or local community could facilitate transmission of zoonotic diseases to human. Despite its rabies-free status, Australia has identified potential areas of rabies incursion and implemented community-based health promotion approach to increase preparedness of the local community[98]. Rabies surveillance including strict monitoring programs is important for prompt control measures as potential cases are identified to halt spreading[97].

Conclusion and future recommendation

Since the description of rabies by the historical records, humans have made a long way in the discovery and development of vaccines for RABV. In actual, thorough research into molecular virology, immunology and epidemiology have provided remarkable understanding of the circulation of rabies virus. Even though the current inactivated vaccine may seem to be working well, it is still far from "perfect" with some studies found inadequate antibodies titre among veterinary students at 2 years after pre-exposure rabies vaccination; the levels of antibodies was independently influenced by several variables including gender, vaccine type or manufacturer, BMI and interval between first and third vaccine doses[105,106]. Furthermore, the currently approved vaccine for rabies needs to be refrigerated, complicating the logistics problem which can lead to delay in treatment time[107].

Global burden of rabies infection is notable, but the zoonotic diseases as such is preventable. Collaborative efforts from several countries have played an important role in improving public health and relieving the economic burden. As part of the drug discovery process, researchers have been studying the potential of small molecules or even peptides expressed by microorganisms and plants to be used to prevent and/or treat infectious diseases including RABV[108–117]. Among these studies, tobacco mosaic virus (TMV) isolated from chimeric plants expressed spherical particles (i.e. coat protein of alfalfa mosaic virus fused with antigenic peptides of RABV) that can improve rabies vaccine protective properties due to the presence of RABV antigenic peptides[108,109]. Yusibov and team showed that using plants as tool to produce antigens to be used in vaccines provide several advantages including lack of contamination of other human pathogens, reasonable ease of genetic manipulation and economical production[108]. After purification steps, spherical particles formed from the recombinant AIMV CP was used to immunize mice and subsequently resulted in an antigen-specific humoral immune response, accompanied with VNA. Apart from that, nucleoside analogs are potential antiviral compounds because they can act as competitive inhibitors to interfere nucleic acid biosynthesis during replication of viral genome. For example, small molecule drugs such as ribavirin (which is an antiviral drug against respiratory syncytial virus) and favipiravir (i.e. antiviral drug against influenza) have been shown to be effective against RABV as well by acting as competitive inhibitors that interfere with nucleic acid biosynthesis during replication of viral genome[110–113]. Along with these, there is also potential use of these molecules in combination and/or as adjuvant (booster) to increase the efficacy or performance of the vaccine. However, further studies on the pathogenic mechanism of rabies virus and therapeutic approaches are still required to prevent the deathly infection following clinical manifestation. Having that said, integrated interventional strategy emphasizing human health and animal health is essential and via the collaboration between health authorities and the public, it is highly possible to control and prevent further spread of zoonotic disease like rabies.

Author Contribution

The literature review and manuscript writing were performed by R-AA and H-LS. H-LS and VL provided vital guidance and support as content expert and proofread of the writing.

Conflict of Interest

All the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Review Article

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COVID-19: Are Malaysians embracing or suffering the new normality?

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Abstract: The COVID-19 pandemic has inevitably rendered a paradigm shift in peoples' day-to-day normality. The pandemic has precipitated various reaction and responses from people across the globe especially with the enforcement of preventive measures initiated by their respective government forces. The movement control order (MCO) was one of the drastic measures taken in Malaysia adhering to the guideline released by World Health Organization (WHO) and has been made effective since 18th of March 2020. The execution of MCO in a developing setting like Malaysia certainly impacts its people on several fronts, especially those from low-socioeconomic background. It creates a domino effect from an economical to psychological aspects at both societal and individual levels. Subsequently, a conditional MCO (CMCO) has been introduced during midway through Phase 4 of MCO with eased restrictions, particularly considering economic downturn. CMCO is followed by recovery MCO (RMCO) phase. In this article, we aim to share some insights while highlighting the impacts of COVID-19 with an emphasize on the psychosocial aspect, particularly during MCO phases, which has thus imposed a new normality on Malaysians.

Keywords: COVID-19; Malaysia; movement control order (MCO); impacts; new normality

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Introduction

A coronavirus disease, known as COVID-19, is a deadly manifestation of a novel virus which came to light in December 2019 when pneumonia of unknown cause was first reported in China[1–4]. The outbreak took a downward spiral when World Health Organization (WHO) subsequently declared it as a pandemic in early March 2020 with death approaching nearly 1000 in the European Region[5,6]. To date, a whopping total of approximately 770, 000 deaths and close to 22 million confirmed cases of COVID-19 have been reported globally[7]. Amongst Southeast Asia countries, Malaysia records one of the highest numbers of confirmed cases with over 6000 cases and 103 total deaths by early May 2020, however this scenario gradually changed as the vigorous measures taken by the authorities through MCO managed to reasonably arrest the rapid spread of Covid-19 infection in local setting. Since June 2020 until the present, the reported number of new cases remains within 2 digits which inevitably reflects the effective countermeasures executed by the local authorities[8,9].

As part of the federal government's initiative to combat the rapid spread of COVID-19 with an alarming surge in new cases amounting to 190 cases on 15th of March 2020, a movement control order (MCO) has been issued effective from 18th of March 2020 till 12th of May 2020[10]. The initial order for a period of 2 weeks has been gradually extended to a total period of 8 weeks, adhering to the WHO's guideline[8,11]. Each phase of MCO, lasting for 2 weeks respectively, sees a gradual modification on the restriction rules, depending on the reported number of new cases and recovery rate. The strictest rules were observed during phase 2 and phase 3 of MCO starting from 1st of April 2020 till 28th of April 2020. During these phases, travel distance was limited to within 10km of radius from home, only one representative from each family allowed to travel at a time to buy essentials, and business hours and delivery

time were limited to 8am till 8pm. There were some areas in major cities subjected to complete lockdown as well. Almost no tolerance was given to MCO violators where 9090 offenders were arrested while 4036 compounds were issued as of 13th of April 2020[12,13]. As soon after implementation of MCO, the number of new cases peaked at most 235 cases per day in March end with a more vigorous screening process taking place across the nation. The trend has been generally decreasing since then, however, with some fluctuations in number of new cases reported on a daily basis. The recovery rate has been reported as high as 70%[8]. During midway through phase 4, from 29th of April 2020 till 12th of May 2020, a conditional MCO has been introduced as Phase 5 starting from 4th of May 2020 by easing some of the restrictions mainly catering to reopening of selected economic sectors involving essential services and manufacturing of critical products. The psychosocial well-being of general public has also been considered by allowing two family members to travel in a car, beyond 10km of radius from home for health needs, groceries and food, and some outdoor sports activities were also allowed while strictly exercising social distance and self-hygiene[11,14–16].

Challenges and difficulties inevitably accompany the execution of this obligatory mandate at numerous aspects as far as the public is concerned. However, the same amusingly unfolds some fundamental values which hold the society together amid an unfathomable crisis. This article intends to explore some insights and impacts relating to the COVID-19 situation in Malaysia from a societal perspective during the MCO phases particularly.

Measure of societal awareness and readiness

The announcement of MCO has inevitably provoked an alarming sense of fear and insecurity amongst the public, however, at varying concerns amongst the different strata within the society, largely influenced by individual socioeconomic status. Panic-buying is one of the resulting occurrences which happened at early phase of MCO where people flocked to the supermarkets, grocery stores and pharmacies, especially in major cities to stock up on provisions for their homes[17]. Essentials were absurdly purchased in larger than usual amount with sole aim to survive the foreseen restriction due to MCO. This is probably an immediate but fading gratification considering the mismatch between the needs and the wants in a long run and poorly understood terms of MCO.

The wearing of face mask is another aspect which becomes a highly critical measure of societal awareness and knowledge. Mask has become one of the most sought-after essentials during this phase. While the usage of mask becomes a matter of debate, it has been made compulsory to wear a face mask upon entering any outside premises currently. Although this was not one of the mandates by the government at the initial stage, starting from 1st of August 2020, it has been made compulsory for everyone to wear mask in public domains[18,19]. Given the asymptomatic yet possibly contagious phase of COVID-19 incubation period, the wearing of mask is merely recommended for everyone being outside[20,21]. However, the paranoia of wearing a mask to the extent of making own masks from random cloths or even tissue paper, and stealing masks from hospitals while neglecting other preventive measures only point to the lack of comprehensive understanding on the rationale behind proper usage of personal protective equipment (PPE) amongst the public[22–24].

While panic-buying and face mask obsession became the highlight during the initial phases of MCO, the announcement of conditional MCO with eased restrictions triggered an unexpected response from 130, 000 over Malaysians who signed a petition to call it off and continue with the existing MCO rules. A fear of repeating mistakes of other countries by easing restrictions only to see another wave of COVID-19 has provoked such a response amongst the public[18]. Resonating with the public's fear, an increased number of new cases was seen during phase 4 after easing some movement restriction, with 94 cases reported on 29th of April 2020, followed by another peak at 105 cases on 2nd of May 2020[8].

Chain of impacts and remedies from a societal perspective

The MCO period has created a new normality for the entire nation at a liability yet to be fully apprehended. The new normality in Malaysian context became apparent with the enforcement of MCO when strictest restrictions were introduced during critical phase 2 and phase 3. The glaring impacts from economical and psychosocial fronts remain a huge concern given the prolonged period of MCO with uncertainties regarding its further extension.

A recent survey conducted by The Department of Statistics, Malaysia, participated by 168,182 respondents aged 15 years and above, revealed that almost 50% of the self-employed workers have lost their job while 94.8% reported reduced monthly income. It further revealed that more than 50% of the working community is not financially prepared for a total lockdown[25]. Another group detrimentally affected by the restriction is the daily wage workers, more so those with families to support due to complete loss of income[26]. Malaysians residing in the southern-most state of Johor who travel across the causeway for work in Singapore were also impacted by the MCO issued by both the countries. Some barely had enough savings to support their prolonged stay in Singapore while some were asked to go on an unpaid leave[27].

The psychosocial effect resulting from the restrictions imposed via MCO differ from one stratum of the community to another to some large extent. These differences are probably attributable to the differences in economic capacity of a stratum. For an instance, panic-buying, is probably an incongruent description of the economically disadvantaged stratum of the society for buying capacity becomes a disheartening matter of concern. People who have been charged for violation of MCO include joggers and golfers at one end of the spectrum and fishermen who were in dire need to feed their families at another[26,28,29]. The markedly differing concerns and priorities divide the psychosocial effects within the different strata of community. Paradoxically, the virus does not have the capacity to be selective of its sufferer, thus a social dilemma

arises when it comes to the execution of the general order like MCO in a developing setting.

Befrienders, a non-profit organization which provides a 24/7 helpline for emotional support, reported an increase of 13% of calls during the initial phase of MCO with 9% expressing concerns and anxiety due to COVID-19 and MCO[28]. 'Talian Kasih', a 24/7 helpline by government body dedicated to providing support related to welfare and community, reported a 57% of rise in number of calls during MCO. Concerns were expressed regarding an increase in domestic violence cases and further predicts a possible rise in child abuse and incest during MCO[30].

Loss of job and income without a financial backup while being confined in a space with families to support would impact the psychological well-being of any affected individual even for a short period. On the other hand, those who have the privilege of continuing work from home requires a minimum of well-equipped networking facilities and an ideal environment free from distractions. This becomes a challenge in a rural setting, particularly in some parts of East Malaysia, where there is no internet viability or basic amenities to support work-from-home alternative which depends on internet[31]. Individuals who are committed to families, especially with younger children or family members who need extra care and attention, equally have to juggle between work and family, especially when interrupted.

The closing of academic institutions and postponement of major public exams become another form of distress to parents, educators and students themselves. Online and home-based learning has been highly encouraged and advocated, however, students from rural background with no access to internet and proper coaching would undoubtedly jeopardize students' academic performance in a long run[32–34]. It would be an unrealistic expectation to have all the school children remain motivated to study throughout the MCO period. Boredom, agitation and stress could not only affect the children, but also the parents or caregivers who equally have the responsibility to ensure an adequate and healthy home environment for their children.

From another perspective, in a common ground of psychosocial effects, the most difficult situation comes when one has to grapple with the loss of their loved ones, be it due to COVID-19 or other causes. It becomes a painful bid-adieu to these souls for the sorrowful memory it creates[35]. There were people arrested and fined for attending funeral of loved ones during MCO which makes the grieving even more painful[36]. Perhaps no remedy is capable of recovering one from the loss of loved ones in such circumstances.

As remedial measures tackling financial aspect, Malaysians were given financial aid through the 'PRIHATIN Economic Stimulus Package' where a one-off amount was credited into accounts of eligible applicants based on net monthly household income of no more than MYR 8000. The 'B40 group', with net monthly household income of less than MYR 4000 for married individuals and less than MYR 2000 for singles, was eligible for the highest aid fund of MYR 1600 and MYR 800 respectively[37]. Other funding aids, including deferment of loan payment up to six months and 15–50% discount on electricity bills do provide a temporary relief [38]. The temporary diversion due to MCO may possibly mask the actual extent of economic burden imposed on both individuals and nation. Nevertheless, it certainly signals a forewarning on a long term and lasting detrimental consequences considering a recent estimate of Malaysian Ringgit (MYR) of 2.4 billion loss per day owing to economic shutdown during MCO[39].

In the face of countless issues and concerns, the forthcoming of several non-governmental organizations (NGOs) and individuals to reach out to the needy including the homeless and refugees is commendable. Free food distribution, shelter to the homeless and fund-raising via social media are all some of the noteworthy deeds[40]. The nation's frontline healthcare workers could not have become more visible for their unremitting labor in fighting COVID-19. The public took it to social media to express their unwavering gratitude for all these selfless heroes while ceaselessly reminding one another to stay home and obey the MCO rules[41]. Enduring eternal claustrophobic hours in PPE suit, heavily drenched in sweat, heading home with exhaustion and fear of possibly carrying and passing on the infection to their loved ones only to call it a day could not be described as nothing but a precious call to perform their clinical duties in this most needed time of crisis. The fellow countrymen are probably more aware than ever the value of a timeless noble service like this and that it deserves far a greater recognition and salutation in days to come.

The four phases of MCO were probably the strictest and tightest lockdown period Malaysians have had endured to date. Following MCO, CMCO was introduced in Phase 5 till 9th of June and subsequently recovery MCO (RMCO) up to 31st of August 2020[42]. Restrictions were eased and economic sectors were gradually allowed to operate at staged phases. Standard operating procedure (SOP) was administered across all the operating business outlets. A mobile app known as 'MySejahtera' has been introduced by the Ministry of Health (MOH) Malaysia as a mean to monitor Covid-19 outbreak. This app provides its user with an updated information on Covid-19 status in Malaysia and health screening facilities, individual risk status and QR code reader to enable individual check-in at each visit to various locations. This app channels the information to the MOH, thus paves the way for effective countermeasures which marks the importance of digital health application in the midst of public health crisis[43]. As of 15th of August 2020, Malaysia has recorded a total number of 9175 positive cases with 96.25% or 8831 cases of discharges, and 1.36% or 125 number of total deaths (Figure 1). Since late May, not long after CMCO was introduced, the number of active cases based on daily record has remained within 2 digits and this marks the successful execution of Covid-19 measures in Malaysian setting[44]. Heavy penalties will be imposed on individuals who disobey the set SOPs, including a fine not exceeding RM1000 or imprisonment up to six months [16]. Although the battle to combat Covid-19 is still ongoing, Malaysians continue to hope for brighter days ahead while recognizing the applaudable measures taken by the respective authorities.

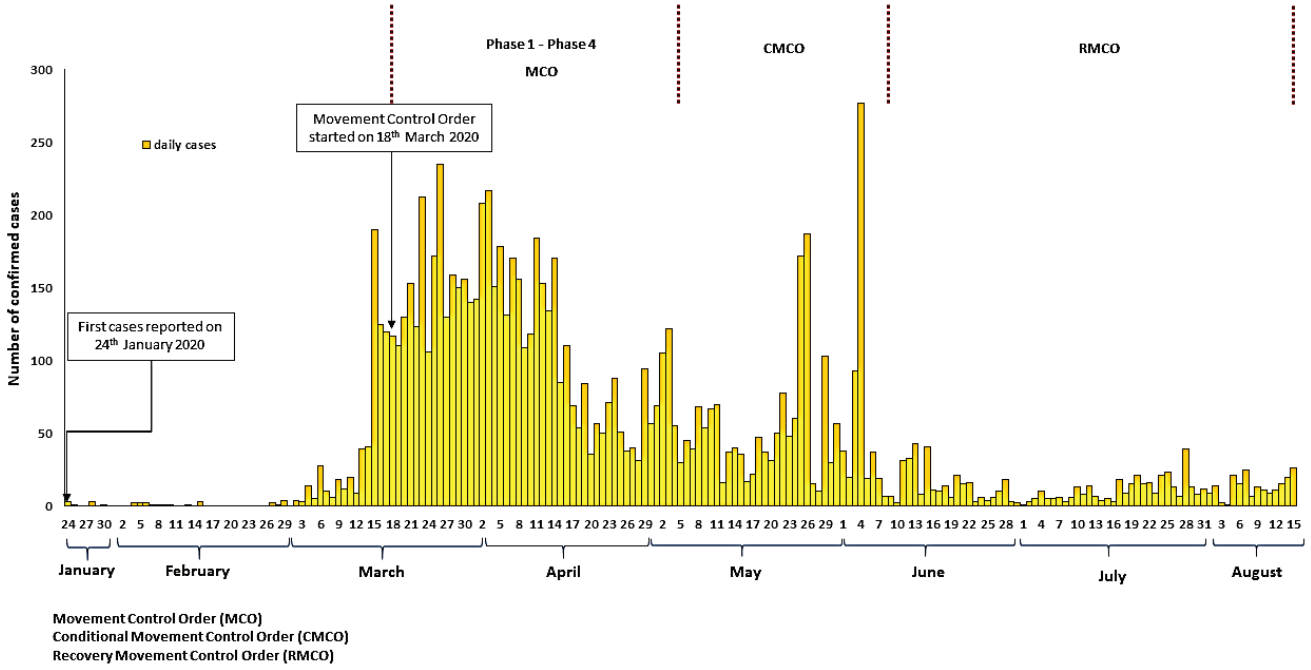

Figure 1. The daily confirmed COVID-19 cases reported in Malaysia up to 15 August 2020 based on Ministry of Health Malaysia. The movement control order (MCO) was implemented on 18 March 2020 in four phases followed by conditional movement control order (CMCO). Malaysian were restricted under recovery movement control order from 10 June 2020-31 August 2020.

Conclusion

The gigantic wave of COVID-19 has awakened the entire nation and calls for a bigger reformation in days to come at both individual and national fronts. Economic downturn becomes a huge concern especially in developing countries like Malaysia. Psychosocial effect is influenced by economic capacity of an individual to a great extent; however, it becomes a borderless effect when emotional sufferings and trauma are considered. Despite various limitations and concerns given the poorly anticipated circumstances, a responsible member of the nation should remain focused on working hand in hand to eradicate the egregious conquest of an invisible creature. The rapid spread of COVID-19 leaves no space for any excuse to violate the governmental mandates. The miserable and dampening economical and psychosocial repercussions of COVID-19 could only be revived by a robust and healthy citizen who survive this phase with deepest sense of conscience and responsibility. Every individual would shoulder even a greater responsibility in curbing the pandemic as well as recovering from personal losses after cessation of MCO. A continuous spread of awareness and reminders are necessary to ensure strict compliance to preventive measures. Technology can never be more accommodating than now to keep the momentum going. All said, let this phase not be taken for granted, for a profound sense of humility has been awakened in the face a natural calamity.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

The literature search and data collection were performed by DJ. The manuscript was written by DJ. Technical supports and proofreading were contributed by SC-HE, HDJ and VL. VL set up the research project.

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Review Article

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Systemic Lupus Erythematosus (SLE): A review on the prevalence, clinical manifestation, and disease assessment

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Abstract: Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease with a varying clinical phenotype. This disease occurs when the body's tissues are attacked by its own immune system. The aetiology of SLE is not fully understood, but both genetic predisposition and ecological triggers are thought to be involved in the disease manifestation. The study on the prevalence of SLE allows us to identify potential risk factors associated with the disease for the disease and allows proper management and treatment in response to overall disease burden. Hence, this review aims to discuss the prevalence, mortality, clinical manifestation and disease assessment of SLE.

Keywords: Systemic Lupus Erythematosus; autoimmune; aetiology; mortality; assessment

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Introduction

Systemic Lupus Erythematosus (SLE) is a multi-systemic autoimmune disease which can cause chronic inflammation and damage to tissue and almost every organs in our human body[1,2]. The exact aetiology of SLE remains unknown, however, it has been hypothesized that genetic, environmental, and other hormonal factors are likely to play a vital role in the occurrence of SLE[3]. There are various immunological faults that have been described in the development of SLE which include T- and B-Cell abnormalities, and the failure to clear autoantibodies that leads to generation of immune complexes[4]. In addition, an association between SLE disease onset and age, sex, geography and race have been also reported.

SLE affects women more commonly than men with a ratio of 9:1[5]. However, male patients tend to have more severe disease manifestation than the female patients[6]. It is because the occurrence of lupus nephritis among men is more than women and more likely to develop to end-stage renal disease (ESRD) than women[7]. SLE could occur at any age, however, more prevalent in people between the ages of 10 and 50. In terms of ethnicity, the disease affects African American, Asians, Hispanic and Native Americans more frequently than other races in the world. This could be due to genetic and geographical influences which are thought to play a role in the development of SLE. The overall survival rates for 5-year and 10-year was recorded as 82% and 70%, respectively, whereas survival for 1-year was 93%[8]. On the other hand, the overall mortality rate among SLE patients was reported as 20.2%[9]. The cause of mortality among SLE patients differs from one to another individual, due to demographical changes including country or region of patient's origin, age, gender and ethnicities.

The exact cause/aetiology of SLE remains elusive, however, the fundamental defect in SLE clearly elucidate the presence of various autoantibodies against self-constituent due to failure in mechanisms that maintain self-tolerance[10]. In recent years, the mortality rate of SLE has been declining which possibly due to early detection and pharmacological advances in controlling and managing the disease progression. This review aims to discuss the prevalence, mortality, clinical manifestation and disease assessment of SLE.

Prevalence of systemic lupus erythematosus

Worldwide, the disease affects a minimum of 5 million people. The current prevalence of SLE is 20 to150 cases per 100,000 people[11]. In the United States (US), the prevalence of SLE among the Caucasians is approximately 51 per 100,000[12]. The prevalence of SLE amongst the African-American women is three times higher than the Caucasian women[13]. Based on a study in the US, the prevalence of definite SLE within the US community is 54.3 per 100,000 people whereas the suspected cases of SLE is 108.6 per100,000 people[14]. It is reported that the prevalence of SLE among African-American women was 286 per 100,000 people, a figure nearly twice as high as the prevalence among white woman[15]. The incidence rates reported in North America and South America range from 2 to 8 cases per 100,000 people per year. One study by Naleway and colleagues reported the incidence of SLE amongst the US population is 5.1 per 100,000–1.9 per 100,000 in adult men and 8.2 per 100,00 in adult women[16]. Previous study reported an overall incidence of SLE amongst the US community was 5.56 per 100,000 people[17].

Looking into the European Continent, an epidemiological research reported an increase in SLE prevalence from 64.99 per 100,000 people in 1999 to 97.04 per 100,000 people in 2012[18]. The incidence of SLE during the same study period was 4.91 per 100,000 people with a yearly decline of 1.8%. Approximately, 60% of SLE populations were women. The highest incidence was reported between the ages of 50 and 59. In terms of ethnicity, Africo-Caribbean people had 2.3-fold higher rate of prevalence and incidence which could be due to regional variation[18]. In terms of gender, the females had 10-fold higher prevalence than the males. The same study also stated that the Africo-Caribbean SLE population had a higher tendency to develop complication such as renal disease and end-stage renal disease (ESRD) approximately 40.5% and 15.3% respectively as compared to Caucasians in the same SLE population whose proportions were only of 18.8% and 4.5% respectively [18]. In another study, it was reported that the Caucasians of England origin had a lower prevalence compared to Afro-Caribbean, Hispanics and Asians[19]. In addition to the above, Table 1 shows the prevalence rate for other countries in the European Continent.

Table 1. Summary of SLE disease prevalence for European states.

