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Environmental Metagenomic Analysis of "ESKAPE" Pathogens in the Pediatric Intensive Care Unit of General Hospital Yogyakarta Indonesia

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Abstract: Healthcare-associated infections (HAIs) are infections that occur while receiving health care, develop in a hospital or other healthcare facility, and first appear 48 hours or more after hospital admission, or within 30 days after having received health care. HAIs are linked to high mortality rates, prolonged stays, increased hospital overhead costs, and financial burdens on patients. Bacterial transmission from medical personnel or the environment, or patient-to-patient contact are all potential causes of these infections. A molecular epidemiology approach is needed to examine the contribution of risk factors and the distribution of "ESKAPE" pathogens within the hospital environment. In this study, we conducted a comprehensive analysis of the distribution of ESKAPE bacterial pathogens in the environment of pediatric intensive care units over a 30-day time interval using shotgun metagenomics. We collected samples from handwashing sinks, the floor around patients, and ventilator screens and tubes in the pediatric intensive care unit (PICU) of General Hospital, Yogyakarta, Indonesia in March 2022. We determine taxonomic profiles and also detect resistome, and virulome distribution of ESKAPE pathogens on various environmental surfaces through shotgun metagenomic sequencing. The microbiomes of the floor, sink, and mechanical ventilator exhibit a diverse composition of microbial communities, featuring significant species richness based on Shannon and Simpson's index. These microbiomes encompass a wide array of microbial species, including ESKAPE bacterial pathogens, as well as profiles related to resistome and virulome. ESKAPE pathogens, especially *Acinetobacter baumannii*, predominated in the PICU environment. Most virulome have been associated

with metabolism/nutrition and adhesion. Noteworthy findings include resistome genes characterized by mechanisms like efflux pumps (MDR) and alterations in antibiotic targets.

Keywords: ESKAPE pathogensl; Hospital environments; Resistome; Virulome; Pediatric Intensive Care Unit (PICU); SDG 3 Good health and well-being

1. Introduction

Numerous cases of healthcare-associated infections (HAIs) have been documented after surgery and the implantation of invasive medical devices in Indonesia. Phlebitis was 2.6% common, surgical site infection (SSI) was 1.8%, Urinary Tract Infections (UTIs) were 0.9%, and septicemia was 0.9% in the first study conducted in Indonesia [1]. Especially for critically ill individuals, HAIs might have serious consequences. The severity of this problem is highest in the Pediatric Intensive Care Unit (PICU). Because of their weakened immune systems and the severity of their illnesses, PICU patients are more likely to get HAIs. The use of intrusive equipment and several monitors increases the danger associated with the use of life-supporting devices [2]. HAIs have been associated with multidrug-resistant bacteria and the formation of biofilm, increasing the risk of infection in critical care units. These bacteria, whether they are pathogens or present on contaminated surfaces, elevate the risk of HAIS in critical care units, especially in areas with previous infections [3].

Pathogens can survive in healthcare environments for extended periods, depending on conditions like temperature and humidity. *Clostridioides difficile* spores, Vancomycin-resistant *Enterococcus* (VRE), Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Acinetobacter baumannii* have all been recovered after 4-5 months in healthcare environments, with endospore-forming bacteria typically lasting longer than vegetative bacteria [4]. The Infectious Disease Society of America reports that ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Escherichia coli*) are commonly found in the hospital environment [5]. Cross-transmission of these pathogens can happen when healthcare workers touch contaminated surfaces in the surroundings or direct contact with patients [6]. A previous study conducted at the PICU in the same setting found that the proportion of HAI-related bloodstream infections among patients in the PICU was predominantly caused by Gram-negative multidrug-resistant (MDR) bacteria, specifically *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [7].

Hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) have different molecular and clinical epidemiologies. HA-MRSA is linked to

serious, invasive diseases, while CA-MRSA may replace HA-MRSA due to cross-contamination between hospitals and communities [8].

Virulence factors play a significant role in interconnecting ecosystems and facilitating the movement of antimicrobial resistance (AMR) genes between niches. They achieve this by promoting the formation of biofilms, which, in turn, make it easier for bacteria to infect other cells [9]. Biofilm is a bacterial cell population enclosed in a self-contained structure, exhibiting distinctive growth rates, gene expression, and metabolism, which enhances their resistance to antibacterial agents [10].