Country	Prevalence	Reference
Lithuania	16.2/100,000 (0.016%)	[20]
South Ireland	21.7–39.5/100,000 (0.022%–0.039%)	[21]
Norway	47.6–57.9/100,000 (0.048%–0.058%)	[22]

For Oceanic continent, looking at Australia, although there is a lack of published information on SLE, one identified study has reported that two ethnic groups, namely Aboriginal Australian and Caucasians were known to have SLE. Whereas, a study reported in 1998 stated that the prevalence of SLE in Queensland, Australia was 45.3 per 100,000 people[23]. In another study conducted at the central part of Australia from the period of 1996–1997, the prevalence of SLE was 92.8 per 100,000 people among the Aborigines ethnic group[24]. Besides that, one study carried out at the Australia Northern territory from the period of 1986–1990, reported SLE prevalence of 19.3 per 100,000 and 52.6 per 100, 000 people amongst the Australian Caucasians and Aborigines community respectively. Another study reported the incidence of SLE amongst Aborigines people was 11 per 100,000 people at the Northern Territory of Australia[25].

A study based in Canada reported that the prevalence of SLE in Canada falls within the range of 22.1 and 51.0 cases per 100,000 people[26,27]. In addition to that, another study reported that the disease generally affects 0.05% of Canadian adults and it is 10 times more prevalent amongst women than men[28]. One study from Brazil population showed that the prevalence of SLE is 3 per 100,000 people with predominantly women[29]. Similarly, another study in Brazil reported SLE prevalence of 20 to150 cases per 100,000 people[30]. In Saudi Arabia, the prevalence of SLE amongst its nationals was estimated at 19.28 per 100,000 people[31].

Amongst Asians, SLE prevalence has been estimated to be between 30 and 50 per 100,000 individuals. It has been reported that SLE is more common among the Chinese population in Asia[32]. Another study reported three cases of SLE out of 4192 adults registered for rheumatic disorder in the north of China (Beijing), and one out of 5057in the south of China (Shantou) from Han population, to have SLE disease[33]. Similarly, the prevalence of SLE amongst the Han population in China was estimated at 37 to 70 cases per 100,000 people[34]. These data show the Chinese population has a higher prevalence of rheumatic disease including SLE in comparison to the Caucasians. In Taiwan, the prevalence of SLE among paediatric population was reported at 6.3 per 100,000 people. The prevalence in girls were 11.2 cases per 100,000, which is 6.2 times higher than that in boys (1.8 cases per 100,000)[35].

In India, a study conducted in Northern India demonstrated a prevalence of 3.2 per 100,000 people with SLE, which is at lowest compared to other Asian countries[36,37]. Moreover, studies also reported that there were no SLE cases found amongst adults from certain rural and urban areas of Jammu, India[38]. Amongst Malaysian populations, consisting of three major ethnic groups; Malays (55.1%), Chinese (24.3%) and Indians (7.4%) out of a total population of 22 millions of people, SLE prevalence has been reported to be 43 per 100,000 people[37]. Amongst the three communities, Chinese population represents the highest prevalence rate of SLE of 57 per 100,000 followed by Malays 33 per 100,000 and Indians with only 14 per 100,000 people.

Survival and mortality of SLE

Generally, the survival rates of SLE can be categorized into three groups. The categories are survival rates at 1 year, 5 years and 10 years. The overall survival rate at 1 year fall

within 93 to 98%, followed by at 5 years ranging from 60 to 97% and 70 to 94% at 10 years. Moreover, with the advancement in pharmacological therapy and treatment, the overall survival rate of SLE is expected to improve.

The cause of mortality amongst SLE patients varies from one individual to another partly due to demographical variances. The demographical differences include geographical origin of patients, age, gender and ethnicity. These factors are considered crucial as some studies have proven that they have major roles in the pathogenesis and survival of SLE. Socioeconomic status often determined by occupation and residing area of patients have also been found to effect survivability and management of SLE disease[39,40]. Besides that, the disease duration also determines the mortality rate amongst the patients.

Several studies have reported that generally, active disease and multiple kind of infections are the most common factors leading to early death among SLE patients. During active phase of SLE, patients receive numerous immunosuppressant which may have negative impact on their natural immune system, thus paving the way for opportunistic infections and even death in severe cases. On the other hand, death in later stage of SLE is usually related to vascular events such as cardiovascular disease[41], thrombotic events[42], non-Hodgkin lymphoma, lung cancer and renal disorders such as lupus nephritis[26]. However, these underlying causes of death vary according to geographical location and ethnicity. For instance, amongst the US populations, the African-American, American Indians and the Asians have higher mortality rate compared to the Caucasians[41]. It was also found that the causes of death amongst the younger and elderly patients were mainly due to infections in the former and cardiovascular or renal complications in the latter.

Another study conducted in the US reported the SLE mortality rate is higher among the African Americans, American Indians and Asians compared to the Americans[41]. It is noted that ethnicity and socioeconomic could be the possible cause of higher mortality rate among the African Americans. Based on medical intervention and clinicians, infections are the main cause of death among the younger patients, whereas cardiovascular or renal complications are the clinical manifestation among the elderly group.

A study conducted in Malaysia on the mortality patterns amongst Malaysian SLE patients, reported that 30% of patients died of infection, followed by 15% of renal disorders, 14% of pulmonary disorders, 7% of cardiovascular diseases, 5% of central nervous system, 1% of malignancy, 1% for acute anaphylaxis, 27% of unknown reason and remaining 19% died due to SLE as a contributory factor of death. The study also mentioned that the Chinese community represents the majority of those who live with SLE at approximately 73%, followed by Malays at 18% and Indians the least at 9%. The average age at death reported was 28.6 years[43].

Clinical manifestation of SLE

Systemic lupus erythematosus is known to be a gradually worsening multi-systemic disease with mild signs and lesser number of affected systems in the beginning stage. After some years or with a rapidly progressive condition, over a few weeks or months, it continues to involve and affect more systems severely. Over time, there will be various clinical and laboratory manifestations. Autoantibodies are the hallmark of laboratory manifestation for SLE diagnosis. Fatigue is the one of the most common complaints of SLE patients. However, it has a broad association with many other diseases such as fibromyalgia, hypothyroidism, depression, anaemia, pulmonary or cardiovascular diseases. Moreover, some other intrinsic signs of an active lupus that may commonly occur amongst the patients include fever, anorexia, lymphadenopathy and weight loss. However, all of the above symptoms cannot be attributed to lupus alone until infection and malignancy causes are ruled out. The stage of involvement and severity based on every organ system is further elucidated as below.

Mucocutaneous manifestations / Dermatological Features

Dermatological features amongst SLE patients account for four out of 11 revised criteria based on the SLE classification tool portrayed in The American College of Rheumatology (ACR). The skin involvement is claimed to be as high as 85% among the SLE patients[44]. One of the most commonly identified signs amongst the SLE patients are the photosensitivity rashes and the butterfly or malar rash on the face. Other familiar mucocutaneous signs in SLE patients include mouth ulcers and alopecia or hair loss. Hair loss can be categorized into three types which include localized diffused alopecia, “frizz” or frontal hair loss and severe diffused alopecia with minimal sign of new hair growth[45]. Discoid lesion (DL) and maculopapular lesion, splinter haemorrhages, dilated capillaries at the nail base, bullous lesions, angioneurotic oedema and livedo reticularis are also some of the identified dermatological manifestations[46]. The discoid lesion repeatedly leads to scarring and older lesions may result in pigmentary changes, either hypopigmentation or hyperpigmentation. Alopecia scaring may appear in relation to discoid rash on the scalp region. Ulcers in mouth, nasal and genital have also been associated with SLE but they are less common. Raynaud’s phenomenon is one of the mildly described signs amongst SLE patients. It can be linked to severe digital ischaemia and maturation of gangrene. Raynaud’s is a disease affecting the blood vessels at the fingers and toes. It will make blood vessels to be narrowed around the area in cold or stress conditions. This feature is difficult to be recognized amongst the dark skinned individuals unless there is sparing of some fingers[47]. Vasculitis is usually identified at the nail folds and finger tips[44]. When it occurs, it may develop into a tender, deep, frank ulceration which can either take months to heal or lead to secondary infections. The other more frequently found lesion is shingles, which caused by herpes zoster and often occurs in patients taking immunosuppressant. Maculopapular rash is a type of rash that appears flat and red on the skin and covered with small confluent bumps which can become infected in the SLE patients.

Musculoskeletal Involvement

Arthralgia is defined as an inflammatory joint pain which develops along with morning stiffness or gelling that

happens after a period of rest. It occurs approximately in 90% of SLE patients[48]. Synovial effusion is less common. However, only small volume is present even if it happens. In addition, non-erosive arthritis with joint tenderness and swelling may also form[49]. About 10% of SLE patients have been found to develop Jaccoud's arthropathy, whereas among rheumatoid arthritis patients the deformities occur along with joint erosions. It has also been pointed out that SLE patients may develop osteoarthritis as they age. Bouchard's nodes that occur at the proximal inter phalangeal joints and Heberden's nodes that occur at the distal interphalangeal joints caused by osteophytes in osteoarthritis is different from acute synovitis that occurs due to lupus flare. Tenosynovitis is an early symptom of SLE associated with tendon rupture found on patellar tendon, Achilles tendon, the long head of the biceps, the triceps and the extensor tendons of the hands. It has been estimated that the, muscle involvement among SLE patients is up to 30–50% of patients[50].

Respiratory/ Lung Involvement

Lung is one of the vital organs in human body and it is highly susceptible to secondary infections caused by bacteria, viruses and fungi which may lead to pneumonia in SLE patients. Pain on deep inspiration is one of the frequent complains amongst SLE patients which is caused by pleurisy. Generally, the immunosuppressive agents taken by the SLE patients may result in an immunosuppression which may trigger infections. Pleural effusion is a sign found in approximately half of the SLE patients specifically during disease flares. Pulmonary fibrosis, pulmonary haemorrhage, oedema and pulmonary embolism caused by lupus pneumonitis are other rare manifestations[51].

Cardiovascular Features

The most prevalent cardiovascular sign in SLE is chest pain. A pericardial rub is more common than a significant pericardial effusion amongst SLE patients. Myocardial involvement in SLE is less common than pericardial disorder. Studies have reported that arthrosclerosis increases the risk of cardiovascular events amongst SLE patients. Systolic murmurs are also commonly found sign, affecting 30% of SLE patients, whereas diastolic murmurs are less common[52,53].

Neuropsychiatric Features

SLE can affect both the central and peripheral nervous systems. Some of the most frequently observed central nervous system features are headache (benign intracranial, hypertension), seizure disorders, psychosis, myelopathy, movement disorders, mood disorder, demyelinating syndrome, cognitive dysfunction, cerebrovascular disease, anxiety disorder, aseptic meningitis and delirium[54]. At some instance, drugs could possible precipitate some of the above-mentioned conditions. Steroids are known to induce psychosis in some SLE patients. Acute inflammatory demyelinating polyradiculoneuropathy (Guillain-Barre-Syndrome), autonomic disorder, mononeuropathy (single or multiplex), myasthesia gravis, neuropathy (cranial) and plexopathy are some of the manifestations involving peripheral nervous system[55]. Severe neuropsychiatric condition of lupus is known as neuropsychiatric SLE (NP-SLE), the third leading cause of death amongst SLE patients[56].

Gastrointestinal Involvement

Various non-specific gastrointestinal manifestations have been reported in SLE patients. Nausea is one of the common symptoms followed by abdominal pain in SLE patients. Vomiting and diarrhoea are less common symptoms but may occur with active SLE. When perforation occurs, necrotizing vasculitis can be seen pathologically. Ascites, dysphagia and pancreatitis are other rare gastrointestinal manifestations[57].

Ophthalmic Involvement

The recurrent factors that leads to red eye in lupus are episcleritis and sicca (dry eye). Approximately 8% of SLE patients develop inflammation of the retinal artery during the early days of disease course. Retinal vasculitis is an active systemic disorder which can lead to visual loss besides optic neuritis. Uveitis is less common and affects less than 1% of patients. Vaso-occlusive disorder that affects the retina or choroidal vessels and causes anterior ischaemic optic neuropathy may also lead to a vision loss. Myositis of eye muscles that lead to diplopia and / or proptosis is a rare occurrence amongst SLE patients[58].

Haematological Abnormalities

It is very common among SLE patients to have haematological abnormalities such as anaemia, thrombocytopenia, and leukopenia during the course of disease[59]. Autoimmune haemolytic anaemia in SLE is another distinguishing symptom amongst SLE patients reflecting decreased level of serum iron and iron binding capacity. Multiple mechanisms lead to iron deficiency which include excessive usage of steroidal and non-steroidal anti-inflammatory drugs resulting in gastrointestinal bleeding[57]. Renal insufficiency, blood loss, dietary insufficiency and infections may lead to anaemia. Another persistent and typical feature of SLE is leucopoenia ($<4.0 \times 10^9$/L) which is found in over 90% of SLE patients. Autoantibody deposition that diminishes the function of immune cells and compliment activation is also partly involved in the development of haematological abnormalities amongst SLE patients. Moreover, immunosuppressive agents also contribute to severe leucopoenia[60]. Thrombocytopenia (platelet count $<100 \times 10^9$/L) is one of the common laboratory findings in SLE patients. It can appear in either chronic or acute form. Chronic form is related to mild disease, whereas acute form is similar to idiopathic autoimmune thrombocytopenic purpura. Platelet dissociation is known to be directed by anti-platelet antibodies (APL). APL is also associated with thrombocytopenia and thrombosis[61].

Renal Involvement

More than 70% of patients with SLE have been estimated to have Lupus Nephritis (LN) at some stage of their disease progression. The World Health Organization

(WHO) classification for Lupus Nephritis has been published to facilitate a more accurate description of renal histopathological biopsies by the International Society of Nephrology and the Renal Pathology Society (Table 2)[62].

Table 2. Classification of lupus nephritis.

Classes	Description
Minimal mesangial lupus nephritis	Normal glomeruli at light microscopy Mesangial immune deposits on immunofluorescence
Mesangial proliferative lupus nephritis	Mesangial hyper-cellularity or expansion with mesangial immune deposits in light microscopy Some subepithelial or subendothelial deposits on immunofluorescence or electron microscopy
Focal lupus nephritis	Involves less than 50% glomeruli Active or inactive lesions with subendothelial deposits
Diffuse lupus nephritis	Involves more than 50% glomeruli Active or inactive diffuse, segmental, or global endo- or extracapillary glomerulonephritis with subendothelial deposits. Divided into diffuse segmental when <50% of involved glomeruli have segmental lesions and diffuse global when >50% of involved glomeruli have global lesions
Membranous lupus nephritis	Global or segmental subepithelial immune deposits by light microscopy and immunofluorescence or electron microscopy, with or without mesangial changes. Class V lupus nephritis may occur in combination with class III or class IV disease, in which case both are diagnosed. Class V disease may show advanced sclerosis
Advanced sclerosis lupus	>90% of glomeruli scleroses without residual activity

Obstetric-related Issues

Although, SLE is not directly associated with infertility, it has been associated with pregnancy, particularly in the second or third trimester which is caused by lupus flare. Moreover, in SLE patients who develop active Lupus Nephritis (LN) during conception, either as new onset or flare, increases the risk of preterm delivery, pre-eclampsia, maternal mortality, foetal / neonatal demise and intrauterine growth restrictions. Other common conditions in SLE pregnant women are cutaneous disease (25–90%), arthritis (20%) and haematological involvement which include thrombocytopenia (10–14%)[63].

Laboratory Criteria

The evolution of autoantibodies in human body has been associated with the manifestation of SLE disease. One of the classic, non-specific antibodies that are present in approximately 96% of SLE patients is anti-nuclear antibodies (ANAs) (Manson and Isenberg, 2003). ANAs are antibodies or immunoglobulins that bind to one or more antigens expressed within the nucleus of human cells in relation to connective tissue disease or infections. Anti-double stranded DNA (AdsDNA) are more specific anti-nuclear antibodies that only target double stranded DNA. Studies have reported that anti-dsDNA antibodies have a strong link with glomerulonephritis in SLE patients. Anti-dsDNA is also used for diagnosis purpose and to monitor the disease progression and anti-Smith (anti-Sm) antibodies[4]. Anti-Smith (anti-Sm) antibodies identify extractable nuclear antigens (anti-ENA) which include anti-Ro and anti-La antibodies. Other antibodies that have been found to be associated with SLE disease include anti-RNP antibodies, Rheumatoid factor, IgG anticardiolipin antibodies, IgM anti cardiolipin antibodies and lupus anti-coagulant. Moreover, complement activation plays a vital role in the deposition of immune complexes when autoantibodies bind to their target antigens. Low level of complements, C3 and C4 are highly associated with lupus nephritis and vasculitis[64].

Non-Specific Features

Lymphadenopathy is defined by changes in number, characteristics or size of the lymph node. In SLE, this is a benign condition which can be diagnosed at any stage of the disease development. Studies have l reported that this sign is commonly found in younger patients with cutaneous involvement[65]. Other sign including spleen enlargement or known as splenomegaly is estimated to happen in 10% of SLE patients.

The disease assessment

One of the challenging and difficult aspects in SLE is measuring the disease activity due to the complexity of disease nature which affecting multiple organs and the clinical outcomes from one to another SLE patient. Number of studies attempted in order to find definition for disease activity in SLE and how it should be measured. Measuring the disease activity in SLE is one of the three domains of SLE assessments that include the measuring of damages caused by SLE and the quality of life[66].

Generally, corticosteroids and immunosuppressive that is used in controlling SLE symptoms plays a vital role in disease activity. This is due to their pharmacological properties producing various side effects such as diabetes,

osteoporosis, arterial hypertension and neoplasia among SLE patients. Various indices or disease assessment tools were discovered with the objective standardizing SLE activity assessment. The commonly used indices are as follows: LAI (Lupus Activity Index); SLAM (Systemic Lupus Activity Measure); ECLAM (European Consensus Lupus Activity Measurement); BILAG (British Isles Lupus Assessment), SLEDAI (Systemic Lupus Erythematosus Disease Activity Index); SELENA-SLEDAI (Safety of Estrogen in Lupus Erythematosus National Assessment-SLEDAI) and SLEDAI-2KG[67].

Among the few listed instruments above, SLEDAI that been introduced in 1985 was proven to be the most reliable and reproducible apart from being sensitive to change in a patient condition when used by various investigators. It is an index that measures disease activity by considering the organ affected and includes 24 clinical and laboratory parameters of nine organ systems in its evaluation. SLEDAI assesses disease activity in the previous 10 days. It assesses 16 clinical features and 8 laboratory indices. The scores of the descriptors range from 1 to 8. Active disease indicated by scores greater than 8 and the total possible score for all 24 descriptors is 105[68]. Later SLEDAI-2K was introduced in 2002, a modified version of original SLEDAI but there was one limitation. This revised index does not account for severity within each descriptor[69]América The Systemic Lupus Activity Measure-revised, the Mexican Systemic Lupus Erythematosus Disease Activity Index (SLEDAI. However, it was proven to have best discriminative validity in which able to differentiate active patients from inactive ones and lowest cost. Later another novel tool was developed in order to measure lupus disease activity index known as SLEDAI-2K GCS (SLEDAI-2KG) which outlined disease activity while considering on glucocorticosteroid (GCS) dose and having same descriptors as SLEDAI-2K but with different weight scores based on the dose of GCS[70].

SLAM (systemic lupus activity measure), was first reported in 1986 and later revised in 2001 based on consensus of the lupus council of the American College of Rheumatology[71]. The difference between SLAM and SLEDAI is illustrated in Table 3. SLAM measured the disease severity in the domains of 9 organ systems and 7 laboratory measures as follows; constitutional, integument, eye, reticuloendothelial, gastrointestinal, cardiovascular, pulmonary, neuromotor, joints and laboratory parameters. The total SLAM score ranges from 0–84, with each organ items scored 0–3 points manifested within one month (30 days) period, in which severity having highest score by item[72].

Table 3. Difference between SLEDAI and SLAM scoring system.

SLEDAI	SLAM
Reliable, validated, sensitive and responsiveness to adapt with time	Reliable, validated, sensitive and responsiveness change over time
Very practical and widely used for clinical and research purpose.	Measures disease severity within one month
Scored within last days if present with symptoms	Laboratory studies needed
None of the SLEDAI version captures on improving or worsening and do not include severity within an organ system. Thus, this is less sensitive to compared to other instruments [73]	Responsiveness to patient care: highly responsive
Laboratory information required.	Excellent sensitivity as compared to other instruments [74]
Responsiveness to patient care [69]	

Conclusion

Systemic lupus erythematosus (SLE) is an autoimmune disease that effects the quality of life for many people. This disease gradually worsen over the time and patients are on medications for life long. Moreover, the disease manifestation and complications vary from one to another individual, taking into the consideration of their ethnicity and geographical region where they are origin. It is a daunting task to determine the cause of the disease in one population. Hence, continuous monitoring and surveillance is required to manage the disease progression.

Author Contribution

The literature search and manuscript writing were performed by MS. MA, AM-S and MA provided vital guidance and support as content expert and proofread of the writing. The project was founded by SA-N.

Conflict of Interest

All the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Review Article

Progress in Microbes and Molecular Biology

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Mycobacterium ulcerans and *Mycobacterium marinum*: Pathogenesis, Diagnosis and Treatment

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Abstract: Skin and soft tissue infections are common presentations for non-tuberculous mycobacteria (NTM). The cutaneous infections caused by NTM may cause localized or diffuse lesions. *M. ulcerans* is one of the most identified pathogens that involves in the skin and soft tissue mycobacterial infections. Meanwhile, *M. marinum*, as an NTM has also become important emerging causal agents of cutaneous disease in various geographical regions. Although having common ancestry and highly similar in genetic makeup, *M. ulcerans* and *M. marinum* have differential impacts on the host innate immune system. In term pathogenesis, prolonged cell exposure to exotoxin mycolactone produced by *M. ulcerans* could lead to Buruli ulcer. Meanwhile, like most pathogenic mycobacteria, *M. marinum* evades the host immune responses by invading and replicating inside host cells and it is capable of modulating host immune responses. This article aims to provide a general overview and comparisons between the pathogenesis, diagnosis, prevention and therapeutic strategies for *M. ulcerans* and *M. marinum*.

Keywords: *Mycobacterium ulcerans*; *Mycobacterium marinum*; diagnosis; pathogenesis; treatment

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INTRODUCTION

Mycobacteria are a group of aerobic, acid-fast bacteria which are slender, non-flagellated and rod in shape. They possess waxy cell wall composed of mycolic acid, enabling them to be resistant to decolorization even with the use of acidified alcohol. The acid-fastness property of mycobacteria can be demonstrated by the Ziehl-Neelsen stain[1]. In general, the genus *Mycobacterium* is broadly categorized into three main groups which include the *Mycobacterium tuberculosis* complex (MTC), *Mycobacterium leprae* and non-tuberculous mycobacteria (NTM). Being the two major human pathogens, *M. tuberculosis* and *M. leprae* cause tuberculosis and leprosy, respectively.

Unlike the other two groups which are pathogenic to human, NTM, or atypical mycobacteria, encompasses a variety species which commonly inhabit the aquatic and terrestrial environments. More than 170 species of NTM has been discovered and the list keeps increasing. These mycobacteria form biofilm that contributes to their survival in diverse ecological niches[2], including soil, water (such as household water) plants, animals and food products. Although NTM disease is not notifiable in most countries, the rise in the prevalence of NTM disease has become a growing health concern in the recent years. The reasons include the aging of the population, the increasing number of immunocompromised patients and the increased awareness of the disease. The NTM can cause pulmonary as well as extrapulmonary diseases (lymphadenitis, cutaneous disease, disseminated disease), that often inflicting immunocompromised individual and patients with pre-existing conditions. Generally, NTM infections are acquired from environmental exposures via inhalation (e.g. aerosol) or inoculation (e.g. trauma, plastic surgery, acupuncture) [3,4].

Skin and soft tissue infection is one of the common

presentations for NTM. Mycobacteria responsible for most skin disease include *M. ulcerans*, *M. marinum*, *M. chelonae*, *M. fortuitum*, *M. avium-intracellulare, M. tuberculosis* and *M. leprae*. The cutaneous diseases caused by mycobacteria usually manifest as nodules with characteristics of crusting, ulcers and hypo- and hyperpigmentation. Furthermore, cutaneous infections associated with these mycobacteria may cause localized or diffuse lesions. *M. ulcerans* is one of the most identified pathogens that involves in the skin and soft tissue mycobacterial infections. Meanwhile, *M. marinum*, has also become the emerging pathogens causing cutaneous diseases in people from various countries. Although having a common ancestry and they are highly similar in terms of genetic makeup, *M. marinum* and *M. ulcerans* exhibit differential impacts on the innate host immune system. The production of mycolactone plays a main role of *M. ulcerans* in the pathogenesis of Buruli ulcer disease. Meanwhile, *M. marinum* is similar to most pathogenic mycobacteria where the bacteria evade the host immune responses by invading and replicating inside host cells and are capable to modulate host immune responses (Figure 1).

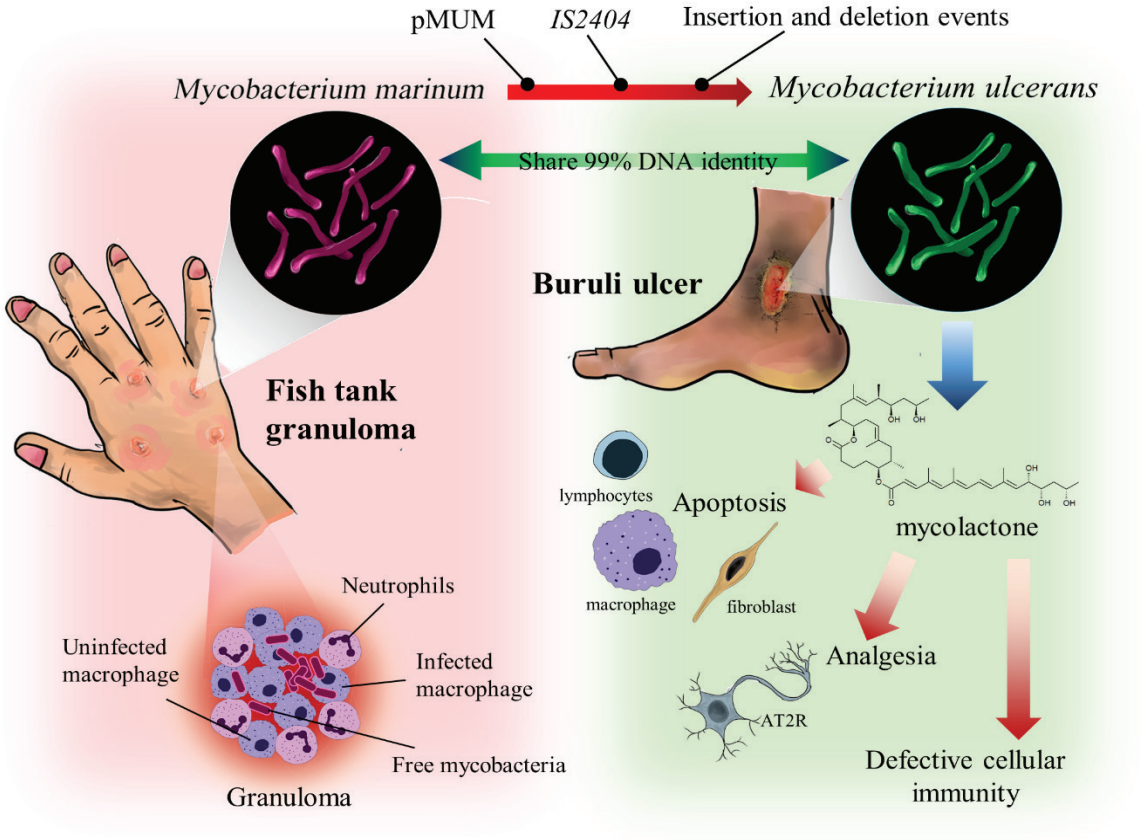

Figure 1. The differential clinical presentations and the pathogenesis between *M. marinum* and *M. ulcerans* despite they share high genomic similarity.

The aim of this review is to provide an overview on both *M. ulcerans* and *M. marinum* as the two major human pathogenic mycobacteria species commonly implicated in cutaneous diseases. Besides that, the pathogenesis and diagnosis of *M. ulcerans* and *M. marinum* infections are summarized and compared. The prevention and ideal strategies to control the diseases are also discussed in this review.

MYCOBACTERIUM MARINUM VERSUS *MYCOBACTERIUM ULCERANS*

Both *M. marinum* and *M. ulcerans* are known to be the two opportunistic mycobacterial pathogens, also named as the mycolactone-producing mycobacteria (MPM), that secrete plasmid-encoded mycolactone exotoxins[5]. Mycolactone is a cytotoxic polyketide metabolite produced by MPM, essential for bacterial virulence, to induce Buruli ulcer-like lesions characterized by extensive necrosis and void of inflammation in intradermal administered animal models[6]. The mycolactone molecule is known to cause apoptosis of mammalian cells, especially more toxic toward the anchorage-dependent cells leading to cytoskeletal rearrangements and detachment in *in vitro* experiments. Interestingly, studies also indicate that mycolactone alters the primary role of innate immunity, including immune cells trafficking and TLR-induced cytokine production.

M. marinum was first isolated from salt water dead fish by Aronson (1926)[7] and was considered as an opportunistic human pathogen after its retrieval from granulomatous skin lesions from Swedish swimmers in the year of 1951[8]. M. marinum is categorized under the Runyon's Group I photochromogenic NTM that are commonly found in non-chlorinated fresh or salt water[9]. Being an opportunistic human pathogen, *M. marinum* causes zoonotic infection in individuals who had exposed through direct-contact with the bacterium from fishes, especially when handling the contaminated aquariums[10]. In general, *M. marinum* infections manifest as superficial skin infections that marked by granuloma and lymphangitis. Thus, the infection of M.

marinum is also termed as the 'fish tank granuloma' or 'aquarium granuloma'. Meanwhile, more severe infections that can spread in a sporotrichoid pattern[11] to deeper tissue inflicting tendinitis, arthritis and osteomyelitis may occur especially in immunocompromised host[12,13].

M. ulcerans is the causative pathogen of a neglected tropical disease, Buruli disease, being one of the most common mycobacterial disease worldwide after tuberculosis and leprosy[14]. Buruli disease is a chronic, necrotizing skin disease with cutaneous tissue destruction and large ulcerations. The Buruli disease cases primarily occur in central Africa, and other regions, including Asia, South America, the western Pacific and Australasia[15]. *M. ulcerans* strains possess a large circular virulence plasmid named pMUM which contains 3 genes encoding polyketide synthases (*mlsA1, mlsA2* and *mlsB*) responsible for the synthesis of the lipid toxin mycolactone[16]. Interestingly, *M. ulcerans* is genetically closely related to the *M. marinum*, thereby they share 99% DNA identity in which *M. ulcerans* exhibit reduced genomes. The comparative whole genomic studies suggest that the emergence of *M. ulcerans* has recently evolved from a *M. marinum* progenitor via acquisition of the virulence plasmid pMUM and subsequent reductive evolution[16]. Acquisition of this plasmid has been considered to be the main contributor for Buruli ulcer in humans[17]. The reduced genome of *M. ulcerans* was subjected to substantial gene loss due to DNA deletion s, DNA rearrangements mediated by insertion of IS*2404* and IS*2606* elements for niche adaptation[18,19].

Both *M. ulcerans* and *M. marinum* strains have optimum growth temperature around 32°C[20], but they grow poorly at 37°C and above, thus reflecting the preference of both strains for the skin and their limited systemic dissemination[21]. Considering both pathogens belonging to the group of slow-growing mycobacteria, *M. marinum* has longer doubling time than *M. ulcerans* when grown in microbial culture medium[22]. In Australia, a mean incubation period of four and half months was identified for *M. ulcerans* infections[23]. Meanwhile, the incubation period of *M. marinum* was approximately ~3 weeks but can be prolonged up to 9 months prior to symptoms onset[24]. Nevertheless, both *M. marinum* and *M. ulcerans* infections may resolve by host immune responses while long-term antibiotic therapy is required on an established infection.

DIAGNOSIS OF *M. ULCERANS* AND *M. MARINUM*

Rapid identification and differentiation of *Mycobacterium* species are crucial to determine the appropriate therapeutic regimens. However, a definitive diagnosis of cutaneous mycobacterial infections can be challenging to make in the clinical routine. There are several diagnostic methods available include histology, microbiological cultures and molecular detection. Histologically, *M. marinum* infections manifest non-specific acute or chronic inflammation as well as positive for tuberculous granulomas and abscesses. However, the detection of acid-fast rod-like bacteria is unusual. Meanwhile, *M. ulcerans* infections are associated with septal subcutaneous necrosis of adipose-rich tissue and positive for acid-fast bacteria detection[25].

The conventional microbiological detection of mycobacteria can be done in specific solid or liquid medium to assist in devising successful antibiotic regimens. The cultivation of NTM is greatly varied depending on the type of pathogen. Incubation of both solid and liquid media at both 30°C and 35°C are usually done to optimally recover the NTM. Different media compositions and conditions according based on specific NTM metabolic needs are also required for their isolation, hence the suspected mycobacterial species could be suggested by the clinicians based on source of exposure and the clinical symptoms to the microbiologists[25].

Being one of the most commonly used molecular-based method, polymerase chain reaction (PCR) has been used for rapid detection of pathogens based on specific target DNA sequence[26-28]. Specific polymerase chain reaction (PCR) assays are available and have been established as the gold standard for the identification and discrimination of NTM that representing public health hazard[29]. PCR can be performed on different clinical samples, including swabs, punch biopsies and fine needle aspirates from nodules, plaques as well as edematous lesions that have not ulcerated yet. Several target genes or sequences are commonly used to differentiate *Mycobacterium* sp., including the *16S rRNA*, internal transcribed spacer (ITS), *23S rRNA*, *hsp*-65, *recA* and *rpoB* genes. Among the different genes, PCR targeting the insertion element IS*2404* has shown to be highly sensitive and specific for *M. ulcerans* detection as it is present in high copy numbers (>200 copies) in the *M. ulcerans* genome[30] Timothy. A highly specific real-time qPCR assay was demonstrated to confer enhanced sensitivity of 10-folds higher than the IS*2404* PCR and provide quantitative assessment of *M. ulcerans* dissemination in Buruli ulcer lesions[31]. Meanwhile, molecular detection technique for *M. marinum* is limited due to its high homology with *M. ulcerans.* Typically, molecular identification of *M. marinum* was performed by analysing 16S rRNA or other conserved gene related to a week-growth of photochromogenic colonies[29].

Although PCR has high sensitivity, sophisticated laboratory infrastructure and well-trained personnel are required to obtain reliable PCR assays with strict quality control[32]. Meanwhile, these criteria are not available in endemic communities. Recently, loop-mediated amplification (LAMP), an isothermal amplification technique, has been proposed as an efficient diagnostic tool for detection of *M. ulcerans*. Being a promising alternative to PCR, this technique offers readily readable results within a short turnaround time and without the need of a thermocycler, hence extending molecular diagnosis in fieldwork and at the point of care[33].

PATHOGENESIS OF *M. ULCERANS* AND *M. MARINUM*

M. ulcerans causes an ulcerative skin infection, namely Buruli ulcer, which is a destructive infection of subcutaneous tissues that result in ulcerative lesions in skin, soft tissue and even the bone. *M. ulcerans* differs from many other mycobacteria in term of its implications for pathology and immune response in human. *M. ulcerans* is found to be distributed extracellularly around the coagulative necrosis regions which is different from other mycobacteria that are

intracellular macrophage pathogens. This observation led to early proposal that *M. ulcerans* produces an exotoxin[34]. The cytotoxic molecule 'mycolactone' was successfully isolated and purified from the acetone soluble fraction of lipid extracts of *M. ulcerans* in 1999[35]Kathleen. Principally, the pathogenesis of *M. ulcerans* is mediated by the production of mycolactone which is uncommon among other bacterial exotoxins. Mycolactones consist of a group poorly immunogenic polyketide-derived macrolides that have strong cytotoxic effects against most of the immune cells and skin cells. There are different variants of mycolactone molecules have been identified[36,37]. The typical mycolactone A/B occurs in Africa while mycolactone C is found in Australia. In *in vitro*, mycolactone A/B is more toxic than type C, the clinical significance of these differences remains elusive.

The paramount role of mycolactone in the pathogenesis of *M. ulcerans* was first established via the administration of the purified mycolactone into the skin of experimental animals which resulted in cell death but devoid of acute inflammatory response[38]. The major role of mycolactone in Buruli ulcer pathogenesis was further fortified by the infection of laboratory animals with *M. ulcerans* mutants which lack of mycolactone production. In contrary to the extracellular infection induced by the wild-type *M. ulcerans*, an intracellular inflammatory infection identical to that of *M. marinum*[39] was resulted by the mycolactone-negative mutants[40,41].

Mycolactone has three major adverse implications on the host in mediating the pathogenesis of *M. ulcerans* infection. The chief destructive outcome of mycolactone is its apoptosis and necrosis inducing effects on an array of cells, including the immune cells. Mve-Obiang *et al.* (2003)[42] revealed the potent cytotoxic effect of mycolactone A/B, as low as 0.1 ng/mL was sufficient to induce cell death associated with apoptosis[38] and necrosis[43]. Recently, mycolactone was demonstrated to induce Bim-dependent cell apoptosis via the $mTORC_2$-Akt-$FoxO_3$ axis[44]. Secondly, a down-regulation of overall host immune defence due to the impairments on the production of tumor necrosis factor (TNF) and other secretory proteins via the blockade of Sec61 by mycolactone. Sec61 is a heterotrimeric complex responsible for the transport of all secretory and integral transmembrane proteins into the endoplasmic reticulum in eukaryotic cell. The blockade of Sec61 activity affects the production of interferon-gamma (IFN-γ) and IFN-γ in activated lymphocytes as well as nitric oxide synthase production in macrophages[45]. Thus, an effective immune response is failed to be activated by the host to act upon the underlying mycobacterial infection. Thirdly, mycolactone also causes impairment of pain sensitivity by targeting the type 2 angiotensin II receptors (AT2R) to mediate its analgesic effect. The mycolactone was suggested to induce analgesia by direct cytotoxicity against sensory neurons and Schwann cells, hence resulting in nerve damage[46,47].

As for *M. marinum*, this bacterium causes tuberculosis-like infection in the ectotherms and induces caseating granulomas in zebrafish that are similar to those in humans[48]. *M. marinum* is an opportunistic intracellular pathogen that multiplies in non-acid phagosome of macrophages prior to phagolysosome fusion[49]. Within the cells, *M. marinum* acquires the ability to escape from the phagosome into the cytoplasm to actively stimulate actin-polymerization, resulting to direct spread into adjacent cells via actin-based motility. The translocation of *M. marinum* into the host cell cytosol depends on an intact Region-of-Difference-1-locus (RD1) which encodes a Type-VII secretion system (ESX-1) that plays a role in mycobacterial virulence[50,51]. Thus, this mechanism confers immune evasion for *M. marinum* by spreading from cell to cell, contributing to permanent infection. *M. marinum* was further found to employ the nonlytic spreading mechanism where the mycobacterium is ejected from the cell via the ejectosome, a F-actin based structure, enabling the transmission to naïve host macrophages[52]. Then, the macrophages migrate into deeper tissue, where they start to form the pathological granuloma-like aggregates after phagocytosis of *M. marinum*. Moreover, *M. marinum* was shown to harbour the ESX-5 system of mycobacteria that responsible for the production of various proline-proline-glutamic acid (PPE) and proline-glutamic acid (PE)-polymorphic GC-rich repetitive sequence (PGRS) proteins. These proteins were demonstrated to interact with host immune system and evade the innate immune response via antigenic variation[53], thus contributing to persistent infection[54,55]. For instance, the expression of PPE38 protein on the cell wall of *M. marinum* was shown to involve in bacterial surface properties and pro-inflammatory effects on infected macrophages[56].

In the view of granulomas as host-beneficial protective structures that has long been a tenet of medical and immunology textbooks, studies employing the zebrafish embryo model of *M. marinum* infection have challenged the idea and provided evidence that the granulomas may be harnessed by mycobacteria for their dissemination and proliferation[57,58]. The study revealed that *M. marinum* employs the ESX-1-dependent early macrophage aggregate to promote spread and growth[57]. On top of that, the mature and established granulomas are found to be porous, where newly infecting mycobacteria can infiltrate and persist within[59].

PREVENTION AND TREATMENT STRATEGIES

An utmost priority to curb Buruli ulcer disease is to enhance our knowledge on the transmission pathway of *M. ulcerans* to human that could aid in the preventive measures focusing on early detection and administration of effective treatment. However, the greatest challenge in Buruli ulcer control is that the reservoir and transmission of *M. ulcerans* are unclear. Exposure to water sources near endemic villages has been shown to increase the risk for developing Buruli ulcer, but it is a challenge to reduce the exposure, particularly in children, to such sources in rural West Africa[60]. The development of an effective vaccine to confer protection has enormous significance in areas of high endemicity for Buruli ulcer. However, there is no effective vaccine specifically targeting *M. ulcerans* is available clinically. The Bacillus Calmette-Guérin (BCG)

vaccine is the only licensed vaccine against mycobacterial infections approved clinically that used to prevent tuberculosis. Although the BCG is cross-protective against *M. ulcerans*, it has only been associated with delaying the onset of disease and short-lived protection in small trials. Collectively, outreach programs to educate communities in endemic areas to recognize early stage of Buruli ulcer is extremely crucial for prevention of severe forms of the disease.

Although there are no vaccine and effective protective strategies, antimicrobial therapy has showcased effective treatment of the disease and lowered the recurrence rate. Given that single-drug treatment led to relapse of mycobacterial disease due to the emergence of drug-resistant mycobacterial strains, multi-drug treatment regimens have been employed for mycobacterial infections. It is a common phenomenon that drug-resistant mutants repopulate the lesions following monotherapy, especially in both tuberculosis[61] and leprosy[62]. Therefore, a second companion drug should be combined with the highly active core antimicrobial agent to prevent treatment failure and relapse.

In 2004, WHO recommended a combination antibiotics alone for small early lesion or as an adjunct to surgical resection for large lesions[63]. A randomised controlled trial reported similar efficacy between the use of either rifampicin and streptomycin (8 weeks) or rifampicin and streptomycin (4 weeks) followed by rifampicin and clarithromycin (4 weeks) which resulted in high recovery rates of exceeding 90% for patients inflicted with early (<6 months) and small lesion (<10 cm)[64]. Recently, a fully oral rifampicin and an extended release formulation of clarithromycin has shown comparable effectiveness for treatment of early and limited Buruli ulcer[65].

According to Center of Disease Control and Prevention, the public water facilities such as swimming pools, spas and hot tub are advised to maintain adequate concentrations of free chlorine, ranging between 0.4 to 1 mg/liter in swimming pool and 2 to 5 mg/liter for spas and hot tub[66,67]. Frequent sanitation and disinfection, and removal of infected fishes are the main control strategies of *M. marinum* infection in fishes. The maintenance personnel for the aquarium should use waterproof gloves to prevent any potential upper limb skin lesions exposure to the pathogen during fish tank-related activities. Proper training is also essential for high-risk populations, such as fishermen and marine-life handlers, to identify signs of *M. marinum* in fish or human in order to facilitate more prompt treatment[68].

To date, there is no clinical trial available which could suggest optimal management of *M. marinum* infections. Furthermore, there is no standardized treatment for cutaneous infections due to *M. marinum*, the therapeutic choice is mainly based on the severity of the infection and the immunocompetency of the patient[9]. Principally, rapid recovery from *M. marinum* in man requires the proper treatment and prevent further progression to deeper tissues. Based on retrospective case studies, single agent antibiotic therapy was shown to successfully treat majority of the limited cutaneous *M. marinum* infections. These single antibiotic agents include minocycline, doxycycline, cotrimoxazole-trimethoprim and clarithromycin have demonstrated positive outcomes in the treatment of *M. marinum* infections[9,69]. Besides that, the combination use of 2 active agents including ethambutol, clarithromycin/azithromycin or rifampicin for 3 to 4 months has been reported to be effective adjunct therapy together with surgical debridement for invasive *M. marinum* infections[70]. Other antimicrobials used for treatment of *M. marinum* infection include ciprofloxacin, moxifloxacin, isoniazid and protionamide[71]. Nevertheless, there were reported cases that yielded negative therapeutic outcomes[72,73]. Several reports also described the worsening of *M. marinum* infection in patients receiving anti-TNF-α therapy[74,75]. Thus, it should be recommended to halt the use of TNF-α inhibitor or other immunosuppressive therapy in *M. marinum* infected patients who are under the course of antibiotics.

Surgical debridement remains a controversial therapy option for *M. marinum* infection and it should be limited to cases that fulfil certain criteria, including cases that associated with poor prognosis involving deep lesions, persistent drainage of sinus and chronic pain[76]. There are also other therapeutic modalities such as local hyperthermic therapy, photodynamic therapy, electrodessication, cryotherapy and X-ray therapy have been recommended to treat *M. marinum* infection[9]. Bacteriophage therapy represents an interesting strategy to be developed for the management of *M. marinum* infection despite only phage therapy using mycobacteriophage D29 for treatment of Buruli ulcer is available at the moment[77].

CONCLUSION

Research on both *M. ulcerans* and *M. marinum* is vital for much needed advancement in the prevention and management of Buruli ulcer and fish tank granuloma, which are both challenging diseases that have been largely neglected. A clearer view of the exact innate and adaptive immune mechanisms leading to protection from *M. ulcerans* and *M. marinum* infections will greatly propel the development of new strategies for effective vaccine design. Moreover, future research focusing on clinical applications and epidemiology is essential to advance our knowledge of mycobacterial pathogens that cause cutaneous infection and improve our capability to control and treat these infections with optimal medical interventions.

Authors Contributions

The literature review and manuscript writing were performed by LT-HT and JW-FL. PR and LC-M provided vital insight and performed proof-reading. The research project is conceptualized by LT-HT and JW-FL.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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Review Article

Progress in Microbes and Molecular Biology

HH PUBLISHER

An overview of the human immune system and the role of interferon regulatory factors (IRFs)

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Abstract: The immune system consists of a dynamic network of cells, proteins, tissues, and organs that communicate to provide adequate defense responses against pathogenic agents. The immune system divide into the non-specific (innate) and the specific (adaptive) components, where the interactions between these two arms are intricately regulated. To deploy effective immune responses, immune systems comprise various cells and molecules that communicate with each other via signaling pathways coordinated by gene regulatory networks. The interferon regulatory factors (IRFs) are critical regulators of both the immune system's development and activation of different cells. To better understand the essential components of the normal immune system, this review essentially aims to cover the current knowledge of individual components of the immune system and the important role of IRFs in regulating the immune system.

Keywords: Interferon regulatory factor; innate; adpative; transcription factor; T cells

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INTRODUCTION

An effective host defense system depends on prompt recognition and response against invaders. Thus, the immune system produces various cells and molecules that communicate with each other via signaling pathways coordinated by gene regulatory networks. In this regard, the immune system, which is the host defense mechanism, is controlled by transcription factors and other elements that activate or repress their target genes in determining cell fate or effector state to ensure effective immune responses[1]. This gene regulatory network is controlled by transcription factors such as Interferon Regulatory Factors (IRFs), comprised of 9 family members (IRF1-9) in mammals. Although this family was initially identified in the type I interferon system, subsequent studies have revealed much broader functions performed by IRF members in host defense. The IRFs play crucial roles in the regulation of immune responses[2]. IRFs function as central molecules that mediate different signaling pathways to induce the expression of interferons and inflammatory cytokines.

Furthermore, IRFs also regulate immune cells' development and activation and act as a bridge between innate and adaptive responses. The IRFs family possesses a turn-helix turn motif that recognizes DNA consensus, known as the IFN sensitive response element (ISRE), which can be found in the promoters of many genes involved in immune responses[3]. The immune responses and pathogenesis of certain diseases correlate with the balance of Th1 and Th2 responses[4,5]. For instance, an imbalance of Th1/Th2 responses, with Th1 bias linked to autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). On the other hand, Th2-dominated responses are associated with allergic conditions such as asthma. Interestingly, many IRFs are involved in T helper (Th) differentiation. For instance, IRF1, IRF2, and IRF8 are mainly engaged in Th1 differentiation[6,7]. Meanwhile, IRF4 that shares several similar biological activities with IRF5 is critical for Th2 cell development [6, 7]. IRF4 and IRF5, which are both involved in MyD88-dependent Toll-like Receptor (TLR) signaling, found to interact with each other in the induction of proinflammatory cytokines and type I interferons[8]. Furthermore, both of these transcription factors are shown to directly regulate Blimp1, a master regulator of plasma differentiation[9,10]. Therefore, we aim to provide an overview of the current knowledge of their roles in immune responses and immune cell development.

THE HUMAN IMMUNE SYSTEM

Immune responses involve recognition of any "non-self" substances, including pathogens. These foreign pathogens or modified particles present in the host body, resulting in activation of cascade complex events

known as the inflammatory process to eliminate the non-self-substances[11]. Following the inflammatory process, the immune system initiates restoration mechanisms involving a series of cellular and molecular events that mitigate the restoration of tissue homeostasis and resolve the inflammatory process[12]. On that note, immune responses are tightly regulated, involving various mechanisms to ensure normal homeostasis. When there is an imbalance of the immune system activity, it can lead to diseases such as autoimmune, chronic inflammatory, and cancer that can potentially be life-threatening[13]. In general, the immune system can be subdivided into two forms of protection known as innate and adaptive immune responses that work closely together to provide effective host defenses (Figure 1).