Staphylococcus aureus, responsible for HAIs linked to medical implants, produces biofilms that evade antibiotics, contributing to 60-80% of hospital infections [11]. A study found that biofilm formation in *Acinetobacter baumannii* significantly contributes to device-related infections, with 89.47% of 16 isolates producing biofilms, and a strong correlation with antibiotic resistance [12].

Shotgun metagenomics sequencing (SGS) provides a valuable database of hospital environment microbiomes, aiding in understanding the relationship between HAIs, the environment, and risk factors, and aiding in the analysis of antimicrobial resistance and virulence genes. For example, SGS of metagenomic DNA can be employed to investigate the presence of clonal types within a species on hospital surfaces by exploring the same sites at different time points.

A molecular epidemiology approach to real-time surveillance of inpatient rooms and environments helps prevent HAIs by analyzing bacterial profiles using SGS [13]. Hence, our objective was to assess the temporal and spatial variations in the taxonomic profiles, also detect resistome, and virulome distribution of environmental isolates obtained from the floor, sink, and surfaces of the mechanical ventilator in the PICU at General Hospital in Yogyakarta, Indonesia.

2. Materials and Methods

2.1. Sample collection and processing

The study was conducted at the General Hospital Yogyakarta Indonesia in the pediatric intensive care unit (PICU). The General Hospital Yogyakarta, Indonesia, served 3 million people in Yogyakarta and the southern part of Central Java, Indonesia. In the PICU unit, there were 12 beds with 10 ventilator machines and 3 sinks. Subsequently, we swabbed the entire surface of the ventilator screen and the ventilator tubing for ventilator samples. We

selected three ventilators for sampling without disrupting their function or use for patients and without touching the patients or the surrounding environment. Three sink locations in the PICU area include (1) the sink near the entrance, (2) the sink next to the toilet, and (3) the sink in the PICU bathroom. We uniformly swabbed all surfaces, including the inside and outside of the sinks and sink taps. We took three floor samples from procedural rooms and two from around patient beds.

We placed all samples in a sterile tube containing microbial DNA purification kits and labeled them after collection. DNA samples were extracted using microbial DNA purification kits, *ZymoBIOMICS DNA Kits*® according to the protocol. These kits were designed to purify DNA from different sample inputs and ensure that the obtained DNA was immediately suitable for microbiome or metagenome analyses. Importantly, the ZymoBIOMICS DNA Kits did not contain growth media that could facilitate the growth of diverse microorganisms. Consequently, the microbiome composition remained unchanged, accurately reflecting the environmental surfaces.

The sampling took place in stages, starting with the collection of environmental floor, sink, and surface samples of ventilator machines of 2 m^2 at three different sites as replicates, then pooled as one sample representing each location. A cotton swab was employed to wipe the entire indicated square's surface. It was subsequently put into a transport medium tube with its labels "FLOOR-01," "SINK-01," and "VENT-01" on March 1 as Day 0 and "FLOOR-02," "SINK-02," and "VENT-02" on March 31, 2022 as Day 30. DNA was extracted using the *Zymobiomics-D4300* (Zymo Research®) following the provided protocol. RNA and protein contamination were checked in the extracted DNA samples using the NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific®). Gel electrophoresis was performed on 2 uL of DNA on a 1% agarose gel stained with ultrapure ethidium bromide (ThermoFisher Scientific®). Finally, a Major Science® SmartView Pro 1100 Imager System documented the gel. According to the manufacturer's instructions, a Qubit 3.0 fluorometer evaluated sample DNA concentrations using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific®) [14].