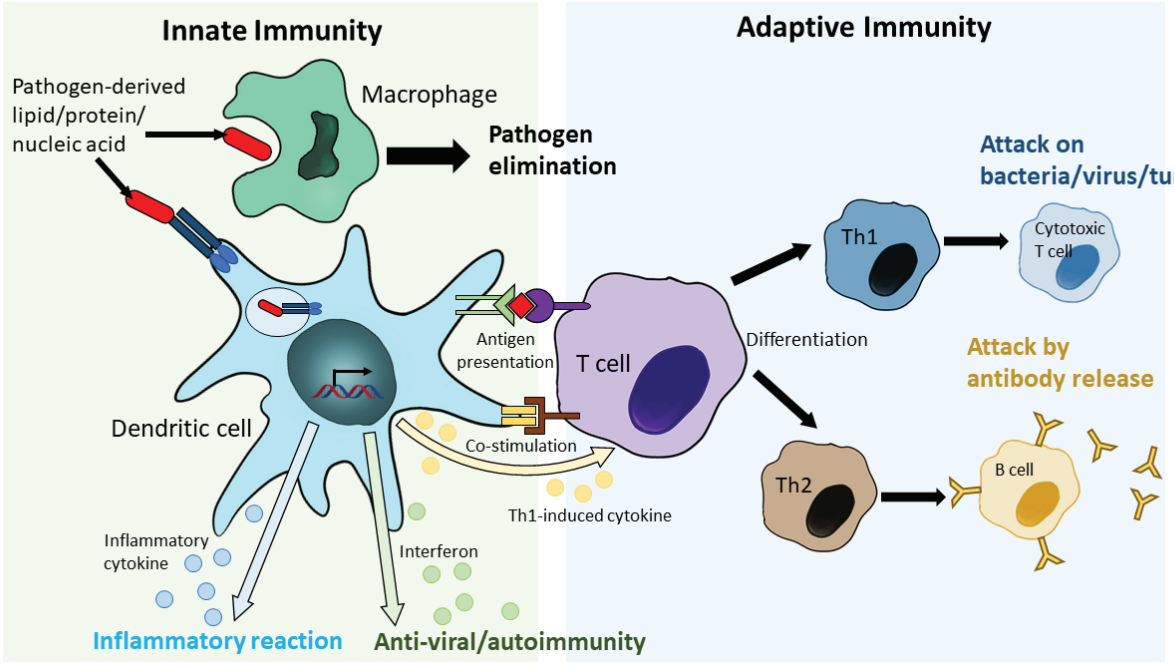

Figure 1. The innate and adaptive immunity in human.

Innate Immunity

Innate immune response acts as the first line of defense. It is also known as non-specific defense mechanism that responds immediately upon recognizing a diverse array of microbial or "danger" signals due to changes in the homeostasis via the pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), Nucleotide oligodimerization domain (NOD) -like receptors (NLRs) and Retinoic acid-inducible gene 1 (RIG-1) - like receptors (RLRs), which recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular pattern (DAMPs)[14]. These recognition systems elicit distinct cellular responses depending on the nature of stimuli and responding cells. The significant roles of innate responses involve; a) prevent the entry of any foreign substances, b) initiate complement pathway for getting rid of pathogens, c) generation of local inflammatory responses, d) induce phagocytosis and cytotoxicity activities, e) facilitate wound healing and tissue repair and, f) activation of adaptive immune responses through antigen-presenting cells[11,15–17]. The innate immune system components consist of physical and chemical barriers, immune cells, and soluble factors that orchestrate rapid immune responses[18]. The physical barriers include epithelial layers of skin and the mucosal membrane that protect the external environment and exposure to foreign substances, including pathogens[18]. Any breakdown or defect in the physical barrier increases the susceptibility of infection and leads to immune responses. Chemical barriers consist of anti-microbial proteins and peptides, such as defensins permeable to microbes and induce cell death [18]. Thus, physical and chemical barriers both play a crucial role in preventing the entry of foreign substances and infection. When pathogens breach protective physical and chemical barriers, they are combated through innate immune cells and soluble proteins. Upon recognition of foreign molecules via the PPRs, innate cells are activated following a cascade of signaling pathways that trigger transcription factors and other proteins to regulate various soluble factors' gene expression to mount defense responses against the pathogens[18,19].

One of the key soluble factors of immune responses is cytokines. Cytokines are peptides that act as mediators in cell communication and signaling. The role of cytokines is not isolated for innate immune responses, as they also play vital roles in adaptive immune responses. Cytokines are broadly classified based on their immune responses and often have overlapping functions[17]. Most cytokines are primarily but not exclusively produced by leukocytes called interleukin (IL)[20–22]. Interleukins can amplify their production in an autocrine or paracrine manner and induce or inhibit other cytokines' production. They bind to their receptors on the cells that produce them (autocrine) or other cells (paracrine) and modulate the transcriptional program in determining the cells' fate[20]. A distinct subset of cytokines named chemotactic cytokines or chemokines is predominantly involved in trafficking activation of

leukocytes to inflammatory sites[17,22,23]. Concerning immune cells, the cells are developed from hematopoietic stem cells (HSCs) within the bone marrow[24]. As these cells mature, they can be differentiated into two main lineages, myeloid progenitor cells (neutrophils, basophils, eosinophils, monocytes, macrophages, mast cells, and dendritic cells) and lymphoid progenitor cells (B cells, T cells, and innate lymphoid cells)[24].

Some of the primary innate immune cells include the granulocytes that contain granules sacs containing enzymes and inflammatory proteins (neutrophils, basophils, eosinophils, and mast cells)[24]. Among these cells, neutrophils are the most abundant leukocytes whose primary function in host defense is to patrol and guard the immune system against invaders[17,24]. While basophils and eosinophils are responsible for defense against helminth and allergic-related diseases through the degranulation process[20,24]. Mast cells primarily reside in the tissues rather than in the circulatory, unlike the other granulocytes[20]. Mast cells play an important role in triggering inflammatory response as well as participate in wound healing [24].

Meanwhile, another group of innate cells; monocytes, macrophages, and dendritic cells, belong to the mononuclear phagocyte system that plays multiple roles during inflammation[25]. Monocytes circulate in the circulatory with a rather short life span and may differentiate into tissue macrophages or dendritic cells depending on the surrounding stimuli[17,25]. Both macrophages and dendritic cells are also known as the antigen presenting cells (APCs), as they capable of processing and presenting foreign protein-based molecules (antigens) to lymphocytes[20,26]. In terms of protection against virally infected cells and tumor cells, other innate cells known as Natural Killer (NK) cells are well known for its function in killing infected and transformed cells that managed to escape T cell recognition[24].

Adaptive Immunity

The adaptive immune response is highly specific. It involves recognizing antigens (foreign agents and particles) via the receptors bound to the surface of B lymphocytes and T lymphocytes, which are unique to different antigens[23]. This second defense mechanism is a contingent of the innate immune system and initiated in the later onset of infection. It provides excellent defense responses against persistent infection and, importantly, possesses immunological memory[15,23]. When the same or closely related antigens are encountered, the immunological memory program is activated, and the adaptive immune system provides rapid and enhanced protection. The adaptive immunity is broadly divided into humoral-mediated immune responses and cell-mediated immune responses coordinated by B cells and T cells, respectively[21,23]. Humoral mediated immune responses involve the production of antibodies by B cells against soluble antigens such as extracellular microbes and toxins. On the contrary, cell-mediated reactions involve activation of effector T cells such as cytotoxic T cells that kill intracellular microbes and tumor cells. These are inaccessible to antibodies and T helper cells that produce cytokines for modulating other immune cells' function to mount against the antigens.

The principal cells involved in the adaptive immune response are APCs, B, and T-lymphocytes[23]. APCs refers to the specialized cells that internalize and process antigen, concomitantly presenting the antigen as peptide within MHC (also known as human leukocyte antigen, HLA, the term designated for humans) on their cell surface[16,23]. There are two types of MHC complexes. MHC class I is expressed on all nucleated cells and present peptide antigens derived from intracellular antigens (e.g., viral proteins, autologous proteins, and tumor antigens)[21,23,27]. On the contrary, MHC class II expression is predominantly restricted to APCs and presents peptide antigens synthesized from extracellular antigens (e.g., extracellular microbes, toxins, and allergens). Examples of prominent APCs are DCs, macrophages, B cells, and thymic epithelium[27]. Antigens processed by dendritic cells are displayed on their MHC class I and class II are capable of activating naïve T cells into helper T cells ($CD4^+$) or cytotoxic T cells ($CD8^+$) subsets, respectively[15,26,27]. Macrophages and B cells can also serve as APCs, presenting antigens to T cells during different type of immune responses[26]. A subset of DCs known as follicular dendritic cells can present antigens to B cells to establish the humoral immune response[20].

As mentioned previously, B cells and T cells are the lymphocytes which developed from lymphoid lineage originated from the hematopoietic stem cells (HSCs) that share the same common lymphoid progenitors with innate lymphoid cells (e.g., NK cells). These lymphocytes undergo complex maturation by which they express surface receptors that dictate their functions and phenotypes. Upon recognizing and binding the antigen-specific to their surface receptors, B and T cells undergo activation, proliferation (clonal expansion), and differentiation to effector cells and memory cells[11]. Unlike B and T cells, innate lymphoid cells are not clonally expressed for specific antigens, serving the innate defense system[23].

B cells comprise several subsets classified based on their ontogeny and anatomical location in[28]. For example, B1 and B2 B cells are associated with antibody productions and regulatory B cells (Bregs), which are essential for suppressing autoimmune and inflammatory responses. B cells' developmental and maturation process involves structural and functional rearrangement of their receptors within the bone marrow[29]. B cells express membrane-bound immunoglobulin (Ig) receptors on their surface and produce soluble antibodies of the receptor's same antigenic specificity. Mature B cells (express membrane-bound IgM and IgD) migrate to peripheral lymphoid organs or lymph nodes via the circulatory, where they encounter antigens to establish humoral immunity[28,29]. Depending on the nature of antigens encountered and the subset of B cells involved. These B cells can be activated either with the involvement of activated helper T cells that express CD40L and the cytokines produced by them (T-dependent B cell activation) or without the involvement of helper T cells (T-independent B cell activation), which is usually facilitated by TLR stimulation[29,30].

Following the activation of B cells, the B cells undergo

clonal expansion and differentiate into plasma cells that produce IgM and IgD type antibodies designed to mount against the particular antigens[28]. Besides, most of the B cells become effector cells, plasma cells that further interact with other stimuli such as cytokines in the local microenvironment. While the plasma cells capable of producing different classes of antibodies other than IgM and IgD (IgA, IgG, and IgE) through the process called immunoglobulin class switching[28,29]. Some of the B cells become memory cells that preserve the "information" for those successful antibodies generated against the antigen and provide robust protection if the same antigens are encountered[29]. The functions of antibodies are to neutralize virulence factors of antigens and enhance the complement pathway and phagocytes' activation to eliminate the antigens[11,18,23]. However, antibodies can also contribute to autoimmune diseases' pathogenesis due to disrupted self-tolerance mechanisms leading to the generation of autoantibodies as a consequence of B cells reacting against self-antigens (particles of host body)[28]. Unlike B cells that can recognize antigens in the extracellular spaces, T lymphocytes have restricted specificity for antigens as they identify and respond to surface-bound antigens displayed on the MHC of APCs.

INTERFERON REGULATORY FACTORS (IRFs) FAMILY

Transcription factors are key players during gene expression as they facilitate when and what gene is "turn on or off" by binding to DNA sequences, acting as co-activator or co-repressor of the gene response to various signals[31]. Structurally they comprise of two domains: a) DNA binding domain that recognizes and bind to DNA sequences, b) activation (effector) domain which interacts with cofactors or other transcription factors[32–34]. Some of them also have an additional domain (signal-sensing domain) that binds to ligands to modulate their activity response to environmental cues[32,33].

Transcription factors are categorized into several groups based on their DNA binding domain structures and their interaction with DNA sequences[33–35]. Some of these transcription factors are known as general transcription factors (GTFs), which are ubiquitous and essential for initiating transcription in the protein-coding gene[31,34]. Other transcription factors are either constitutive or inducible and are specific to certain cell types and stages of organism development[31,34]. Several of these transcription factors function as master transcriptional regulators in controlling signal responses and specifying cells' lineage[31,36]. Also, transcription factors play a crucial role in interacting with histone proteins, which influence chromatin state and establish the environment that allows interactions for activation or repression of gene transcription[33,37]. One group of transcription factors that have been extensively studied for their crucial role in regulating gene networks in the immune system is the Interferon Regulatory Factors (IRFs), which possess the turn-helix turn motif[38].

The first IRFs member, IRF1, was initially identified as a factor that binds to the human interferon's upstream regulatory region (IFN) β gene and induced its expression[39]. Over decades, the family of IRFs has been expanded to ten members, to which IRF1 to 9 are found in mice and humans, whereas IRF10 is only found in chickens[2]. Also, several viral IRFs that interact with cellular IRFs have been identified[40]. All IRFs consist of two domains, the N-terminal (DNA binding domain) and C-terminal (regulatory domain). The N-terminal binding domain is enriched with five tryptophan repeats that are well conserved among all IRF. This region recognizes a DNA consensus, known as IFN sensitive response element (ISRE) found in the promoters of type 1 IFN, IFN-inducible genes, and many other genes involved in immune responses[3]. On the other hand, the C-terminal consists of the IRF association domain (IAD) responsible for homo- and heteromeric interaction with other family members or transcription factors[38,41]. IRFs functions are not limited to, regulating innate and adaptive immune responses. These transcription factors also play a crucial role in controlling type 1 IFN induced by viruses and pathogens involved in the cell cycle, apoptosis, and oncogenesis[2,3,38,41]. Some of these IRFs regulate immune cell development and functions[2,6,7,42].

Accumulating data from several studies has shown that IRF5 is a critical mediator in developing Th1 responses associated with the pathogenesis of various diseases, including autoimmune, metabolic, and infectious diseases[43]. Conversely, studies have also demonstrated that IRF5 plays a role in inducing Th2 responses[44,45]. Strikingly, most studies have attributed IRF5 in regulating Th1/Th2 reactions by altering antigen-presenting cells (e.g., macrophages and dendritic cells) rather than IRF5 intrinsic properties in T cells. Perhaps because early studies reported that expression of IRF5 is barely detected in T cell[46]. Nonetheless, recent studies have detected elevated IRF5 expression in parasitic and viral infected T cells[47,48] and have highlighted its direct role in a T cell subset during chronic parasitic infection[49]. Fabié *et al.* (2018) [49] demonstrated that TLR-7 mediated activation of IRF5 promoted IFNγ^{+} CD4 T cell death because of its ability to enhance death receptor 5 (DR5)-induction cell apoptosis in CD4 T, thus promoting persistent *L. donovani* infection. However, the role of T cell-intrinsic dependent on IRF5 in modulating Th cytokine production remains uncertain.

Roles of IRFs in T helper differentiation

Over the years, many IRFs are involved in T helper differentiation either by modulating the functions of antigen presenting cells or directly altering the transcription of cytokine genes of Th cells[7]. IRF1 is a crucial factor in promoting Th1 differentiation and its absence results in predominant Th2 responses as best characterized using experimental mice disease model. For example, IRF1 deficient mice (*Irf1*$^{-/-}$) were vulnerable to *Leishmania major* (*L. major*) and *Listeria monocytogenes* (*L. monocytogenes)* because the host could not control the parasitic infection due to a lack of Th1 responses, which is needed for protection against the intracellular parasitic infections[50,51]. Collectively, IRF1, IRF2, and IRF8 directly participated in Th1 differentiation predominantly by regulating the production of IL-12, the Th1 signature cytokine.

On the other hand, IRF4 is critical for Th2 cell development. Mice lacking IRF4 showed to be able to mount Th1 immune response against *L. major,* but the Th2 development was impaired[59]. Also, $CD4^+$ T cells of *Irf4*$^{-/-}$ mice were unable to differentiate into Th2 cells and displayed impaired production of Th2 cytokines; IL-4, IL-5, and IL-13[60]. Moreover, overexpression of IRF4 in human Jurkat T cells activated Th2 cytokines' expression in response to mitogenic stimulation[61]. Consistently, in the absence of IRF4, IL-4 failed to induce Th2 cytokine, instead induced Th1 response by upregulating IFNγ and TNF expression, thereby implying the importance of IRF4 in Th2 differentiation[62]. In accordance with the role of IRF4 in Th2 differentiation, Honma *et al.* (2008)[63] reported that IRF4 plays a dual role in different stages of $CD4^+$ T cells. IRF4 inhibited Th2 cytokine production in naïve $CD4^+$T cells but induced Th2 cytokine production in effector/ memory $CD4^+$ cells. However, IRF4 function is restricted to Th2 cell differentiation since several studies have shown that IRF4 is vital in generating Th9, Th17 and Tregs cells[6]. Taken together, IRF4 is able to differentially control the Th subsets and act as Th modulator. On the other hand, IRF5 plays a pivotal role in modulating T helper responses by altering the functions of antigen-presenting cells such as macrophages and dendritic cells and influencing the cytokine production drives the Th differentiation as well as participates in activation downstream of TLR-mediated signaling[3,64].

CONCLUSION

The immune system protects against invaders such as microbial pathogens, toxic agents, and transformed malignant cells. The crosstalk between innate and adaptive immunity ensures an effective host defense system, mediated by various cells and molecules that work dynamically. In terms of T cells, upon activation, naïve CD4+ T cells acquire the ability to differentiate into several subsets, including T helper 1 and T helper 2 cells mediated by specific cytokine signaling and transcription factor. The positive vital regulators involve in Th1 differentiation are T-bet, IFNγ/STAT1, and IL-12/STAT4; meanwhile, for Th2 differentiation are GATA3 and IL-4/STAT6. Besides the master transcription factor, several other transcription factors such as the RUNX3, Hlx, IRF1, Dec2, IRF4, NFAT, Gif-1, c-Maf, and Jun-B are known to cooperate with the subset specific master transcription factor that collectively involved in establishing Th1/Th2 differentiation. Although specific cytokines expressions and transcription factors of T helper subsets are exclusive to each subset, they can still present in both states and fine-tune the T helper cells capabilities for polarization when the situation requires. This can be exemplified clearly by IL-2, which is essential for both Th1 and Th2 differentiation. Collectively, the immune cells orchestrate a complex network made of transcription factors, in cooperation with other proteins involved in cytokine signaling. In which subsequently influences the chromatin state of the targeted genes, resulting in activation or repression of the genes, thereby eliciting the required immune responses in a given state.

Authors Contributions

AK and C-MF performed the literature review and manuscript writing. C-MF conceptualizes the research project.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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Review Article

Progress in Microbes and Molecular Biology

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Insights into quorum sensing (QS): QS-regulated biofilm and inhibitors

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Abstract: In the environment, bacteria can communicate with a known mechanism called quorum sensing (QS). These bacteria will communicate in a group for social interactions like a multi-cellular organism. It provides significant benefits to the bacteria in host colonization, the formation of biofilms, defense against competitors, and adaptation to environmental changes. The bacteria that organize in biofilms are difficult to control and manage, resulting in a higher dosage of antibiotics to clear the infectious biofilms. Also, many QS-controlled activities are involved in virulence and pathogenicity. Hence, understanding the details of quorum sensing mechanisms, its phenotype regulation (biofilm), and QS inhibitors (which attenuate virulence/pathogenicity) may open a new avenue for controlling bacterial infections.

Keywords: Quorum sensing; biofilm; inhibitors; virulence; infections

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INTRODUCTION

Bacteria are a group of microorganisms that can interact with each other and their surroundings via quorum sensing. Quorum sensing is a bacterial cell-to-cell communication process that depends on the release and response to extracellular chemical signaling molecules known as autoinducers[1]. These autoinducers will increase in concentration in a synchronized manner with the density of the bacterial cell population. Thus, detecting a minimum threshold concentration of signaling molecules could stimulate an alteration in gene expression[2, 3]. In other words, QS regulates gene expression as a result of changes in the cell population density. Several types of autoinducers have been identified, and they consist of small peptides, quinolones, and acyl homoserine lactones (AHL). The AHL-based QS system is the most studied among other methods, and AHL molecules are the primary QS signals utilized by Gram-negative bacteria[4]. Briefly, a typical AHL-based QS system comprises two primary proteins: LuxI-type protein (cytoplasmic AHL synthase) and LuxR-type protein (AHL-responsive DNA-binding transcriptional regulator)[5,6]. The bacterial cells generate AHL signals (synthesized by LuxI-type AHL synthase) at a low basal rate, which can penetrate the cell membrane without using a receptor. Once the threshold concentration of AHL signals is achieved, the signs are sensed by LuxR-type transcriptional regulator protein and thereby produce a LuxR/AHL complex that alters gene expression upon binding to *lux* box DNA - a conserved site in the promoter region[6–8].

The AHL is known to be involved in regulating different phenotypes, which is strain-dependent through QS[9,10]. In the natural environment, bacteria can sense the cell population density and regulate a wide range of physiological processes, including expressing essential phenotypes such as bioluminescence, biofilm formation, virulence factor production, swarming motility, chemotaxis, toxin secretion, and antibiotic resistance[1,2,9]. These QS-regulated phenotypes are also essential for bacteria to successfully establish a symbiotic (beneficial or pathogenic) relationship with higher organisms. This review aims to provide insights

into the quorum sensing mechanisms, phenotype regulation (biofilm), and the QS inhibitors in which attenuate bacterial virulence/pathogenicity.

BACTERIAL QUORUM SENSING AND BIOFILM DEVELOPMENT

The attachment to surfaces is the first step for bacteria forming communities (known as biofilm) that enmeshed in a self-produced polymeric matrix[11,12]. The majority of bacterial infections in humans (more than 80%) involve biofilm development[13]. Notably, biofilm formation is one of the phenotypes which is closely related to QS. The development of biofilm *in vitro* involves five stages. First, the reversible attachment of bacterial cells to the surface will turn into irreversible attachment mediated by exopolymeric material[14,15]. Fibrinogen and fibronectin-binding proteins are usually found to play a role in this attachment process. Next, microcolonies are formed, and this indicates the beginning of biofilm maturation. The mature biofilms engineered varies, from flat, homogenous biofilms to highly structured 3-dimensional biofilms. The matured biofilm contains cells that are packed in clusters with channels in between to allow water and nutrient transportation and waste removal. The architecture of developed biofilm is often influenced by motility, rhamnolipid production, and extracellular polymeric substance matrix production. AHL-based QS has been shown to affect biofilm formation at the maturation stage. Labbate and colleagues (2004)[16] proved that a mutation in *S. liquefaciens* acyl-synthase gene, *swrI* results in thin biofilms that lacked aggregates and filaments as compared to its wildtype's biofilm, which is heterogenous that consist of an aggregation of long filaments of cells. This is further substantiated by work on *Burkholderia cepacia* H111 with mutations in either *cepI* or *cepR*[17]. Both mutants showed defective in biofilm maturation and were only arrested at the microcolony stage of growth compared to the robust biofilms covered with attachment surface formed by the wildtype.

Additionally, the maturation of biofilm is influenced by the LuxS-based QS other than the AHL-dependent pathway[18,19]. In *Streptococcus mutans*, the mutation in *luxS* resulted in a mature biofilm with decreased biomass as compared with its wildtype. The final stage of biofilm involved aggregation and detachment, dissolution or dispersal of cells from the biofilm to initiate a new biofilm formation. The dispersed cells showed similarity with planktonic cells, which is non-adherent. This dispersal process allows bacteria to colonize new surfaces and spread its virulence effectively within a closed environment. In this final stage of biofilm formation, the cell dispersal was also found to be QS controlled. In *Rhodobacter sphaeroides*, the mutation in its AHL synthase resulted in hyper-aggregation of cells; but QS's role in this bacteria still remains unknown[20]. Other than that, *yspR* mutant of *Yersinia pseudotuberculosis* resulted in increased swimming motility[15].

The complex formation of biofilm provides a "room" with a hydrated matrix of microbially produced proteins, nucleic acids, and polysaccharides that allows the cells to act less as individual entities but more as collective living systems[14]. Biofilm shields the bacteria by significantly increased in resistance to environmental stresses (pH fluctuation, high salt, and nutrient fluctuation) or microbially harmful particles (antibiotics and biocides). The exciting point arises the criteria for determining the role of QS in biofilm formation[15]. Perhaps it is not surprising that QS indeed plays a major role in biofilm formation, evident by increasing the study of mutant construction experiments that produce pleiotropic phenotypes that affect motility, surface attachment expression, or cell chemistry surface, which is later translated into biofilm formation. However, it would be best if the role of QS could be evaluated by monitoring the signaling process *in situ* in a developing biofilm in the parental strain and determine if the onset of QS corresponds to any observable transition in bacterial biofilm development that relates with other phenotypes such as incline of antimicrobial tolerance.