2.2. DNA library preparation and sequencing

For 1 µL of metagenomics DNA samples, genomic DNA screentape was used for the tape station. At least 50 ng/ul of DNA concentration in the samples had been used, of which 1 ul had been loaded into a tape station. DNA 5000 ScreenTape in an Agilent 4150 Tape Station tested library quality. The library qualification criteria were a broad peak in the range

of 200 bp to 48,500 bp, an average size of 350 bp on the Agilent 4150 TapeStation system, Qubit concentrations over 2 ng/mL or 10 nmol/L, and without primers, adapters, or larger peaks. The pooled libraries were shotgun metagenome sequenced using Illumina PE150 (HiSeq). The sequence readings were barcode demultiplexed by bcl2fastq v2.1.9. Checking sequence data quality using FastQC v0.11.9 [15]. Approximately 51, 44, 56, 52, 50, and 60 reads (in millions) were sequenced for "FLOOR-01," "SINK-01," "VENT-01," "FLOOR-02," "SINK-02," and "VENT-02," respectively.

2.3. Taxonomic classification, Ressistome and Virulome

Quality filtering on the sequences was performed using the Taxonomic Classification Service BV-BRC Ver.3.30.19a for taxonomic classification needs. All bioinformatics analyses were performed using the BV_BRC web service (www.bv-brc.org) [16]. Kraken2 was used to taxonomically classify the clean reads, with the NCBI nonredundant nucleotide database serving as a reference [17]. The classification of the readings by Kraken2, as shown in Pavian v1.0 [18]. The BV-BRC's Metagenomic Read Mapping Service uses k-mers aligned (KMA) to align reads against antibiotic resistance genes or virulence factors [16]. KMA is an alignment method that allows for direct alignment of raw reads against entire databases, without the need for similarity reduction [19]. This service accepts reads or a feature group, which is a selection of genes present in the resource that the user has chosen. It aligns the k-mers to nucleotide sequences from the Comprehensive Antibiotic Resistance Database (CARD) for antimicrobial resistance genes or the Virulence Factor Database (VFDB) for virulence factors [16,20,21]. Meanwhile, the analysis of virulence factor genes utilized a reference database from VFDB (Virulence Factors Database) at http://www.mgc.ac.cn/VFs [21]. Our study utilizes Flourish (https://flourish.studio) for all data visualization.

2.4. Ethical Clearance

The Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital approved this research (KE-FK-0311-EC-2022).

3. Results

The microbiomes of the floor, sink, and surface of the ventilator screen are evident from their significant species richness as measured by the Shannon and Simpson index. In all the samples, the Shannon and Simpson diversity index showed similar microbiome diversity (Figure 1). These microbiomes encompassed various microbial types, including ESKAPE bacterial pathogens, along with profiles associated with the resistome and

virulome. We used shotgun metagenomics to determine differences in bacterial community composition, diversity, and abundance of the PICU's floor, sink, and mechanical ventilator surfaces. The difference was insignificant for Shannon diversity but significant for species richness. Correspondingly, using the number of differently assigned features from the metagenomic dataset as a hallmark for microbial richness resulted in significant differences as well (FLOOR_01: 51,956,244; SINK_01: 44,193,856; VENT_01: 56,336,785; FLOOR_02: 52,673,491; SINK_02: 50,911,611; VENT_02: 60,721,212).

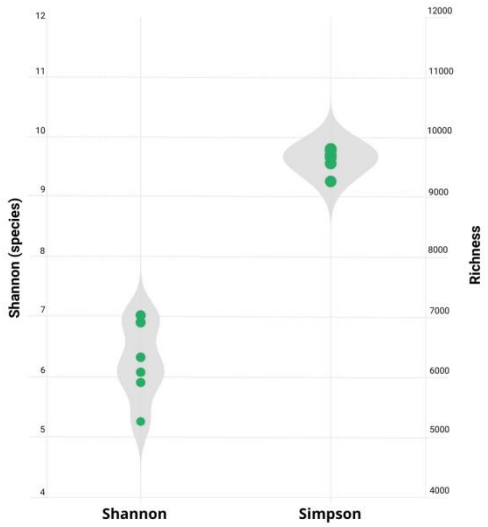

Figure 1. Microbiome species diversity (Shannon Index), and bacterial richness (Simpson Index) in all samples.