USE OF QUORUM SENSING INHIBITORS AS POTENTIAL ANTIPATHOGENS

The pathogenesis portrayed by bacteria is a multi-factorial process regulated by the production of virulence factors, which causes a variety of bacterial infectious diseases[21]. QS could regulate many of the bacterial infectious diseases in humans, animals, and plants. Consequently, QS-regulated biofilm formation plays a vital role in bacterial pathogenesis. This has raised the level of concern in clinical settings and other industrial settings where biofilms pose a significant issue, such as aquaculture, agriculture, wastewater treatment plants, and drinking water processing[22].

The dedication of antibiotics in the early 20th century initiated a new era in treating microbial infections, and they were the most rewarding drug that saves myriad lives[23]. However, antibiotics usage over a long time could cause substantial evolutionary stress on the bacterial population and lead to the emergence of multidrug-resistant strains that possessed defensive mechanisms against these antibiotics[24,25]. Methicillin-resistant *Staphylococcus aureus* (MRSA) [26,27], vancomycin-resistant enterococci (VRE)[28], multi-drug resistant *Salmonella enterica* Subsp. *enterica*[29–31], multidrug-resistant *Mycobacterium tuberculosis*[32], multidrug-resistant *Vibrio parahaemolyticus*[33–42] are some dangerous bacterial species that have emerged due to over usage of antibiotic. The emergence of multidrug-resistant bacteria has caught medical attention, and various approaches are now taken to investigate alternative antimicrobials from different sources (e.g., plants and microorganisms)[24,43–49]. Interestingly, scientists have also considered another approach in recent years by exploring into QS linking to bacterial pathogenicity. The findings into the association of QS and bacterial pathogenicity have been evidently strong as virulence has been greatly reduced in mutants that are defective in QS [21,50,51]. In addition, researchers are actively venture into the investigation of different approaches to interrupt or inhibit QS for the control of bacterial diseases. This inhibition process is generally known as "quorum quenching". Quorum quenching (QQ) can be carried out by the application of enzymatic degradation of autoinducers,

blockage of autoinducer compounds synthesis, and utilization of inhibitor compounds to block the signal detection[52–54]. Therefore, techniques that target the QS pathway could serve as a potential new strategy to attenuate bacterial pathogenicity and inhibition of biofilm formation (Figure 1).

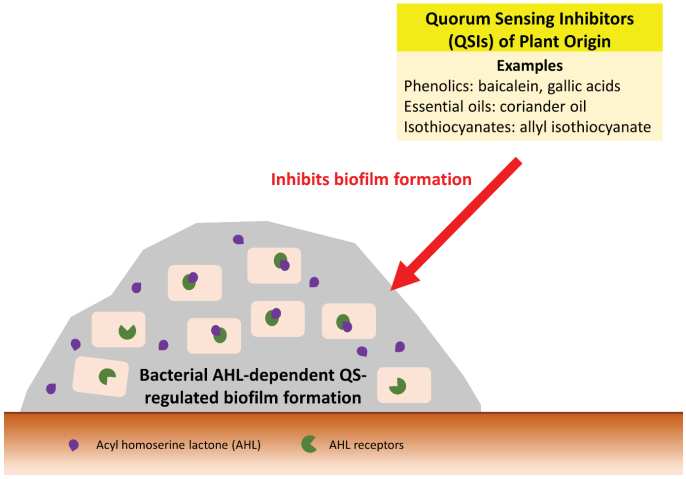

Figure 1. Application of QS inhibitors against biofilm formation.

Halogenated furanone compounds or known as fimbrolides are intensively studied as a group of QS inhibitors[23]. They are isolated from red microalga *Delisea pulchra*, an alga that can produce secondary metabolites that are made up of more than 30 types of furanones. Previous studies had shown that these secondary metabolites could interfere with the AHL-based QS communication circuit. A study performed by Janssens and colleagues (2008)[55] showed that brominated furanones could prevent biofilm formation of *Salmonella* serovar *Typhimurium* at non-growth inhibiting concentrations. Brominated furanones were also found to meddle with the biofilm formation of several other bacterial species including *E. coli*, *B. subtilis*, *P. aeruginosa* and *Streptococcus* species. Another study performed by Givskov and colleagues (1996)[56] evident that 100 µg/mL of furanone extracted from *D. pulchra* could inhibit swarming abilities of *Serratia liquefaciens*. Moreover, Defoirdt *et al.* (2006)[57] also showed that furanone can inhibit bioluminescence of *Vibrio harveyi* strain JMH597 at a concentration of 100 mg/L. However, drawbacks of halogenated furanones are too reactive and could cause toxicity towards human cells.

Thus, researchers exert into finding potential quorum sensing inhibitors (QSIs) from various natural sources. It has been proposed that a potential QSI should fulfill specific criteria[22]: (i) small molecule with high efficiency in reducing QS regulated genes, (ii) high degree of specificity with no adverse effect, (iii) chemically stable and resist to host metabolic system, (iv) longer than AHLs to prevent bacteria resistance, (v) do not affect the host microbiome, and (vi) show no toxicity effects towards the host. To date, numerous naturally occurring QSI is presently well established and grouped into various categories. Besides, several QQ enzymes have been discovered from prokaryotes and animal sources. One of the QQ enzymes is AHL-acylase that cleaves acyl side chain. Acylase produces by *Streptomyces* sp. is similar to acylase I produce by porcine kidney, where both cleaves the acyl chain longer than six carbons[58,59]. Some other QQ enzymes are AHL lactonases that produced by *Bacillus* spp.[60] and mammalian paraoxonases[61] that function to hydrolyze AHL lactone ring. However, researchers have been focusing on exploring potential QSIs from plant extract because it has been anticipated that plant sources are safer for human consumption. These natural compounds are known as secondary metabolites (or phytochemicals), and many classes of these phytochemicals demonstrated their potential as antimicrobials or synergists of other products[62]. Recent studies have promoted the potential of these phytochemicals as potential QSIs. As a result, the active compounds have been extracted from plants and their QS inhibition activity has been evaluated by numerous studies (Table 1). Further toxicology study should be performed on these extracted compounds to validate their safety as biopharmaceutical agents.

One of the QSIs consists of phenolic products or polyphenols, which constitute one of the most abundant and omnipresent as plant secondary metabolites (phytochemicals)[63]. Phenolics are considered potential QSIs because they are used to treat ailments such as diabetes, cancer, or inflammatory diseases besides having antimicrobial properties. Jagani and colleagues (2009)[64] proved that naturally occurring phenolics could act against biofouling of *P. aeruginosa*. Another study conducted by Vandeputte and colleagues (2010)[65] showed that catechin extracted from *Combretam albiflorum* reduces elastase, pyocyanin, and biofilm formation *P. aeruginosa* PAO1. They had selected eight types of phenolics, anarcadic acid, polyanarcadic acid, salicyclic acid, polysalicyclic acid, polyphenol, catechin, epigallocatechin, and tannic acid; all eight compounds showed significant reduction towards *P. aeruginosa* biofilm formation. Flavonoids extracted from citrus species such as quercetin and naringenin hinder the biofilm formation of *E. coli* O157:H7 and *V. harveyi* BB120[66,67]. Another subclass of phenolics, furocoumarins, shows QSI abilities in which purified furocoumarins — dihydroxybergamottin and

berggamottin inhibit autoinducer activities *V. harveyi*[68]. Girennavar and colleagues (2008)[69] further substantiated that furocoumarins from grapefruit juice inhibited more than 95% of autoinducer-1 and autoinducer-2 activities in *V. harveyi*. Other than that, ferulic acid and gallic acid (grouped under subclass of phenolic acids) were found to block bacterial motility, adhesion, and biofilm formation of *E. coli*, *P. aeruginosa*, *S. aureus*, and *Listeria monocytogens*[70]. A study carried out by Plyuta and colleagues (2013)[71] showed that the usage of 200 µg/mL of gallic acid reduced the biofilm formation of *P. aeruginosa* PAO1 to 30 %. Gallic acid has been proven as a potential QSI. Gallic acid at a concentration of 1mM resulted in an 80% reduction of biofilm formation by Eikenella corrodens as demonstrated in the experiment Matsunaga *et al.* (2010)[72]. As for ferulic acid, application at a concentration lower than eight µg/mL found to forbid S. aureus' biofilm formation[73].

Other groups of phytochemicals such as isothiocyanates and essential oils could serve as potential QSIs. Isothiocyanates are products formed during glucosinolate hydrolysis, and they are considered the most critical biological active products in plants[74]. One of the aliphatic isothiocyanates, allylisothiocyanate, interfered with the adhesion-related genes in *S. aureus* in work done by Lee *et al.* (2013)[75]. This compound demonstrated to reduce the *Pseudomonas sp.* planktonic cell growth and the number of cells adhered to the *Brassica nigra*. Likewise, essential oils have proven to be potential QSIs as they are complex mixtures of volatile compounds synthesized from several plant organs[76]. The QS activities of *P. aeruginosa*, *Proteus mirabilis,* and *S. marcescens* — swarming, production of extracellular polymeric substances and biofilm formation were inhibited upon exposing to methanolic extracts of *Cuminum cynimum*, where one of the components is methyl eugenol — an essential oil with an aromatic ring[77]. This plant-based QSIs may not function as bactericidal compounds; however, the infection process could be interrupted by interfering the bacterial QS and this eventually leads to elimination of pathogens by the host immune system.

BIOTECHNOLOGICAL IMPLICATIONS OF STUDYING QS

As the number of bacteria that employ QS systems continues to bloom, the research into QS could span a wide variety of potential applications, mostly controlling bacteria growth and activities by interfering with the signaling pathways[78]. QS cross talk is also another exciting implication as bacteria always exist in the mixed-species population, such as biofilms in nature. This could cause an outbreak of infectious diseases or further health complications[79]. The study into QS paved the way for discovering various QSIs that is feasible as a treatment for bacterial infections in all living organisms. Given the growing numbers of multidrug-resistant strains, the rational strategy is to control these bacteria's outbreak by manipulating QS properties. Nowadays, scientists are exploiting the possible benefits of understanding the bacterial QS system. Ultimately, this could significantly contribute to many fields, such as improving the water treatment process, preventing bacterial diseases in aquaculture systems, and treating human infections[80].

Another interesting fact of QS is that eukaryotes can recognize bacterial QS molecules. This cross-kingdom interaction alters the physiological adaptation in colonized eukaryotes that modify their defense system, immune responses, hormonal responses, or growth responses[81]. Besides creating a pathogenic relationship with higher organisms, the interesting interaction is signaling molecules (AHLs); reported to mediate root growth through biosynthesis of phytohormones. Indole-3-acetic acid (IAA), or known as auxin, is a crucial phytohormone that enhances different developmental processes in plants. IAA production is widely spread among plant-associated bacteria. They can play a critical role in promoting plants' growth and development, especially root elongation[82]. Plant growth-promoting bacteria (PGPB) have been extensively studied as potential bio-fertilizers due to increasing pollution by over-usage of chemical fertilizers[83]. Biosynthesis of IAA by microbial strain is considered one of the essential criteria to be selected as an efficient PGPB. To date, there is an increasing number of reports stating that QS facilitates the PGPB in enhancing plant growth. As previously reported, treatment of *Arabidopsis thaliana* roots with 1–10 µM of C4- and C6-HSL increased the ratio of IAA/cytosine that led to promoted root growth[84]. In their study, they found out that the introduction of C6-HSL did not induce the systemic resistance and priming effect of *A. thaliana*. They further stated that short-chain AHLs might play a better role in promoting plant growth due to the hydrophobicity of long-chain AHLs. A study substantiates this fact revealed that C6-HSL was transported to the leaves of yam beans and barley leaves but not the C10-HSL[85]. Various studies also showed that Rhizobium mutants that were unable to produce AHLs were unable to nodulate legume plants compared to the wildtype strain[86]. These also further support the idea that AHLs could be participating in beneficial plant-bacteria interactions. Thus far, the QS studies could lead us into a different dimension in searching the potential of QS bacteria to contribute beneficially.

CONCLUSION

It is undeniable that QS plays an essential part in the physiological processes of bacteria. Nonetheless, further studies are still required to characterize the function and pathway-related to QS fully. The occurrence of antibiotic resistance and infections reflects the downside of utilizing antibiotics to treat biofilm linked continual bacterial infections. The application of QSIs has exhibited promising results against biofilm formation; however, the utilization of QSIs as an approach to the battle against multidrug-resistant bacteria entails additional investigation. Future work needs to reveal if QSI compounds can be developed as antipathogenic treatment and their successful bacterial eradication mechanisms.

Table 1. Examples of QS inhibitors (QSIs) from plant origin and its effect on QS activity.

Phytochemical Group	QSIs (Phytochemicals)	Effect on QS Activity	References
Phenolics	Ascorbic acids	Reduction in autoinducer-2 activities, spore production, and enterotoxin production in *Clostridium perfringens*	[87]
	Baicalein, Hamamelitannin	Inhibition of biofilm formation increased permeability of vancomycin and reduced production of staphylococcal enterotoxin in *S. aureus*	[88, 89]
	Curcumin	Attenuation of virulence in *P. aeruginosa*	[90]
	Ellagic acids	Inhibit biofilm production in *E. corrodens*; reduction of AHLs production in *E. carotovora*.	[91]
	Epigallocatechin gallate, Catechin	Interference with biofilm formation of *E. coli* and *P. putida*. Reduction in extracellular polymeric substance of *Staphylococcus* sp.	[65, 92, 93]
	Ferulic acids	Inhibition of biofilm in *P. aeruginosa*, interference to the motility of *P. fluorescens* and *B. cereus*	[94, 95]
	Gallic acids	Inhibition of biofilm in *S. mutans*	[96]
	Giganteone A	Reduction of QS-related activity in *E. coli* biosensors	[97]

Phytochemical Group	QSIs (Phytochemicals)	QS Activity	References
Phenolics	Gingerone	Reduction in swarming and biofilm-forming capacity in *P. aeruginosa* PAO1	[98]
	Glycyrrhiza glabra flavonoids	Interference of motility and reduction in biofilm formation in *Acinetobacter baumannii*	[99]
	Malabaricone C	Reduction in pyocyanin production and biofilm formation in *P. aeruginosa*	[100]
	Rosamarinic acid	Influence the protease and elastase production, biofilm formation, and virulence factors of *P. aeruginosa*	[101]
	Salicylic acids	Reduction of AHL production, interference towards twitching and swimming motility of *P. aeruginosa*	[102]
	Tea polyphenols (*Camellia sinensis* L.)	Reduction of proteolytic activity, elastase, swarming motility, and biofilm formation in *P. aeruginosa*	[103]
	Pyrizine-2-carboxylic acid	Inhibition of biofilm formation in multidrug-resistant *V. cholerae*	[104]
	Proanthocyanidins	Reduction in production of QS-regulated virulence determinants in *P. aeruginosa*	[105]

Phytochemical Group	QSIs (Phytochemicals)	QS Activity	References
Essential Oils	Cinnamon oil, Ferula oil, Dorema oil	Interference of QS related phenotypes; production of pyocyanin, alginate, and rhamnolipid in *P. aeruginosa*	[106, 107]
	Cinnamon bark oil	Modification of permeability of outer membrane and inhibition of bacterial QS-activity in *E. coli*	[108]
	Clove oil	Reduction of violacein production in *C. violaceum* and interference of swarming ability of *P. aeruginosa*	[109]
	Coriander oil	Inhibition of biofilm formation and lipid peroxidation in *Campylobacter coli* and *C. jejuni*	[110]
	Linalool	Inhibition of biofilm formation and alteration of the adhesion of *A. baumannii*	[111]
	Oregano oil	Inhibition of violacein production by *C. violaceum*	[112]
	Rose oil, Geranium oil, lavender oil, Rosemary oil	Reduction in violacein pigmentation in *C. violaceum* and AHLs production in *E. coli*	[113]
	Thyme oil	Reduction of flagella gene expression in *C. violaceum* and interference of biofilm formation in *P. fluorescens* KM121	[114, 115]

Phytochemical Group	QSIs (Phytochemicals)	QS Activity	References
Isothiocyanates	Allicin, Ajoene	Renders *P. aeruginosa* sensitive towards tobramycin; inhibition of biofilm	[116, 117]
	Allyl isothiocyanate	Interference of adhesion and motility, inhibition of biofilm formation in *E. coli*, *S. aureus*, *L. monocytogenes,* and *P. aeruginosa*	[75, 118-120]
	Iberin	Interference of rhamnolipid production and gene expression of *lasB* and *rhlA* in *P. aeruginosa*	[121, 122]
	Sulforaphane, Erucin	Antagonists of transcriptional activator of LasR and inhibition of biofilm formation in *P. aeruginosa*	[123]
Stilbenoids	Resveratrol, Piceatannol, Oxyresveratrol	Reduction of violacein in *C. violaceum* CV026; Decreased in production of pyocyanin and swarming motility in *P. aeruginosa* PAO1	[124]

Author Contributions

The literature search, data extraction, and manuscript writing were performed by TW-S, LJW-F, LLN-S, and VL. At the same time, K-GC provided vital guidance, insight, and technical support to complete the project.

Conflict of Interest

The authors declared that research and writing were conducted in the absence of financial and non-financial interest. The funders do not participate or influence any of the experimental design, research, or writing work. This research was completed in the absence of any commercial or financial relationships construed as a potential conflict of interest.

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Genome Report

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Whole genome sequence of MUM116, a *Bacillus* species isolated from intertidal soil

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Abstract: Over the past few years, mangrove-derived *Bacillus* sp. have been characterized frequently for their bioactive potential. *Bacillus* sp. MUM 116 was isolated from mangrove forest in Kuala Selangor which is located on the west coast of Peninsular Malaysia. In order to obtain better understanding of the strain, the genome sequence of MUM 116 was acquired through Illumina MiSeq sequencing platform and yielded 5,720,395 bp along with 165 tRNA and 25 rRNA genes. Based on antiSMASH and RAST annotation, there was one cluster associated with production of bacteriocin. A deeper analysis into the genome sequence of MUM 116 would be essential to exploit the strain for production of bioactive compounds, which could potentially be developed as potent antibacterial agent.

Keywords: *Bacillus*; antibiotics; mangrove; secondary metabolite; genome

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Short Introduction

As a unique ecosystem, the mangrove forest are habitat for many plants as well as microbial populations that highly capable of adapting to fluctuations in temperatures, organic matter content, salinities and oxygen conditions[1,2]. Owing to these factors, some strains came up with adaptation strategies to survive and persist in the environment; one of which is by modifying metabolic pathway by scavenging nutrients available in the environment before converting them into useful, bioactive compounds that improve their survivability (i.e. antibacterials and antifungal)[3–5]. With reference to mangrove forest, Asia represents an ideal "hunting zone" for bioactive microbial strains as this continent has got the largest coverage of mangrove forests, contributing 42 % of the global total[6,7].

Several studies have shown that *Bacillus* sp. derived from mangrove forest have great potential in producing bioactive compounds[8–13]. *Bacillus* sp. MUM 116 was isolated from the west coast of Peninsular Malaysia during a screening program for bioactive microbes[14–19]. 16S rRNA analysis showed that MUM116 showed high similarities (<90%) to some bioactive type strains including *Bacillus ginsengisoli*, *Bacillus niacini* and *Bacillus mesonae*[20]. Given that mangrove-derived *Bacillus* sp. have been demonstrated to possess potential bioactive potential and MUM 116 displayed high 16S rRNA gene similarities with bioactive type strains, the strain was subjected to genome sequencing to uncover its genomic potential.

Data description

The genomic DNA of MUM 116 was extracted using Masterpure™ DNA purification kit (Epicentre, Illumina Inc., Madison, WI, USA) before subjected to RNase

(Qiagen, USA) treatment[21,22]. Genomic DNA quality was evaluated using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a Qubit version 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA)[23,24]. Nextera™ DNA Sample Preparation kit (Nextera, USA) was used to generate DNA library and its quality was examined with Bioanalyzer 2100 high sensitivity DNA kit (Agilent Technologies, Palo Alto, CA) prior to sequencing[25,26]. Whole genome sequence of MUM 116 was obtained via paired-end sequencing on Illumina MiSeq platform with MiSeq Reagent Kit 2 (2 × 250 bp; Illumina Inc., Madison, WI, USA)[27]. The assembly of trimmed seqeuence was done with CLC Genomic Workbench version 5.1 (CLC Bio, Denmark), resulting in 208 contigs and an N_{50} contig size of approximately 52,003 bp. The assembled genome size of MUM 116 consists 5,720,395 bp, with an average coverage of 74.0-fold and G + C content of 38.4%. The genome sequence of *Bacillus* sp. MUM 116 has been deposited at DDBJ/EMBL/GenBank under accession of MLYR00000000.

Table 1. General genomic features of *Bacillus sp*. strain MUM 116.

	Bacillus sp. MUM116
Genome size (bp)	5,720,395
Contigs	208
Contigs N_{50} (bp)	52,003
G + C content %	38.4
Protein coding genes	5,273
tRNA	165
rRNA	25

Annotation of MUM 116 genome was carried out using Rapid Annotation using Subsystem Technology (RAST) [28] while gene prediction was performed using Prodigal version 2.6. The detection of ribosomal RNA (rRNA) and transfer RNA (tRNA) was done using RNAmmer and tRNAscan SE version 1.21, respectively[29–31]. Based on RAST analysis, more than one-quarter of the protein-coding genes were associated with primary metabolism and highest number of genes were related with metabolism of amino acid and derivatives (12%). Furthermore, both RAST and another bioinformatics tools, antibiotics & Secondary Metabolite Analysis SHell (antiSMASH) revealed potential of MUM 116 in producing bacteriocin under the thiazole/oxazole-modified microcins (TOMMs) class[32,33]. Several *Bacillus* sp. have been described to have the potential of synthesizing TOMMs[34,35]. For instance, *Bacillus amyloliquefaciens* FZB42 isolated from plant-pathogen-infested soil was capable of compounds producing not just plant-promoting activity, the strain produced a novel TOMMs — plantazolicin which can suppress growth of bacterial and fungal plant pathogens[35]. Even though *Bacillus* sp. isolated from terrestrial region showed great potential in producing bioactive compounds, several studies have hinted that genomes of *Bacillus* sp. from special environment like mangrove area are generally more "enriched" than those from terrestrial area, as the dynamic environment imposes selective pressure on genomic region associated with adaptation which then promotes production of unique secondary metabolites[36,37]. Altogether, the availability of MUM 116 genome sequences enabled further investigation into its genomic potential, particularly for the production of bacteriocin(s). In future work, more experimental testing is required to optimize production medium and culture conditions for *Bacillus* sp. before exhaustively examine all potential antimicrobials.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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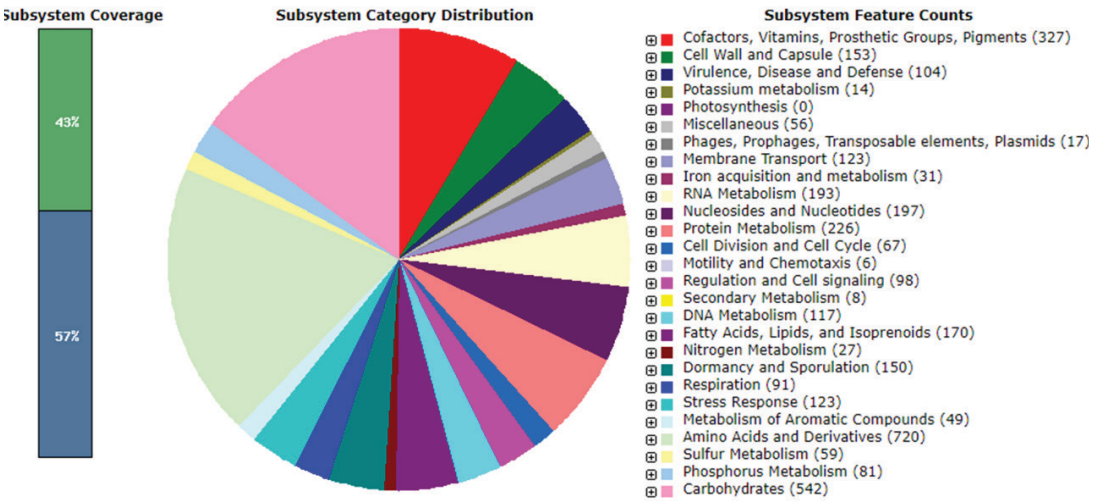

Figure 1. Subsystem category distribution of *Bacillus* sp. MUM 116 (based on RAST annotation server).