The floor microbiome is highly complex, with 69 phyla, 123 classes, 242 orders, 522 families, 1,840 genera, and 6,629 species identified on Day 0. However, in the second sample after 30 days, we discovered 60 phyla, 110 classes, 219 orders, 468 families, 1,586 genera, and 5,704 species. Nevertheless, *Proteobacteria* and *Firmicutes* were the most abundant phyla in the floor microbiome samples. The sink microbiome consisted of 71 phyla, 131 classes, 249 orders, 531 families, 2,011 genera, and 7,142 species found in the initial sample. After 30 days, our sample identified 52 phyla, 102 classes, 207 orders, 420 families, 1,423 genera, and 5,012 species. *Proteobacteria* is the most commonly found phylum in all sink samples.

The surface of the ventilator machine microbiome revealed 69 phyla, 124 classes, 236 orders, 506 families, 1,880 genera, and 6,954 species identified on Day 0. At different times after 30 days, we found 45 phyla, 91 classes, 186 orders, 386 families, 1,172 genera, and 3,854 species. *Proteobacteria* was found to be the dominant phylum in all ventilator machine surface samples.

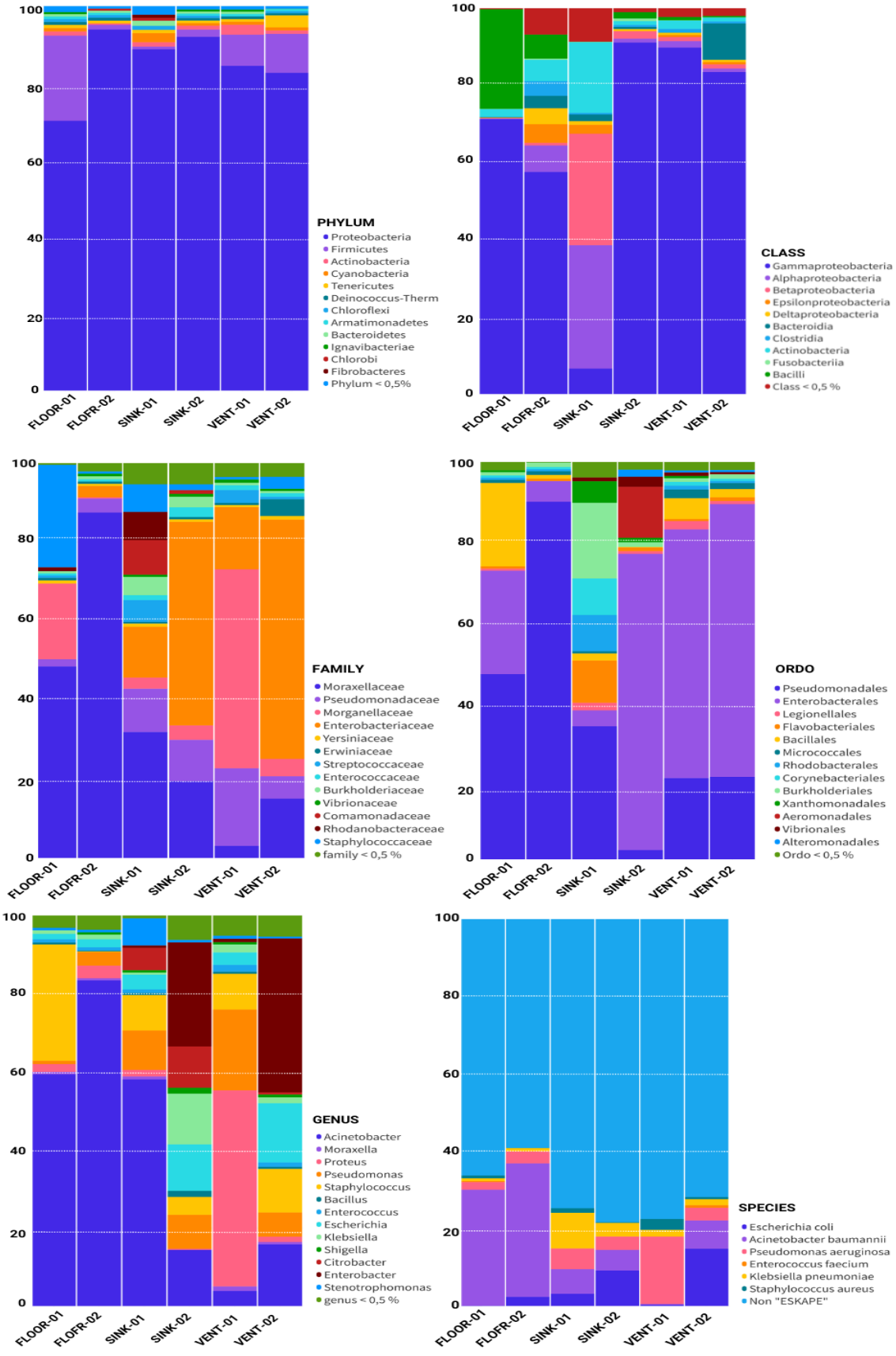

Figure 2. The bacterial composition in terms of relative abundance at various taxonomic levels in all the samples.

Environmental microbiomes like *Pseudomonas* were the predominant genera in Sink 1, Sink 2, and Ventilator 1, whereas *Acinetobacter* was dominant in Floor 1, Floor 2, and Sink 1. *Proteus* was dominant in Ventilator 1, *Enterobacter* was dominant in Sink 2, and *Staphylococcus* was also dominant in all Sink samples (Figure 2). Overall, in all hospital environment samples, *Proteobacteria* was the most abundant phylum. Approximately 80% of the bacterial community that was found in the PICU environment was constituted of *Proteobacteria*. In all the other samples, *Bacteroidetes* were comparatively low (1% to 10%).

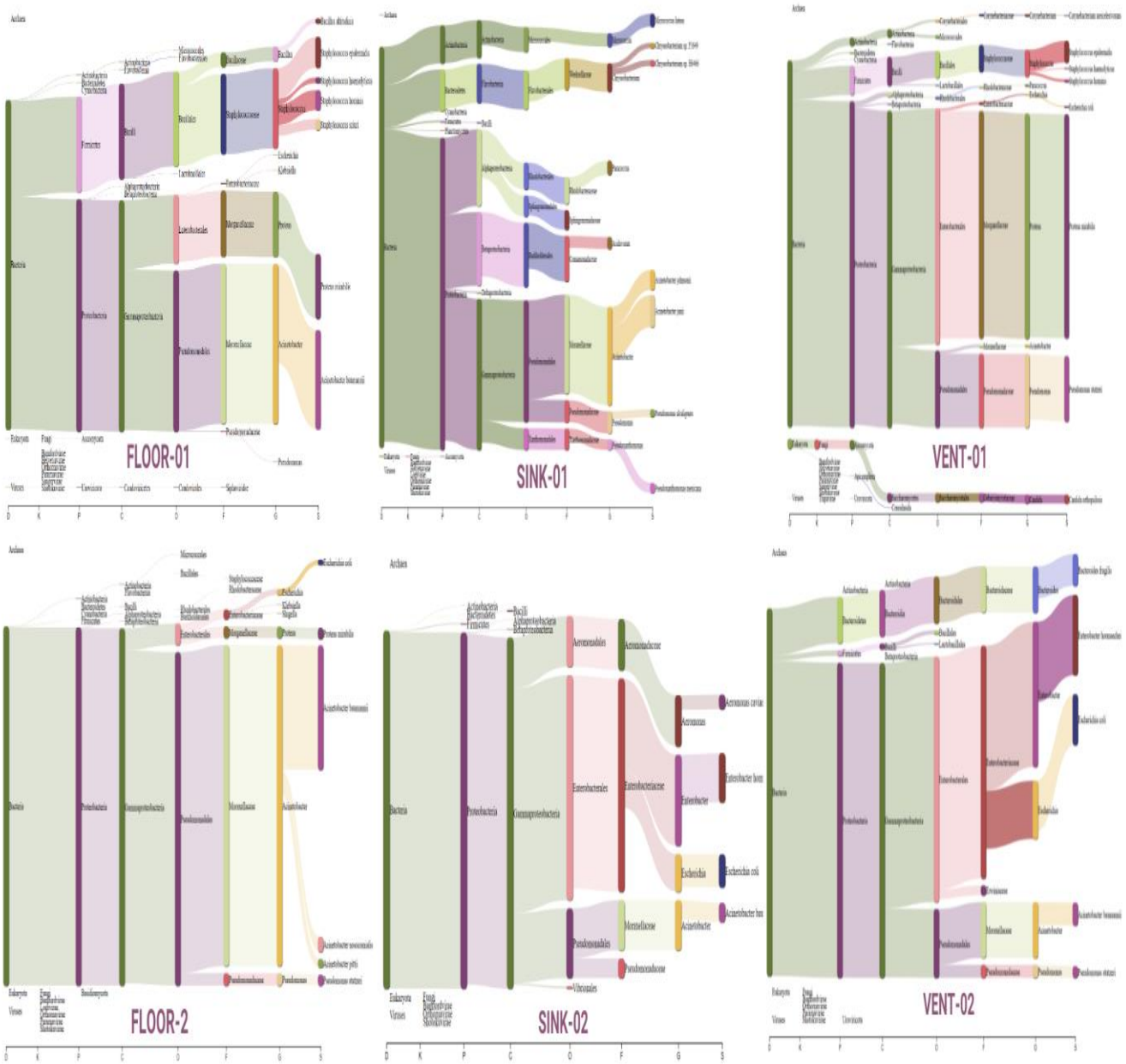

Figure 3. Distribution of ESKAPE pathogens in the bacterial microbiome of PICU environmental samples using Sankey plots.

In total, we identified ESKAPE pathogen determinants using Kraken2, visualized in Pavian v1.0 [18] (Figure 3). Sink 2 and Ventilator 1 had lower abundances of ESKAPE pathogens, compared to all floor samples. We detected the lowest abundance of the ESKAPE pathogens in the sink after 30 days and the ventilator in the first sampling.

Our study reveals that ESKAPE pathogens distribute their virulome on various surfaces such as the floor, sink, and mechanical ventilators. The identified genes predominantly relate to metabolic and nutritional factors, as well as adhesion (Figure 4). This study discloses that ESKAPE pathogens are of concern because of their capacity to acquire, express, and transmit antibiotic resistance, particularly in healthcare environments, including the floor, sink, and mechanical ventilators. The identified genes exhibit a distribution that is not significantly different between Multi-Drug Resistant (MDR) and non-MDR strains. Notably, antibiotic resistance genes are primarily characterized by mechanisms such as efflux pumps and alterations in antibiotic targets (Figure 5).