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Genome Report

Progress in Microbes and Molecular Biology

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Whole genome sequence of *Streptomyces colonosanans* strain MUSC 93J^T isolated from mangrove forest in Malaysia

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Abstract: Under the family *Actinobacteria*, streptomycetes are ubiquitous in nature, producing a wide spectrum of bioactive compounds including antibacterial, antioxidant, anticancer and immunomodulatory properties. During a screening programme in Malaysia, *Streptomyces colonosanans* MUSC 93J^T was isolated as a novel *Streptomyces* sp. from the mangrove soil in Sarawak. The strain exhibited potent antioxidant activities and cytotoxic activity against several human cancer cell lines. Due to these data, the strain was subjected to whole genome sequencing to uncover its genomic potential and further improve the understanding of the strain. The genome of MUSC 93J^T consists of 7,015,076 bp (G + C content of 69.90%), carrying a total of 5,859 protein coding genes. Analysis using a bioinformatics tool, antiSMASH predicted a total of four biosynthetic gene clusters which displayed similarity of more than 70% to known gene clusters and one of which was associated with the production of a natural protectant, ectoine. Displaying selective toxicity that kills only cancer cells, ectoine has showed its potential to be developed as therapeutic agents for humans. Altogether, the current project clearly highlights the importance of under-explored environment like mangrove in natural product discovery. The availability of whole genome sequence MUSC 93J^T warrants subsequent in-depth investigation and optimization for the production of bioactive compounds which can be exploited for the health and wellbeing of mankind.

Keywords: *Streptomyces*; anti-cancer; mangrove; genome; MUSC 93J^T; actinobacteria

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Short Introduction

Streptomycetes are filamentous bacteria that can be found in various ecosystems and most well-known for their ability to produce secondary metabolites which can be exploited for the benefits of mankind[1–7]. For instance, the isolation of streptomycin from *Streptomyces griseus* described by Professor Waksman and his team was a major breakthrough back in the 1950s, being the first effective treatment against the causative agent of the great white plague, *Mycobacterium tuberculosis*[8,9]. Even though more than 60 years have passed, drug discovery studies investigating bioactive potential of *Streptomyces* sp. from various habitat did not regress, but more efforts are now being poured into the investigation of their genomic potential[10–19]. *Streptomyces colonosanans* MUSC 93J^T was recovered from mangrove forest soil located at Sarawak, Malaysia during a screening programme for bioactive streptomycetes[10,20]. Forming light yellow aerial and vivid yellow substrate mycelium on ISP 2 agar which is a typical trait of streptomycetes, MUSC 93J^T was designated as novel species of genus *Streptomyces* which is closely related to *Streptomyces malachitofuscus* NBRC 13059^T (99.2% sequence similarity), *Strep-*

tomyces misionensis NBRC 13063^T (99.1%)*, and Streptomyces phaeoluteichromatogenes* NRRL 5799^T (99.1%) based on phylogenetic analysis using their 16S rRNA genes. Nonetheless, fermentative extracts of MUSC 93J^T displayed potent antioxidant activity and anticancer activity against several human colon cancer cell lines without significant cytotoxic effect against human normal colon cells. The type strain for MUSC 93J^T is available at two culture collection centres with accession of (= DSM 102042^T = MCCC 1K02298^T). Based on the biosystematics study using a polyphasic approach, the strain was selected for whole genome sequencing to explore its genomic potential, particularly the production of bioactive compounds that are responsible for its anticancer and antioxidant activities[10,21,22].

Data description

Genomic DNA of MUSC 93J^T was obtained using Masterpure™ DNA purification kit (Epicentre, Illumina Inc., Madison, WI, USA) and subjected to RNase (Qiagen, USA) treatment[23–25]. Following that, DNA quality check was conducted with NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a Qubit version 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Construction of DNA library was done using Nextera™ DNA Sample Preparation kit (Nextera, USA) and the library quality was checked by Bioanalyzer 2100 high sensitivity DNA kit (Agilent Technologies, Palo Alto, CA). Paired-end sequencing was performed on MiSeq platform with MiSeq Reagent Kit 2 (2 × 250 bp; Illumina Inc., Madison, WI, USA)[26,27]. After trimming, the paired-end reads were *de novo* assembled on CLC Genomics Workbench version 7 (CLC bio, Denmark), which resulted in 166 contigs and an N_{50} contig size of approximately 99,963 bp. The genome size of MUSC 93J^T comprised 7,015,076 bp, with an average coverage of 53.0-fold and G + C content of 69.90 %. The genome sequence of MUSC 93J^T has been deposited at DDBJ/EMBL/GenBank under accession of MLYP00000000.

Table 1. General genomic features of *Streptomyces colonasanans* MUSC 93J^T.

	Streptomyces colonasanans MUSC 93J^T
Genome size (bp)	7,015,076
Contigs	166
Contigs N_{50} (bp)	99,963
G + C content %	69.90
Genome coverage	53.0x
Protein coding genes	5,859
tRNA	66
rRNA (5S, 16S, 23S)	3, 1, 1

The assembled genome was annotated using Rapid Annotation using Subsystem Technology (RAST)[28]. Gene prediction was performed using Prodigal version 2.6, while ribosomal RNA (rRNA) and transfer RNA (tRNA) were predicted using RNAmmer and tRNAscan SE version 1.21, respectively[29–31]. The analysis from RAST revealed 5,859 protein-coding genes, along with a total 71 RNA genes (Figure 1). Based on RAST system, most of the protein-coding genes were shown to be involved in amino acids and derivatives metabolism (9.18%), followed by carbohydrates metabolism (6.21%) and protein metabolism subsystems (4.91%). Further analysis on antibiotics & Secondary Metabolite Analysis SHell (antiSMASH) detected presence of 23 biosynthetic gene clusters in MUSC 93J^T genome using "strict" detection settings (version 5.1.1)[32,33]. Among the four biosynthetic gene clusters which displayed similarity of more than 70% to known gene clusters, one cluster was associated with the production of ectoine (75 % gene similarities). Ectoine is commonly expressed by bacteria to survive in harsh environments, protecting these microorganisms against extreme osmotic stress[34–38]. As a compatible solute, ectoine has been shown to be safe as it does not interfere with the host's metabolism while offering some beneficial effects including antioxidant and protection against ionizing radiation[39–42]. Apart from that, a recent study by Sheikhpour *et al*. (2019) showed that ectoine induced apoptosis in lung cancer cells without affecting normal cells. As a natural protectant, ectoine seems to be a promising protective agent to be developed for human use, particularly against chronic inflammatory diseases and cancer[43,44]. On top of that, there has been many studies reported ectoine-based spray or lozenges showed superior efficacy in treating acute pharyngitis and/or laryngitis, proposing its potential use as adjuvant treatment for anti-inflammatory or anti-infective drugs[45,46]. The detection of this biosynthesis gene cluster within the genome of MUSC 93J^T reflects the bioactive potential of mangrove-derived actinobacteria (including rare actinomycetes and streptomycetes and further highlighting the possible development of this strain as "mini-factories" for the production of protective molecule like ectoine[47–49]. With the emerging role of probiotics in regulating human diseases caused by gut dysbiosis (i.e. imbalance in gut microbial population), ectoine as a osmoprotectant could potentially increase the viability of probiotics in food and prolong its shelf life[50–60]. With the availability of the whole genome sequence of MUSC 93J^T, these data would greatly accelerate the medium optimization process and allow genomic manipulations to maximize the production of bioactive compounds including ectoine.

Conflict of interest

The authors declare that there is no conflict of interest in this work.

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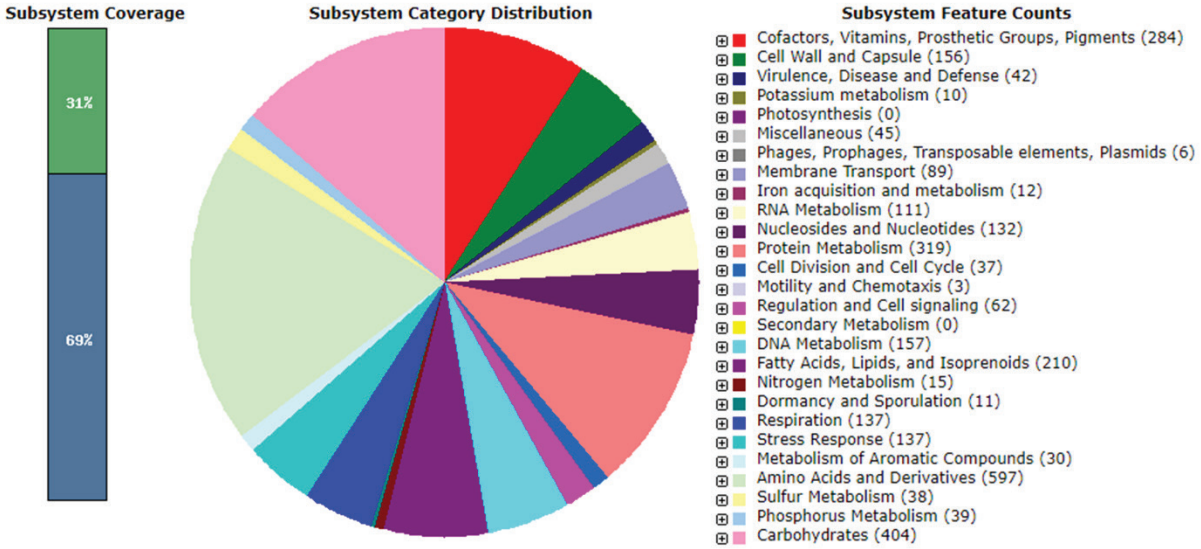

Figure 1. Subsystem category distribution of *Streptomyces colonosanans* MUSC 93J^{T} (based on RAST annotation server).

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Genome Report

Progress in Microbes and Molecular Biology

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Genome sequence of *Vibrio* sp. OULL4 isolated from shellfish

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Abstract: The members of Vibrionaceae family are Gram-negative bacterium are ubiquitous in marine and estuarine environments. This diverse group of bacteria include many pathogenic strains that potentially cause infection to human and aquaculture animals. *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are among the few recognized as a major, worldwide cause gastroenteritis, particularly in countries where seafood consumption is high. The control of these vibrios has been a hurdle due to the rising numbers of antibiotic resistant strains in the environments. We report the genome sequence of *Vibrio* sp. OULL4 isolated from shellfish. The availability of this genome sequence will facilitate the study of its antimicrobial traits, as well as add our knowledge of *Vibrio* sp. diversity and evolution.

Keywords: Vibrionaceae; infection; gastroenteritis; antibiotic; genome

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Introduction

Seafood production has double over the years to meet the rising consumer demand for seafood. This involuntary action has exposed aquatic animals to bacterial infections[1,2]. This situation gets complicated and worsen by the emergence of resistant *Vibrio* sp. strains, which hampers medical care. *Vibrio* sp. is a Gram-negative halophilic bacteria that belongs to the Vibrionaceae family[3–8]. They naturally inhabit the aquatic surroundings and associated with aquatic animals for example crustaceans, molluscs and fish[9–13].

The World Health Organization (WHO) has acknowledged antibiotic resistance as a public health hazard that affects millions of people worldwide[14]. Due to excessive use of antibiotics in the aquaculture sector, the incidence of resistance accelerated, mostly among foodborne pathogens such as *Vibrio* sp.[15–23], *Listeria* sp.[24–26], and *Salmonella* sp.[27–32]. The resistant foodborne pathogens poses a threat and challenge to drug discovery programmes worldwide[33,34]. Therefore, it is important to continuously monitor and manage the resistant *Vibrio* sp. in seafood and environments.

Vibrio sp. OULL4 strain was isolated from shellfish originated from a supermarket in Selangor, Malaysia. The strain presented a large yellow colony on selective media—thiosulphate citrate bile salt sucrose (TCBS) agar. The antibiotic susceptibility test was performed to determine to resistance phenotype of *Vibrio* sp. OULL4 strain. The strain was resistant to 11/14 antibiotics tested, namely the ampicillin, ampicillin/sulbactam, 3rd generation cephalosporin (cefotaxime, ceftazidime), aminoglycoside (amikacin, gentamicin, kanamycin), suphamethox/trimethoprim, oxytetracycline, tetracycline, and chloramphenicol. This is a worrying scenario as the antibiotic resistant profile exhibited by the strain is among the recommended antibiotics agents used in treatment if *Vibrio* sp. infection[35–37]. The *Vibrio* sp. OULL4 strain was selected for genome sequencing to further explore and understand the antibiotic resistant traits.

Data description

The genomic DNA of *Vibrio* sp. OULL4 was extracted using Masterpure™ DNA purification kit (Epicentre, Il lumina Inc., Madison, WI, USA) prior to RNase (Qiagen,

USA) treatment[38,39]. The DNA quality was quantified using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a Qubit version 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Illumina sequencing library of genomic DNA was prepared using Nextera™ DNA Sample Preparation kit (Illumina, San Diego, CA, USA) and library quality was validated by a Bioanalyzer 2100 high sensitivity DNA kit (Agilent Technologies, Palo Alto, CA) prior to sequencing. The genome of OULL4 strain was sequenced on MiSeq platform with MiSeq Reagent Kit 2 (2 x 250bp; Illumina Inc, San Diego, CA, USA)[40]. The trimmed sequences were *de novo* assembled with CLC Genomic Workbench version 5.1 (CLC Bio, Denmark). Contigs with at least 200bp and 30-fold coverage were selected for gene prediction and annotation. The bacteria identity was also checked by local BLAST against NCBI prokaryotic 16S rRNA database. Prodigal (version 2.6.1) was utilized to predict the bacteria gene coding sequence (CDS) from the draft genome[41]. Gene annotation was performed by local BLAST of translated predicted CDS against NCBI-nr database and on Rapid Annotation using Subsystem Technology (RAST) server[42]. Presence of rRNA and tRNA genes were detected using RNAmmer and tRNAscan SE version 1.21[43,44]A total of 59 contigs were generated with N50 size of 201,133bp. The assembled genome size of *Vibrio* sp. OULL4 contains 4,146,642 bp, with an average genome coverage of 54-fold with a G + C content of 45.4% (Table 1). The whole genome project was deposited at DDBJ/EMBL/GenBank under accession MQVK00000000. The version described in this paper is the first version MQVJ00000000. It is composed of 59 contigs and there were 3,743 protein coding genes (out of a total of 3,898 predicted gene) (Table 1).

Table 1. General features of *Vibrio* sp. OULL4 draft genome.

Attribute	Value
Genome size (bp)	4,146,642
G + C content %	45.4
DNA scaffold	59
Total genes	3,898
Protein coding genes	3,743
RNA genes (5S, 16S, 24S)	5, 3, 1
Pseudo genes	55

The analysis obtained from RAST server revealed 493 subsystems (Figure 1). The annotated genome has 63 genes responsible for resistance to antibiotic and toxic compounds including 25 genes for multidrug resistance efflux pumps, one gene for beta-lactamase, and two genes for tetracycline resistance. The presences of these genes in the genome is closely related to the phenotypic resistance exhibited by the strain toward ampicillin, cefotaxime, oxytetracycline, and tetracycline.

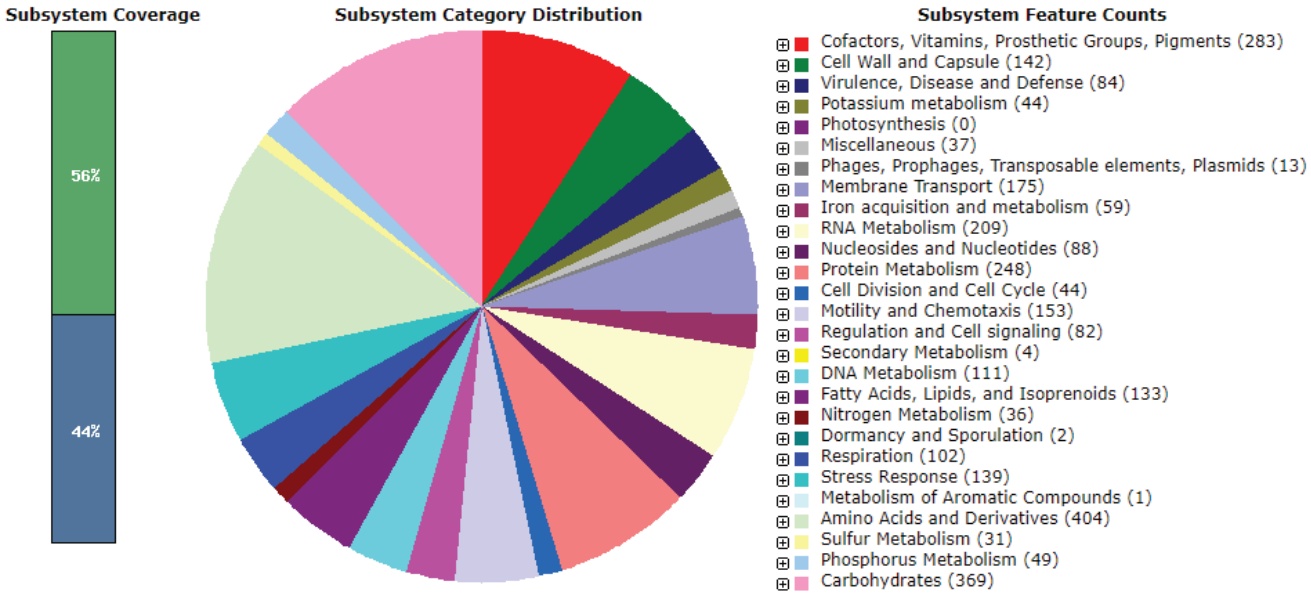

Figure 1. Subsystem category distribution of *Vibrio* sp. OULL4 (based on RAST annotation server).

Vibrio sp. OULL4 is a multidrug resistant strain—resistant to 11/14 antibiotics tested. The resistant phenotype and genes of genome illustrates how extensive antibiotics have been used in aquaculture sector. Some of the resistance phenotype seen in this strain possibly due to the misuse of permitted antibiotics in Asian aquaculture industry namely tetracycline, quinolone, oxytetracycline, sulphonamide, and trimethoprim[45]. Soon, our dependency to antibiotics will eventually deprive the efficacy of clinical antibiotics. We will need to resort to non-antibiotic approach such as bacteriophage application or natural plant antimicrobials to manage *Vibrio* infections in the aquaculture[46–50]. We also could adapt quorum sensing method to understand the various signalling molecules of *Vibrio* sp. These information are useful in the management of virulence traits[51]. In summary, the application of antibiotics in aquaculture should be reviewed and monitored in order to ensure the efficacy of these antibiotics for treatment.

Conflict of Interest

The authors declare that the research was conducted in

the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors Contributions

The research and manuscript writing were performed by VL and W-ST. W-FY and K-GC provided vital guidance and support for the success of the project. The project was founded by VL and K-GC.

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Methods Article

Progress in Microbes and Molecular Biology

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An Optimized Anti-adherence and Anti-biofilm Assay: Case Study of Zinc Oxide Nanoparticles versus MRSA Biofilm

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Abstract: Biofilms form protective layers over bacteria that are associated with a majority of the hospital infections contributing to antibiotic resistance development in susceptible strains. Nowadays, there is a pressing need for developing effective anti-biofilm agents to help address the growing problem of biofilm-producing bacteria associated with antibiotic resistance. In recent years, zinc oxide nanoparticles (ZnO-NPs) has emerged as a prospective candidate for new anti-biofilm agents. The present method paper described an optimized anti-adherence and anti-biofilm assay using ZnO-NPs. The antibiotic-resistant bacteria Methicillin-resistant *Staphylococcus aureus* (MRSA ATCC4330) and vancomycin were used as the growth control and positive control, respectively. The result showed concentration-dependent anti-adherence and anti-biofilm activity. The ZnO-NPs effectively prevented attachment of bacterial cells onto walls of wells with 51.69 ± 2.55% at the highest concentration tested (65.4 µg/mL). ZnO-NPs was also able to break-up 50% pre-formed MRSA biofilm at the lowest concentration of 13.5 µg/mL. Interestingly, ZnO-NPs at lower concentrations demonstrated significantly stronger anti-biofilm activity than that of the positive control vancomycin, demonstrating that ZnO-NPs is a promising anti-biofilm agent. This method could be used as a preliminary screening of transition metal oxide nanoparticles as potential anti-adherence and anti-biofilm agents followed by other specific anti-biofilm assays.

Keywords: Methicillin-resistant *Staphylococcus aureus*; MRSA; anti-biofilm; anti-adherence; Zinc oxide nanoparticles; ZnO-NPs

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Introduction

Over the course of history, nature through its subsidiary plants and microbes, has proven to be an essential player in driving development of future drugs by virtue of their potential in producing secondary metabolites with anti-bacterial, anti-cancer, anti-oxidant and neuroprotective activities[1–18]. Even to this day, nature continues to instill its significance in society as a prominent resource for future antibiotics in treating antibiotic-resistant infections[19]. Despite the use of current antibiotics, infectious diseases acquired either in hospitals or through consumption of foods contaminated by food-borne

pathogens[20–26] remain a major public health problem. Additionally, the unscrupulous practice of antibiotics for various ailments has encouraged antibiotic-susceptible infectious bacteria to form natural defenses against them. One such defense established by these bacteria is the biofilm[19].

Biofilm is a term that describes a community of microorganisms within a self-produced matrix of biopolymers attached on surfaces[27]. Microbes tend to produce biofilm on surfaces evading harmful effects of antibiotics as well as detergents and persists in hospitals causing many internalized hospital-related infections. It was estimated that biofilm contributes to approximately 60 to 80% of hospital infections[28–30]. Given that *Staphylococcus aureus* normal flora is the skin, suggests that it is among the most common causative agent in hospital-acquired infections associated with medical implants[31,32]. Moreover, it was shown that *S. aureus* was tolerant against higher doses of antibiotics and may thus contribute to development of antibiotic resistance in susceptible strains[32].

Recent years has seen a growing interest in the study of biofilm inhibitors acting as adjuvant agents in reducing biofilm layer of pathogenic bacteria[33]. This has led to the use of anti-biofilm assays to identify alternative sources as potential inhibitors of microbial biofilm. Previous studies have highlighted the antibacterial potential of transition metal oxides for crop protection[34] and disease eradication[35–37]. Nanoparticles especially those of metallic nature are one of the newest emerging systems which have great potential in inhibiting the formation of biofilms accredited to their high anti-microbial and anti-bacterial properties. The use of nanoparticles as anti-biofilm agents have found its way in many different sectors such as in healthcare (drug delivery, therapeutics and dentistry) or even in the food industry with a plethora of tailored applications[38,39].

The potency of these metallic nanoparticles in resisting the production of biofilm is high due to its nanoscale size and active participation in most of the stages in biofilm production. If the nanoparticles can successfully prevent adherence of microbes, then cycle of biofilm production is halted from the start. Sometimes, these nanoparticles disrupt the biofilm at the proliferation or even maturation stages, generally through the formation of radicals and reactive oxygen species (ROS) which affects gene expressions and breaks DNA strands[40]. In this context, the ZnO-NPs were chemically synthesized using a zinc nitrate precursor and subsequently characterized to confirm its identity. This includes conducting elemental analysis, Fourier-transform infrared spectroscopy and morphological analysis using electron microscopy. The ZnO-NPs synthesized as an anti-adherence and anti-biofilm agent have nanorice morphologies and have an average size of 250 nm.

The aim of this methodology article is to present step-by-step and optimized anti-adherence and anti-biofilm assays to evaluate the efficacy of ZnO-NPs as anti-adherence and anti-biofilm agents[41] (Figure 1). To validate the test method, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 and vancomycin hydrochloride were used as the control bacteria and positive control, respectively. The experiment set-up consisted of a 96-well plate with a flat bottom, crystal violet as a staining agent and a 96-well microplate reader for quantification of both the anti-adherence and anti-biofilm activities. The result obtained indicate ZnO-NPs has anti-adherence and anti-biofilm properties against MRSA ATCC 4330. Given that crystal violet anti-adherence and anti-biofilm assay is an indirect measure of biofilm biomass, this study could be used as a preliminary screening to investigate the anti-biofilm properties of transition metal oxide nanoparticles prior to studying the mechanism of action of anti-adherence and anti-biofilm properties.

Method Details

Synthesis of ZnO-NPs

A weighted measurement of 1.90 g of zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) is first dissolved in 100 mL of ultrapure water under constant stirring. Subsequently, the pH of the mixture was adjusted to pH 10 using 1M of sodium hydroxide (NaOH) solution. Next, the solution is heated for 1 hour at 85°C under continuous stirring. The white suspension was then centrifuged for 5 minutes at 7000 rpm. Upon removing the supernatant, the residue is washed with distilled water and then subjected to another cycle of centrifugation before removing the supernatant again. The residue was then dried in an aerated oven at 60°C overnight, yielding a white powder.

Anti-adherence and Anti-biofilm Assay

Materials

- Biosafety level 2 cabinet, functional incubator, 96-wells microplate reader
- Sterile disposable consumables: 96-wells microtiter, 15 mL centrifuge tubes
- Bacterial cells (American type culture collection strain- ATCC)
- Bacteria nutrient-rich media. The use of Tryptic soy broth (TSB) which contains glucose and stimulates biofilm formation especially with Methicillin-resistant *Staphylococcus aureus*.
- Aqueous crystal violet (0.1% w/v)
- Glacial acetic acid (30% v/v)
- 1×Phosphate buffer saline
- Multichannel pipette (preferable)
- Vancomycin hydrochloride drug as the positive control
- Zinc oxide nanoparticles (ZnO-NPs) prepared in different test concentrations

Procedure

Bacterial culture preparation

Inoculate 3 to 5 pure colonies of MRSA ATCC43300 from

the culture plate into 15 mL TSB. Revive the bacteria in the shaker incubator at 200 rpm and at 37°C for 18 to 24 hours prior to the experiment so that they are preferably in their log phase of growth. Ensure sterile TSB is used by autoclaving TSB at 121°C for 15 minutes.

Anti-adherence Assay

1. Inoculate 50 µL of ZnO-NPs and vancomycin into designated wells at a series of concentration. (vehicle control e.g. DMSO is also needed to be aliquoted into appropriate wells if used as the diluent for the test substance)
2. Prepare a bacterial suspension (~1 x 10^8 CFU/mL equivalent to UV absorbance reading of 0.08 to 0.1 with wavelength at 600 nm) in 15 mL from a 24-hour bacterial culture. Alternatively, a 0.5 McFarland standard can be used to determine the optimal bacterial suspension. Make a 1:100 dilution in a separate centrifuge tube to obtain a 10^6 CFU/mL bacterial suspension.
3. Add 50 µL diluted bacterial concentration in respective wells using an appropriate multichannel pipette. Using a multichannel pipette is a faster and more efficient mean of adding the bacterial suspension into the wells.
4. Add sterile distilled water to the 4 corners of the microplate to prevent evaporation of water from the test wells. Evaporation of water in test wells can interfere with the results. Alternatively, the microplate can be kept in a container placed with moist filter paper during the incubation.
5. Cover the plate with the lid and place the plate in an incubator at 37°C for 18 to 24 hours.
6. Take the 96-well plate out from incubator and slowly remove the TSB either by decanting or pipetting. Rinse the plate thrice with sterile double distilled water and allow the plate to air dry under the biosafety cabinet. Turn the plates upside down to hasten the process of drying. Ensure it is dry before moving on to the next step.
7. Dispense 100 µL of aqueous crystal violet (1% w/v) into the test wells and let it stain the bacterial cell walls for 10 to 15 minutes. Decant the crystal violet either into a sink or onto clean disposable tissues.
8. Rinse the test wells three times with sterile double distilled water and allow the wells to dry under the biosafety cabinet. Alternatively, the plate can be bathed subsequently with 3 dishes of water.
9. Add 30% (v/v) glacial acid in water to solubilize crystal violet and leave it standing for 15 minutes. Ensure there is clear blue/violet solution with no visible residue in each of the test wells.
10. Read the UV absorbance of all the wells at 570 nm (suggested range would be between 570 to 600 nm)
11. Calculate the anti-adherence activity of test substance and vancomycin using the following formula:

$$\text{Anti-adherence activity\%} = \frac{\text{Absorbance of control-Absorbance of test sample}}{\text{Absorbance of control}} \times 100\%$$

Anti-biofilm Assay

1. Follow the steps stated in bacterial culture preparation and step 2 in anti-adherence assay to prepare 10^6 CFU/mL bacterial suspension. Inoculate 100 µL of the diluted bacterial suspension in TSB into respective well of a new 96 well microplate.
2. Incubate the plate at 37°C in an incubator for 24 hours.
3. Decant the TSB broth completely from the microplate, wash the well gently without disrupting the biomass formed attaching on the bottom and wall of the wells with sterile phosphate buffer saline (PBS) 3 times.
4. Add in 100 µL of freshly prepared sterile TSB broth (control well), ZnO-NPs suspended in TSB with test concentrations and TSB containing the vancomycin in test concentration.
5. Repeat the steps 5 to 10 from the above anti-adherence assay protocol.
6. Calculate the anti-biofilm activity of the test substance and vancomycin using the following formula:

$$\text{Antibiofilm activity\%} = \frac{\text{Absorbance of control-Absorbance of test sample}}{\text{Absorbance of control}} \times 100\%$$

Method Validation

Determination of Biofilm Formation

Based on the protocol, biofilm formation was indicated by the violet stains. This show that TSB media was adequate for biofilm formation whilst 0.1% (w/v) concentration of crystal violet was sufficient for visible observation with the naked eye and quantification by the spectrophotometer.

Assessment of Anti-adherence Assay

This protocol allows the determination of anti-adherence property of ZnO-NPs versus vancomycin using the 96-well plate. The biomass of the bacterial cell was quantitatively analyzed on the microplate reader at absorbance of 570 nm showing a decreasing trend of biomass attachment with increasing concentrations of ZnO-NPs tested. The result show that ZnO-NPs at 65 µg/mL achieved a significant anti-adherence activity of 51.69 ± 2.55%. However, vancomycin at 0.5 µg/mL did not exhibit significant anti-adherence activity when compared to negative control (TSB only) (Figure 2).

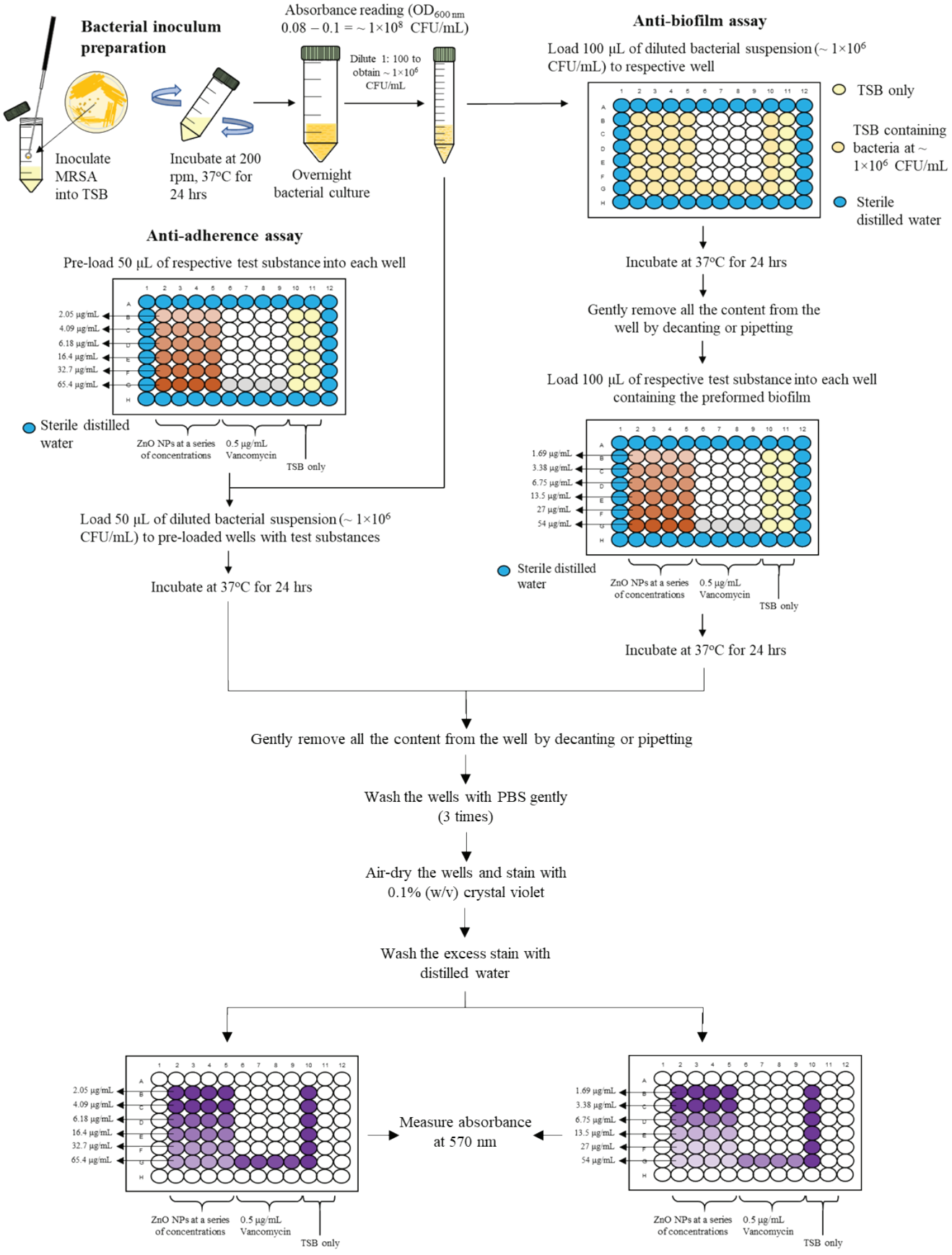

Figure 1. Schematic diagram shows the step-by-step protocol of the optimized ant-adherence and anti-biofilm assays. More detailed protocol should refer to the text.

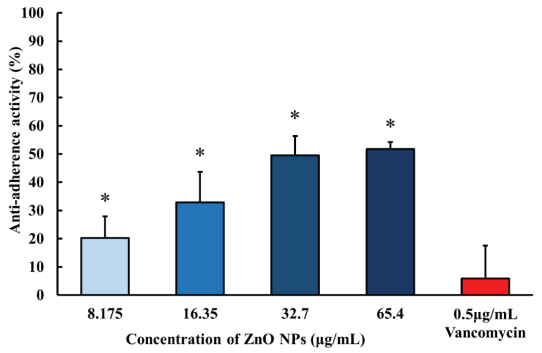

Figure 2. Anti-adherence assay using vancomycin as positive control and MRSA ATCC 43300 as growth control. Experiment evaluated based on quadruplicate results with standard deviation. (n = 4, $p < 0.05$). *indicates significant difference when compared to negative control (TSB only).

Assessment of Anti-biofilm Assay

This method allowed the determination of anti-biofilm property of ZnO-NPs versus vancomycin using the 96-well plate. The biomass of bacterial cell was quantitatively analyzed on the microplate reader at absorbance of 570 nm showing significant reduction of biofilm when increasing concentration of ZnO-NPs used when compared to the control (TSB only). The result shows that ZnO-NPs at 54 µg/mL exhibited significant anti-biofilm activity of 77.35 ± 2.67%. Meanwhile, the anti-biofilm activity of the positive control vancomycin was measured at 40 ± 8.39% at higher concentration of 100 µg/mL tested (Figure 3).

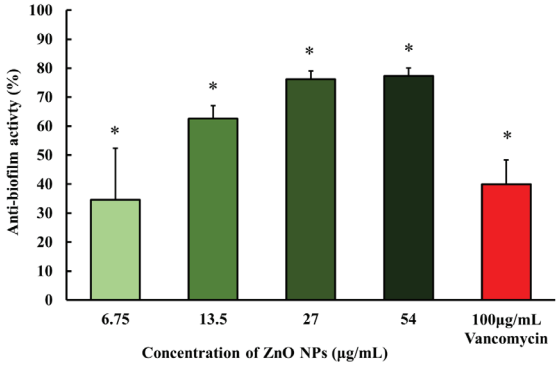

Figure 3. Anti-biofilm assay using vancomycin as positive control and MRSA ATCC 43300 as growth control. Experiment evaluated based on quadruplicate results with standard deviation. (n = 4, $p < 0.05$). * indicates significant difference when compared to negative control (TSB only).

Conclusion

Collectively, the present study shows step-by-step optimized protocol of anti-adherence and anti-biofilm assays which incorporate the crystal violet biofilm staining method of visualization and quantification of biofilm biomass in a 96-well microplate reader. The use of 96-well plates has allowed more samples that can be tested at any one time and is preferable to be carried out post-MIC assessment.

Conflict of interest

The authors declare no conflict of interest.

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Methods Article

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Generation of stably expressing IRF5 spliced isoform in Jurkat cells

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Abstract: Lentiviral transduction enables the generation of gain-of-function of a targeted gene in mammalian cells. Single cell cloning through limiting dilution can establish a population of cells with homogenous transgene expression for exploring protein function. Here, we describe step by step optimized protocols for generating clonal stably expressing using crude lentiviral supernatant in Jurkat cells. Although the protocol is for general use, we will detail how to create stable cell lines based on Jurkat cells expressing IRF5 spliced isoform. These protocols will be broadly useful for researchers seeking to apply overexpression by viral transduction and generation of stable clone to study gene function in mammalian cells.

Keywords: Lentiviral; IRF5; viral transduction; transgene; Jurkat cells

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INTRODUCTION

IRF5 has a diverse role in the induction of pro-inflammatory cytokines and chemokines downstream to various signalling pathways contributing to the pathogenesis of various autoimmune and inflammatory diseases [1-3]. In humans, *Irf5* gene exists as multiple spliced variants that give rise to at least nine isoforms [2-4]. Out of these nine isoforms, four are known as the functional isoforms (v1, v3, v4, v5 and v6), the rest are either transcriptionally inactive or lack certain functional elements, resulting in mutant IRF5 [4-6]. We utilised one of the predominant functional IRF5 spliced isoforms; IRF5 variant 4 (IRF5v4) in our study. The IRF5v4 is among the first to be cloned variant, and it exhibits similar characteristics to another spliced variant, IRF5v3 in terms of identical deletion pattern in exon6, encodes identical polypeptide sequences as well as their functions [4-7]. The IRF5v4 are widely characterised in early studies in defining IRF5 roles in regulating type 1 interferons in response to viral infection [8, 9]. Besides that, IRF5v4 was found to be involved in ubiquitination, a post-transcriptional process critical for the nuclear translocation and target gene regulation of IRF5 [10].

Gene overexpression is a method to produce many targeted gene products for several applications. These include studying gene function, recombinant protein production, screening tools for drug targets, connecting genes in biological pathways, and finding genetic modifiers overexpression phenotypes [11]. The use of mammalian cells as expression systems has been widely established to study the function of gene and production of recombinant protein [12]. Delivery of exogenous genetic materials (e.g., DNA, siRNA) can be accomplished using viral or non-viral vectors that act as vehicles to carry genes of interest into host cells. Viral vectors consist of viruses such as adenoviruses, retroviruses, and lentiviruses, which deliver nucleic acids efficiently into target cells through infection [13-16].

Lentiviral vector derived from HIV has been widely used as a gene delivery tool. It provides stable and long-term gene expression in various mammalian cells, including non-dividing primary cells like neurons [17]. It also serves several benefits, such as carrying large inserts and exhibiting low immunogenicity after some modification in the vector design. Gene transfer mediated by viruses is highly efficient due to their natural ability to infect cells. However, concern over safety and toxicity issues due to the possibility of producing wild-type infectious viruses has led researchers to explore various strategies to improvise the use of viruses as a vector by generating a recombinant viral vector. Generation of viral vector particles depends on multiple plasmid proteins of which the genes are genetically separated, for instance, a) packaging plasmids that consist of *gag, pol*, *tat,* and *rev* genes that are required viral particle formation, b) transfer vector bearing the expression cassette for

transgene insert, c) envelope glycoprotein for infectivity [18-20].

To date, three-generation systems of HIV type 1 based lentiviral vector have been generated. The first generation system closely resembles the wild-type HIV genome except that the packaging systems are modified whereby the helper plasmid that encodes *gal-pol* and the envelope plasmid are driven by heterologous promoter rather than the viral LTR. The HIV glycoprotein (gp120) envelope is usually replaced with Vesicular stomatis virus glycoprotein (VSV-G) to target broader host cells [18, 21]. The second and third-generation vectors are designed so that the necessary components for virus production are split for increased biosafety of the use of lentiviral in the laboratory. In the second generation systems, the accessory genes are removed from the packaging systems, whereas in the third generation, the packaging systems are separated into two plasmids, and *tat* gene is removed; (*gal* and *pol*) and (*rev*) [18, 19, 21, 22]. Besides, the transfer vector in the third generation is modified such that a chimeric 5'LTR is fused to a heterologous promoter such as cytomegalovirus (CMV) or Rovs sarcoma virus (RSV), and the U3 3'LTR is deleted from the viral genome to create a self-inactivating vector which is replication-incompetent [15, 20-22]. Of note, the replacement of a strong viral promoter or enhancer with a hybrid heterologous constitutive promoter reduces insertional mutagenesis and immune genotoxicity because of the absence of virulence factors. The third generation lentiviral systems are considered safe, even to be used in clinical studies [22].

The lentivirus production is accomplished through trans-complementation, whereby by change, all three plasmids get contained into a single cell and together express all the viral proteins that assemble into infectious lentiviral particles [23]. The 293 cells are derived from human embryonic kidney cells, which were transformed by adenovirus type 5 fragments [24] followed by the insertion of a temperature-sensitive version of the simian virus 40 (SV-40) large T tumor antigen [25, 26]. The permissibility of transfection of 293T cells and SV40T, which aids in the extra-chromosomal amplification of HIV-1 plasmid containing SV40 origin of replication, makes it suitable for the production of HIV-1 based lentiviral vector particles [26].

Crude viral supernatant is usually sufficient for in vitro transduction [27-30]. The titer of crude viral supernatants usually ranged from 1 to $5x10^7$ infectious particles [31]. Although several studies have employed the technique of concentrating lentivirus and titer, this is particularly important for some difficult to transfect cells such as primary cells and the use in vivo experiment that requires control of transduction rate [30, 32-35]. Nonetheless, many studies have reported the use of fresh viral supernatant to transduced cells [30, 32, 33, 35]. Moreover, the use of virus supernatant is a faster and simpler approach to transduce cells. Most of the existing protocols used polybrene to enhanced transduction efficiency [32, 36-38]. This is because both cells and virus lipid confers net negative charges, and polybrene a cationic polymer function as counteracting repulsive electrostatic effect, which mediates virus adsorption to the cells, thereby increasing the transduction rate [39].

In this study, we used pLenti CMV GFP Puro (Addgene 17448), a third-generation lentiviral vector to carry the IRF5 cDNA construct into Jurkat cells to achieve stably expressing Jurkat cells. This lentiviral vector is a tat independent and self-inactivating lentiviral vector with enhanced green fluorescence protein, (GFP) under CMV promoter and confers *Puro* gene for puromycin antibiotic selection. To do so, DNA fragment encoding GFP was removed and replaced with IRF5v4. The production of recombinant lentivirus was carried out by transient co-transfection of three plasmids; pLenti expressing GFP or pLenti expressing IRF5v4 was co-transfected with packaging plasmid, psPAX2 (Addgene 12260) and envelope plasmid, pmD2G (Addgene 12259) into HEK 293T cells, mediated by Trans-IT X2 transfection reagent (Mirus Bio LLC). Production of experimental lentiviral constructs of IRF5v4 was done in parallel with lentiviral containing GFP in separate flasks. Unlike the transfer vector that carries GFP in its backbone, no reporter gene was conjugated in these experimental lentiviral constructs of IRF5v4. To ensure that the experimental conditions during this transient transfection experiment were conducive, the expression of GFP was evaluated in the cells transfected with pLenti-GFP. Prior to generating stably expressing IRF5v4 in Jurkat cells, the transduction protocol was optimized using pLenti-GFP.

This methodology article aims to present the optimized protocol for evaluating the use of crude viral supernatant for generating stably expressing IRF5v4 in Jurkat cells. Moreover, this experiment was conducted to investigate whether the virus packaging was an indeed successful and infectious virus was released into the culture medium. For this experiment, the easy-to transduce cells, HEK 293T cells, were used for transduction with lentivirus expressing GFP. Aside from checking the infectious virus produced, we also titer different volumes of the viral supernatant to test an adequate amount for transduction without causing any toxicity. Following the optimized protocol, the transduction of lentiviral IRF5v4 construct was done parallel to lentiviral encoding GFP that served as a positive control. The success of the generation of stable clones of IRF5v4 was validated through western blot analysis. The flow of the experiment is illustrated in Figure 1.

METHOD DETAILS

Construction of lentiviral vector carrying IRF5v4

Primers for human IRF5 coding sequences [8] were designed with restriction enzymes *Bam*HI and *Sal*I, incorporated at the 5' and 3' ends, respectively. Amplified fragments of IRF5v4 (1467 bp) were cloned into pCR Blunt II TOPO vector (Invitrogen) and subsequently cloned into pLenti GFP Puro (Addgene 17448), replacing the GFP fragment.

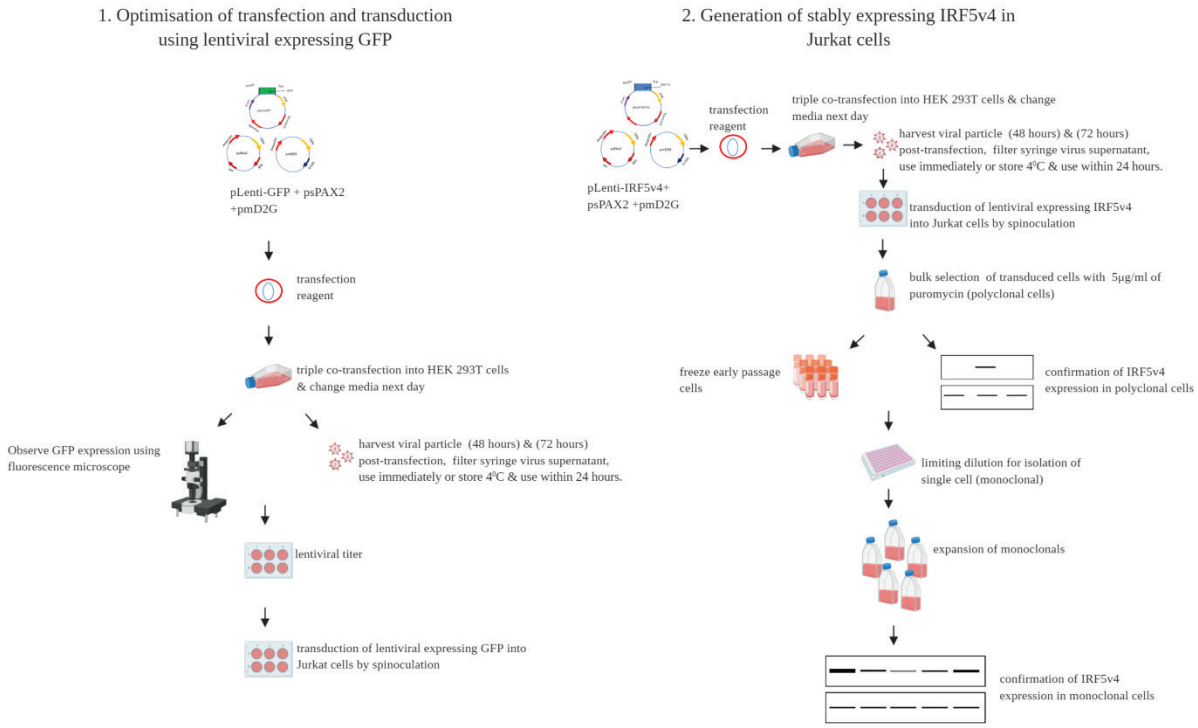

Figure 1. The schematic diagram for the flow of the experiment

Cell line and conditions

Human embryonic kidney (HEK) 293T cells (generously provided by Dr. Leong Chee Onn, International Medical University) were maintained in DMEM (Cellgro Mediatech, US) supplemented with 4.5g/L glucose, L-glutamine, sodium pyruvate, 10% heat-inactivated fetal bovine serum, FBS (Sigma Aldrich), and 1% penicillin/ streptomycin (Gibco, Thermo Fisher Scientific, US). Jurkat cells (obtained from cell culture bank of University of Nottingham Malaysia Campus) were cultured in RPMI medium (Cellgro Mediatech, US) supplemented as above, with an additional component of 25mM HEPES.