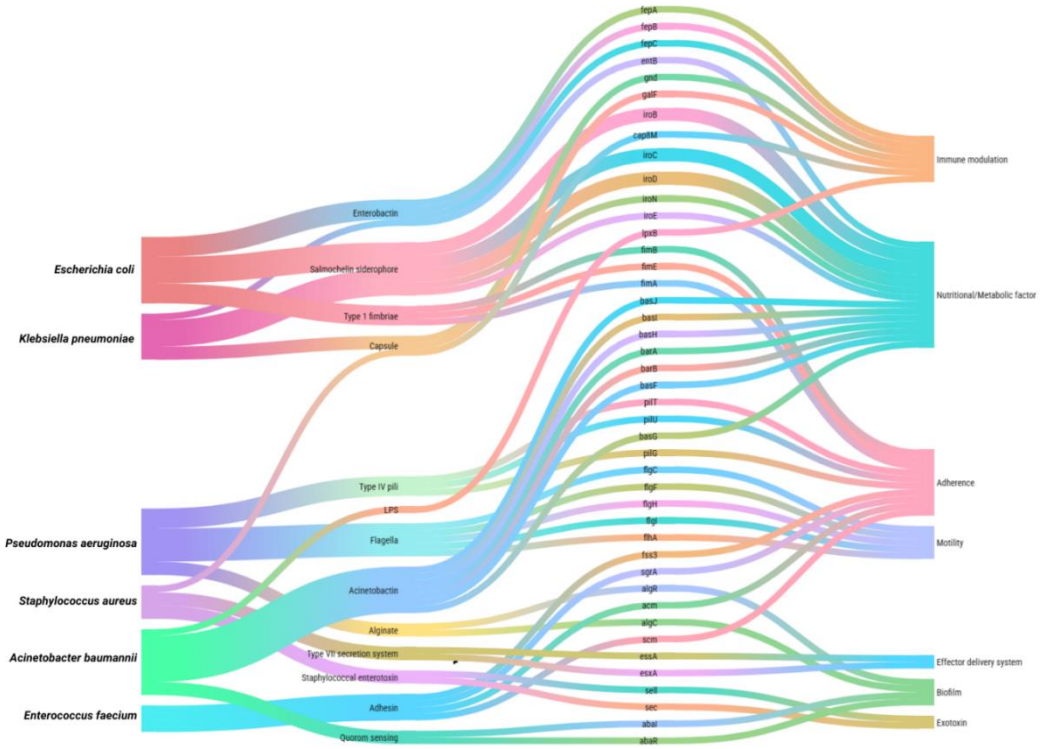

Figure 4. Sankey diagram illustrating the most abundant virolomes distribution of clinically concerning ESKAPE pathogens on the floor, sink, and surface ventilator

A bacterium must produce virulence factors that promote bacterial colonization of the host as well as virulence factors that impair or harm the host to cause infectious disease. Investigation of resistome (ARG) in ESKAPE pathogens within hospital settings is essential, given its potential impact on HAIs and the pathogens' ability to resist antibiotics, thereby increasing morbidity and mortality. The existence of the Multi-Drug Resistant (MDR) resistome in the environment, while not dominant, demands special attention, particularly in averting the transmission of ESKAPE bacteria from the environment to patients.

Implementing hygiene measures based on this understanding is crucial for infection prevention, significantly reducing the risk of pathogen transmission. This is especially vital in hospitals and other healthcare facilities, where stringent infection control measures are mandatory to prevent HAIs outbreaks.

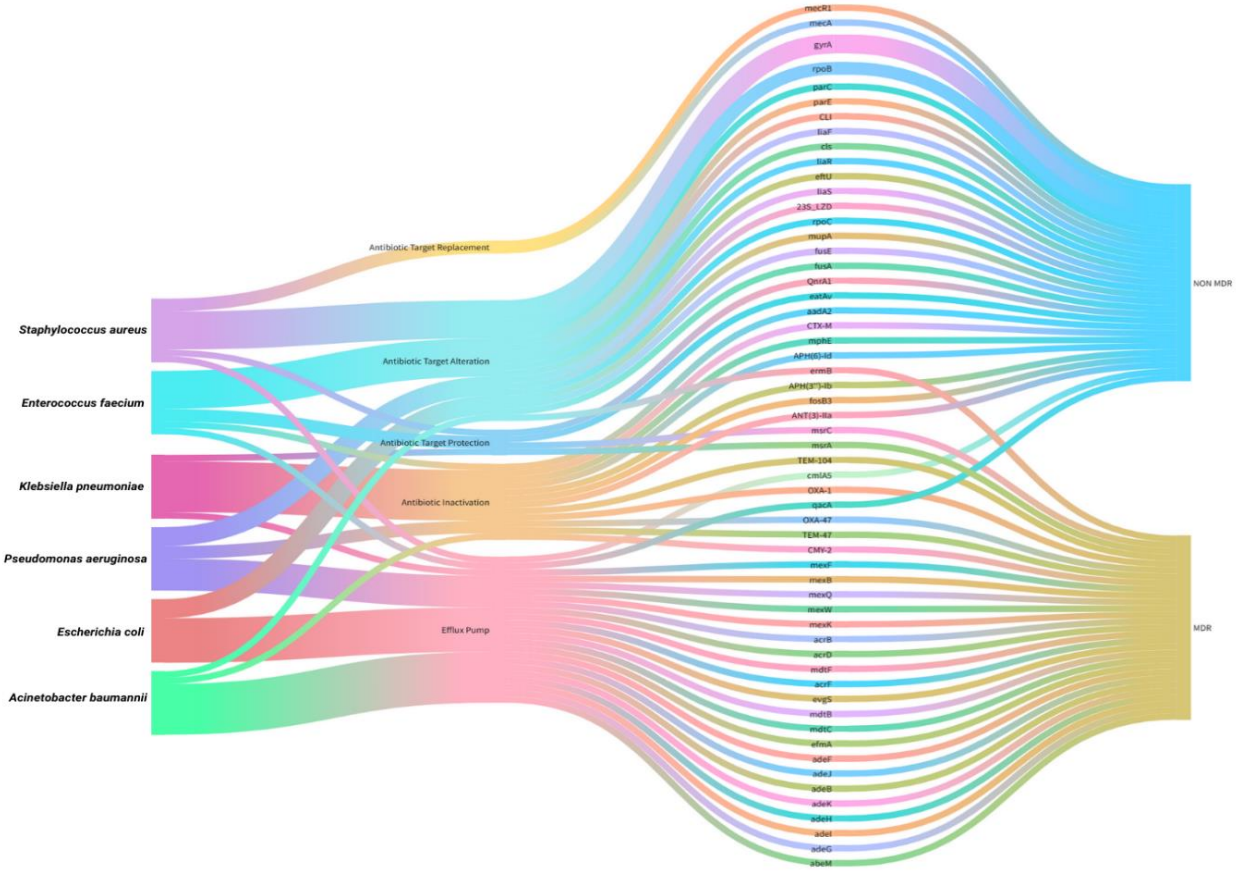

Figure 5. Sankey diagram that illustrates the distribution of the most abundant Multi-Drug Resistant (MDR) and non-MDR resistome in clinically concerning ESKAPE pathogens on the floor, sink, and surface ventilator.

4. Discussion

The primary cause of healthcare-associated infections (HAIs) is linked to ESKAPE pathogens. As per the European Centre for Disease Prevention and Control, 8% of ICU/PICU patients staying for more than 2 days encounter at least one ICU-associated HAI infection. Developing countries bear a heavier burden, with PICU HAIs rates reaching 11.98% [17].

Fomites are inanimate objects that become colonized with microbes and serve as potential intermediaries for transmission to/from humans, making understanding the survival of ESKAPE pathogens on fomites crucial for controlling HAIs. Further, the prevalence of "ESKAPE" pathogens was evident and considered an urgent clinical concern in the PICU

environment. The microbiology laboratory of General Hospital Yogyakarta provided the data for ESKAPE pathogens in 2019–2021. The proportion of ESKAPE from all clinical isolates among positive cultures during 2019–2021 slightly increased from 49.4% to 48.4% to 50.7% each year (P >.05) with data collected from all patients, including PICU patients, but no significant difference was found in PICU data [23]. This study explored the temporal and spatial variation in the taxonomic profile of environmental isolation from the floor, sink, and surfaces of the mechanical ventilator in the PICU at General Hospital in Yogyakarta, Indonesia.