Determination of infectivity and optimal inoculum of crude supernatant for transduction using lentiviral expressing GFP

Materials

- Serological pipettes, pipettor
- Pipettes, tips
- microcentrifuge tubes and rack
- falcon tubes and holder
- DMEM (Cellgro Mediatech, US)
- OptiMEM (Gibco, Thermo Fisher Scientific, US)
- Heat inactivated FBS (Sigma Aldrich)
- 0.25 % trypsin-EDTA (Gibco, Thermo Fisher Scientific, US)
- 1xPBS without Ca^{2+} and Mg^{2+} (Cellgro Mediatech, US)
- Mirus TransIT-X2 Dynamic Delivery System
- Plasmids (pLenti-GFP, psPax2, pmD2G)
- Petri dish
- 30 ml syringes
- 0.45µm PES filter
- T-75 flask
- 6 well plate

a) Procedure for recombinant lentivirus production

1. One day before transfection, seed $5x10^6$ HEK293T cells in T-75 cell culture flask containing 15 ml of growth media, DMEM +10% FBS media (*no antibiotic) to achieve 70-80% of confluency on day of transfection.

   **Notes: Antibiotic may interfere with a transfection complex*

2. Add 1.85 ml of serum-free media OptiMEM into falcon tube followed by all three plasmid DNA as below:

Plasmids	Concentration	Ratio
Transfer vector (pLenti-GFP)	3750 ng	4
Packaging vector (psPAX2)	2812.5 ng	3
Envelope vector (pmD2G)	937.5 ng	1

3. Add 45 µl of Mirus TransIT-X2 by dropwise into the falcon tube and gently mix with the diluted plasmids and incubate for 30 minutes undisturbed for complex formation.

4. Aspirate the mixture gently* and add dropwise into the flask using 1000 µl pipette. Rock flask front-back and sideways to distribute the complex evenly over the cells. Incubate the cells at 37°C, 5% CO_2 incubator for 16 hours.

   **Note: Do not pipette up and down this time as it will dislodge the formed complex between the plasmids and transfection reagent.*

5. After 16 hours of post-transfection, remove media and replace* with fresh 15 ml of complete media. Return the flask into the incubator for 24 hour incubation.

   **Note: It is essential to do all media changes with extreme gentleness, as the cells have been sensitized and can easily detach from the plate. If needed, use two serological pipettes, to add media (first 10 ml and then 5 ml, to prevent cells detaching, insert the serological pipette fully inside the flask and add media to edge of flask, dropwise).*

6. At day two post-transfection (48 hours after transfection). Observe cells for GFP expression using a fluorescence microscope and capture images to evaluate transfection efficiency.
7. Harvest the supernatant containing viral particles by gently aspirating the medium from the flask with a serological pipette into a 50 ml centrifuge tube. Replace 15 ml of complete media into the flask and put back in the incubator.
8. Get rid of debris* from the supernatant containing viral particles by filter syringe. This can be done by gentle aspiration of supernatant from the petri dish with a syringe, invert syringe several times to mix the solution well. Pass supernatant through 0.45 μM filter and drain the filtered supernatant into a falcon tube.

   **Notes: Ideally, there should be no debris of cells in the virus supernatant. If there are some small cells, gentle centrifugation of 1500 rpm for 5 minutes at 4^0C before filtering the supernatant can be done to prevent the filter from getting blocked by the cells' debris.*

9. On day three post-transfection (72 hours after transfection). Repeat step 6-7.
10. Pool the 48 and 72 hours of viral supernatant collection. Use immediately or store in 4^0C for not more than 24 hours and proceed with transduction. Avoid freeze-thaw as it can cause a lower transduction rate.

    **Notes: The ratio of transfer plasmids to packaging plasmid and envelope plasmid used for transfection is 4:3:1 was adapted from previous studies[29,33]. The ratio of plasmids DNA to transfection reagent used is 1:2:4 and the seeding number of cells was determined in preliminary expreiment done in out lab. The steps shown here is for T-75 flask, to scale down to 6 wells, divide by 2.5. Number of cells for 6 wells to use: 0.5x10^6 cells/well.*

b) Procedure of lentiviral titer using crude viral supernatant

1. Prepare complete media containing polybrene to a final concentration 8 μg/ml.
2. Seed 2x10^5 HEK 293T cells into 6 wells of the 6 well culture plate containing 2 ml of growth media and polybrene.
3. Bring virus supernatant to room temperature* and mix gently. Add 0.6ml, 0.8ml, 1ml, 1.5ml, and 2ml of virus supernatant into seeded HEK 293T cells (Figure 2).

   **Notes: Thawing of virus supernatant from 4^0C can be done by keeping it in the biosafety cabinet to reach room temperature. Be careful not to leave the tube containing virus supernatant in the water bath too long as high heat can reduce viral infectivity resulting in poor transduction.*

4. Incubate the cells at 37°C, 5% CO_2 incubator for 24 hours.
5. After 24 hours of incubation, transfer change media with complete fresh media. Incubate cells at 37°C, 5% CO_2 incubator for another 48 hours.
6. At day three post-transduction (72 hours after transduction). Observe cells for GFP expression using a fluorescence microscope and capture images to evaluate transduction efficiency.

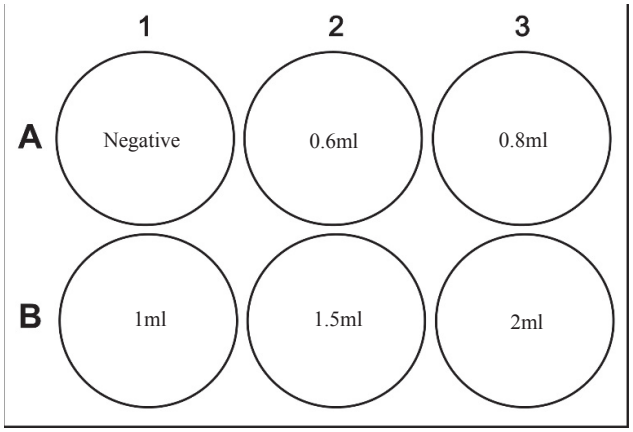

Figure 2. A layout of 6 well- plate for optimization of transduction. A1: Control 1: non-transduced, A2: 0.6ml viral supernatant, A3: 0.8ml viral supernatant, B1:1ml viral supernatant, B2: 1.5ml viral supernatant, B3: 2ml of viral supernatant.

Generation of stable clones expressing IRF5v4

Materials

- Serological pipettes, pipettor

- Pipettes, tips
- Multichannel pipettor
- 1.5 ml microcentrifuge tubes and rack
- 15 ml falcon tubes and holder
- RPMI medium (Cellgro Mediatech, US)
- FBS (Sigma Aldrich)
- Penicillin/ streptomycin (Gibco, Thermo Fisher Scientific, US)
- Hexadimethrine bromide is known as polybrene (Sigma-Aldrich).
- 6 well plate
- T-25 flask

Procedure for transduction in Jurkat cells

1. Prepare recombinant lentiviral particles harboring IRF5v4 coding sequence following steps 1a in parallel with lentiviral particle harboring GFP to monitor experimental conditions.
2. Prepare two sets of 6 well plates. Seed $2x10^5$ Jurkat cells into 6 wells of 6 well plate cell culture flask containing 2ml of growth media and polybrene. In one 6 well plate label (IRF5v4) and another one as (GFP).
3. Bring virus supernatant to room temperature and mixed gently. Transfer 2 ml of virus supernatant into each well. One plate containing lentiviral particles harboring GFP and another plate for lentiviral harboring IRF5v4.
4. Seal the plate with paraffin film and spin the cells* at 1,200 x g for 90 minutes at 25^0C without a break. Incubate the cells at 37°C, 5% CO_2 incubator for 24 hours.
   **Notes*: *Spinoculation can be performed in a falcon tube if a plate adapter for centrifugation is not available. If the falcon tube is used, resuspend the mixture gently and transfer it into the 6 well plates.*
5. The next day, change the media by centrifugation at 1000 x g for 10 minutes at 25^0C and replace it with fresh 2ml of complete media into each well. Incubate the cells at 37°C, 5% CO_2 incubator for another 48 hours.
6. At day 3 post-transduction (72 hours after transduction), observe cells for GFP expression using a fluorescence microscope, and capture images to evaluate transduction efficiency.

Procedure for selection of transduced cells and isolation for monoclonal

1. Collect the transduced cells from each well and pool them, and split into four T-25 flasks, with each flask containing 3ml of cells suspension.
2. Add 6ml (1:3 ratio) of complete media containing 5 µg/ml puromycin into each well and culture the transduced cells * for two weeks to generate polyclonal cells.

   **Notes: Selection of transduced Jurkat cells in puromycin containing medium, typically takes two weeks to achieve the population of cells that contain only integrated transgene as any unintegrated cells would have died. These bulk cells that survived the antibiotic selection are referred to as polyclonal cells.*
3. After two weeks, split the cells and freeze down some polyclonal cells to have early passage cells that serve as storage and back up. Use the remaining cells to check for the presence of the transgene.
4. Using the early passage cells, perform limiting dilution to isolate a single cell (monoclonal) to generate a homogenous population of cells.
5. To do so, dilute $1 x10^4$ transduced cells in 20 ml media.
6. Transfer 5 µl of cells into a tube containing 5ml of complete media supplemented with 5 µg/ml puromycin and 5 ml of self-conditioned media*.

   **Notes: The self-conditioned media is isolated from healthy growing Jurkat cells (non-transduced cells). To spin down the cells and collect the supernatant into a sterile tube and filter using 0.22 µm polyvinylidene fluoride (PVDF) filter.*
7. Transfer 100 µl of the diluted cells suspension into each well using a multichannel pipettor.
8. Incubate cells undisturbed at 37^0C in 5% CO_2 for 2 hours.
9. Inspect wells and label wells containing only a single cell.
10. Incubate plate undisturbed at 37^0C in 5% CO_2 for three weeks with regular inspection for confluency.
11. Once cells reach about 30-50% confluency, transfer the cells to 24 wells and expand to 6 wells and later to T-25 flask.
12. Check the expression level* and freeze down early passage cells to be used in subsequent experiments.
    **Notes: Routinely check expression level by reverse transcription PCR (RT-PCR) to ensure the transgene is not lost over the time of culture, a common gene silencing phenomenon associated with transgene under CMV promoter. If transgene exhibit toxic to cells resulting in extended lag phase cell growth, an alternate viral vector under inducible promoter can be used.*

METHOD VALIDATION

Confirmation of successful transient transfection for recombinant lentivirus

Since GFP fragments were removed in order to construct experimental transfer vectors pLenti-IRF5v4; by inserting IRF5v4 DNA fragment in replacement of GFP DNA fragment, there was no reporter marker to track these constructed experimental vectors during transient transfection for recombinant lentivirus production. Therefore, the production of recombinant lentivirus harboring IRF5v4 was conducted in parallel with pLenti-GFP in separate T-75 flasks to ensure the setting for successful transfection for virus production. As seen in Figure 3(a), co-transfection of pLenti-GFP along with the two helper plasmids (psPAX2 and pmD2G) in HEK 293T cells exhibited about 60-70% of green fluorescence at day 2 of post-transfection, similar to the results obtained in the preliminary experiment conducted as described above. Also, the morphology of the transfected cells was examined under the bright-field view. The photo bright-field of transfected cells shows the presence of syncytia represented by the multi-nucleated cells (in black arrow) that were

caused by the translation of glycoprotein of VSV-G (envelope protein), indicating successful transfection and sign for lentivirus production [35, 40]. The third day of post-transfection, fluorescent microscopy showed GFP was expressed about 80-90%, as seen in Figure 3 (b). Based on these results, it was deduced that the production of lentivirus was successful and ready to be used for the transduction experiment.

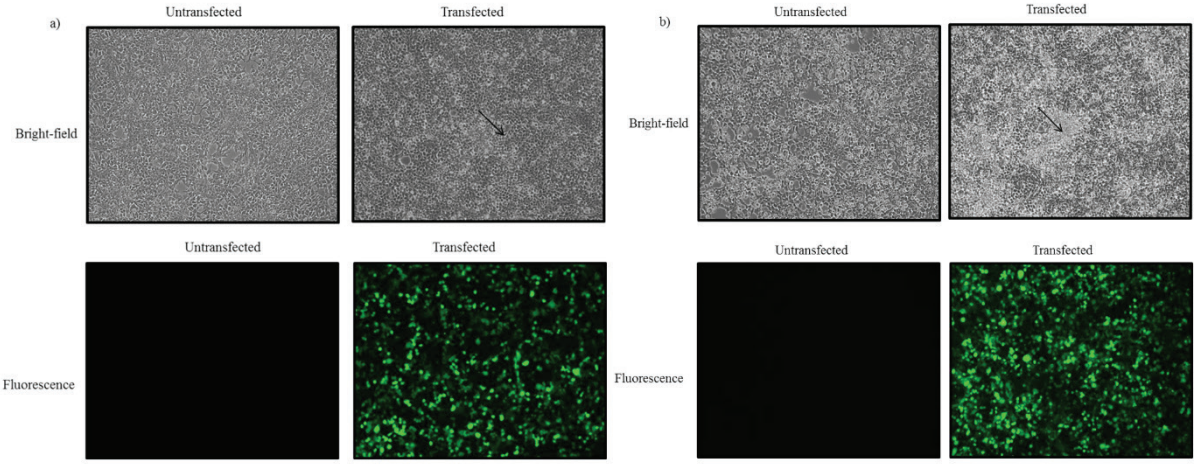

Figure 3. Detection of GFP expressions from recombinant lentivirus expressing GFP from post-transfection days.(a) On day 2, post-transfection. (b) On day 3, post-transfection. Photos were taken under Nikon Inverted Microscope Eclipse TS2R under bright field or fluorescent light (x200 magnification). The exposure time of fluorescent images were 600 milliseconds. The black arrowhead shows the presence of syncytia in transfected cells.

Determination of the optimal amount of viral supernatant for transduction

The presence of GFP expression visualized by a fluorescence microscope after transfection reveals the efficiency of transfection, but it does not display the lentivirus's infectivity. It was necessary to ensure the recombinant lentivirus harvested was infectious for proper gene delivery to target cells [41]. It was also essential to make sure that there was no cell toxicity from a viral infection that could cause massive cell death, resulting in a lower transduction rate. Based on the results, the minimal volume of lentiviral supernatant inoculum that showed some GFP expression was 0.6ml (Figure 4 (b)), and the highest volume, 2 ml tested showed greater transduction efficiency of more than 90% GFP expression seen (Figure 4(f)). Moreover, about 95% of viable cells were observed by evaluating trypan blue, indicating no cell toxicity from a viral infection with the amount of virus supernatant used for transduction. This data provided the evidence that the recombinant lentivirus produced was infectious and good transduction efficiency was achieved with crude viral supernatant.

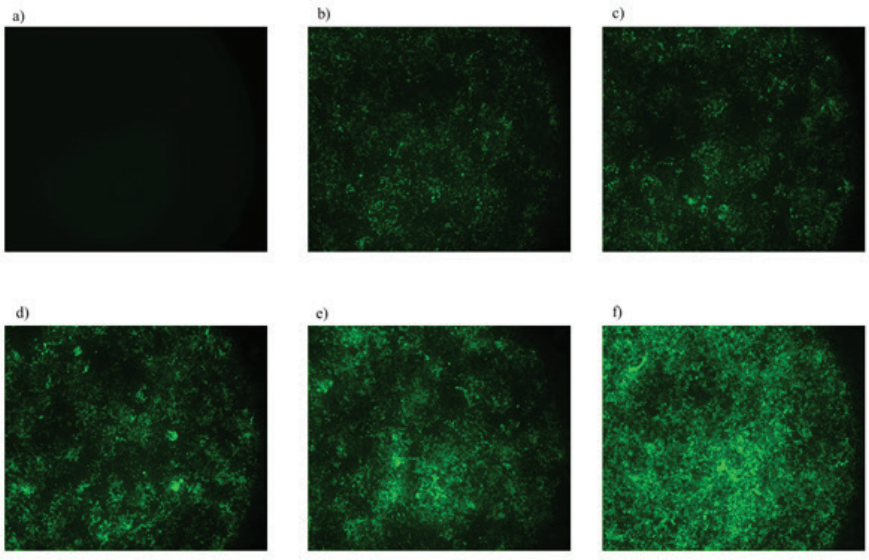

Figure 4. Titers of lentivirus expressing GFP on HEK 293T cells. (a) non-transfected cells, (b) 0.6 ml of lentivirus, (c) 0.8ml of lentivirus, (d) 1ml of lentivirus, (e) 1.5ml and (f) 2ml of lentivirus. Photos were taken under Nikon Upright Microscope Eclipse Ei under fluorescent light (x100 magnification).

Confirmation of successful transduction in Jurkat cells

Transduction is more permissible in the adherent cell line. Thus, spinoculation is not required transduction in HEK 293T cells. But this is not the same for suspension cells like Jurkat cells. Numerous researchers have conducted transduction of Jurkat cells by spinoculation and the presence of polybrene, which have shown to enhance the transduction of lentiviral vector by virus binding to the cells [32, 36-38]. The experiment was conducted in parallel with the control transfer vector, pLenti-GFP in a separate culture flask to track the successful transduction experiment. After 72 hours of transduction, GFP expression was accessed by a fluorescence microscope. As seen in Figure 5, the bright field photo of transduced cells revealed that infection with lentivirus did not alter Jurkat cells' morphology, implying no cell toxicity from the lentiviral transduction. Moreover, Jurkat cells' transduction with virus supernatant obtained from the transfection of pLenti vector expressing GFP displayed about 70-80% of green fluorescence. As expected, no GFP expression was observed in non-transduced cells.

Meanwhile, the transduced cells showed an excellent transduction efficiency of more than 70% of GFP expression accessed by a fluorescence microscope. Furthermore, no cell toxicity from viral infection was noticed as the evaluation of trypan blue observed about 95% of viable cells. From these data, it was deduced that the infectious lentiviral constructs efficiently transduced Jurkat cells.

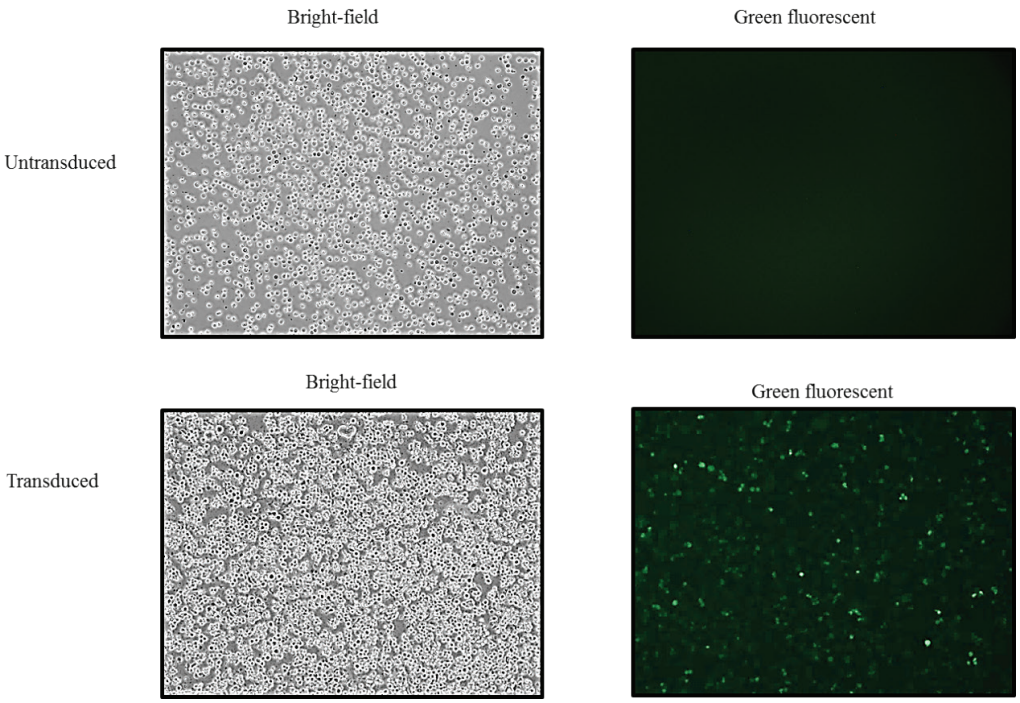

Figure 5. Detection of GFP expressions from transduced Jurkat cells with lentivirus harbouring GFP at day 3 post-transduction. Photos were taken under Nikon Inverted Microscope Eclipse TS2R under bright field or fluorescent light (x200 magnification).

Confirmation of IRF5v4 overexpression in polyclonal Jurkat cells

Puromycin is an aminonucleoside antibiotic produced by *Streptomyces alboniger*. It inhibits peptidyl transfer on both prokaryotic and eukaryotic ribosomes, which cause premature chain termination during translation [42, 43]. The pLenti-GFP and the engineered pLenti-IRF-v4 encode puromycin-N-acetyl transferase gene (Puro) in their plasmid backbone constitutively express it. Therefore, during the antibiotic selection, those cells with stably integrated transgene and constitutively expressed the resistant gene, Puro, will survive when grown in a medium containing puromycin. Those non-transduced cells and cells that uptake plasmid transiently will not survive the antibiotic selection. The presence of IRF5v4 in the polyclonal cells generated from the experimental lentiviral constructs was checked western blot analysis. As seen in Figure 6, no IRF5 was detected in non-transduced Jurkat cells. Similarly, no IRF5 was detected in Jurkat cells transduced with pLenti-GFP, which indicates that infection with lentiviral without IRF5 spliced variant in the backbone did not lead to an upregulation of endogenous IRF5. Therefore, detecting IRF5 in stably expressing cells indicates the *de novo* synthesis of integrated IRF5v4 in Jurkat cells. In line with this, approximately 60 kDa bands corresponding to the IRF5v4 protein were detected from polyclonal cells, as shown in Figure 6. From these results, it was deduced that experimental lentiviral constructs had successfully integrated the transgene of IRF5v4 in Jurkat cells.

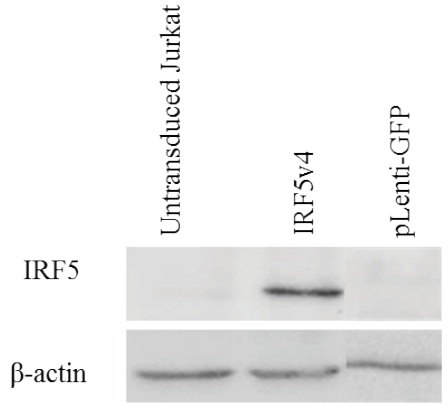

Figure 6. Confirmation of overexpression of IRF5v4 in polyclonal cells. Western blot analysis for detection of IRF5v4 (approximately 60kDa). Housekeeping protein actin act as the loading control (43kDa) was included as part of the analysis. The chemiluminescence signal was captured by Gel Doc imager.

Confirmation of IRF5v4 in monoclonal cells

Polyclonal cells constitute of heterogeneous transgene expression level, which means the population of cells containing both high and low expression levels of the transgene [35]. Over time, the transgene population of polyclonal cells may drop because cells with high transgene levels may have a slower growth rate. Therefore the rapidly growing low-level transgene expression may take over the culture. To avoid the drift effect towards low transgene expression and obtain a homogenously higher level of expression of the IRF5v4, the early passage of polyclonal cells was used to isolate single cells (monoclonal) limiting dilution method. Limiting dilution is a traditional method for obtaining clonally identical cells (monoclonal) of stable expressing transgene derived from single isolated cells cultured in multi-well plates [44]. Several monoclonal cells were isolated and expanded in culture with a complete medium containing 5 µg/ml of puromycin. The clones were screened for the presence of overexpression of IRF5v4 using western blot analysis. From the result seen in Figure 7, two clones expressing IRF5v4 (referred to as B8, F9) were found to have stable high IRF5 expression. While two clones, D7 and E6, showed a substantial level of IRF5v4. Another clone E4 had a low expression of IRF5v4.

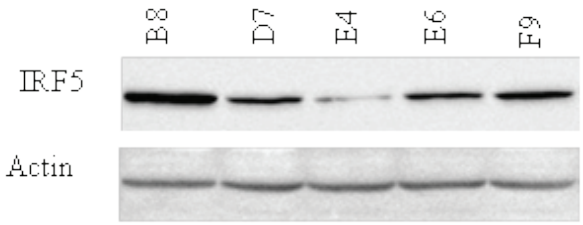

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Figure 7. Confirmation of overexpression of IRF5v4 in monoclonal cells. West ern blot analysis for detection of IRF5v4 (approximately 60kDa). Housekeeping protein actin act as the loading control (43kDa) was included as part of the analysis. The chemiluminescence signal was captured by Gel Doc imager.

CONCLUSION

In conclusion, the present study shows the optimization protocol for generating IRF5v4 stable clones using recombinant lentiviral transduction. Stably overexpressing IRF5v4 was successful achieved through lentiviral transduction. Five monoclonal of IRF5v4 expressing Jurkat cells were obtained. The GFP expression from the control lentiviral vector helped evaluate whether the experiments carried out following the protocol designed were ideal and successful.

Authors Contributions

AK and C-MF performed the literature review and manuscript writing. C-MF conceptualizes the research project.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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