Our investigation, conducted between days 0 and 30, with a 30-day interval, aims to determine the presence of ESKAPE bacteria and assess whether they can survive for an extended period in the routinely cleaned PICU environment. Furthermore, a study (Kramer et al., 2006) indicated ESKAPE pathogens have been shown to persist in the hospital environment for variable lengths of time (from days to months) acting as reservoirs of infection leading to further contamination, via staff hands or patient-to-patient transmission [24].

ESKAPE pathogens have an amazing ability to survive in clinical settings, where they change their genomes quickly and dynamically. They acquire antibiotic resistance genes (ARGs) under selective environmental pressure, contributing to heightened mortality, morbidity, and prolonged hospital stays in the PICU. ESKAPE pathogens, for instance, demonstrate significant persistence on floors, sinks, and glass, with capsulation identified as a crucial factor in their persistence. Biofilm-forming strains of ESKAPE pathogens have been found to survive considerably longer (over 35 days) on glass, ceramic floors, synthetic fibre, cotton, and mattresses than non-biofilm-forming strains (15 days) [25].

In this study, the dominant ESKAPE pathogen bacteria were found on the floor surrounding the patient. A previous study observed that the hospital floor is a frequently contaminated area but is frequently not considered an important source of the spread of pathogens for reasons rarely touched. However, the floor is often touched by objects that are then touched with hands (eg, shoes, socks, slippers) [4].

A previous study discovered that *E. faecium* could live on glass slides for more than 77 days and that it could live on different materials (cotton, terry, blend, polyester, and polyethylene) for more than 80 days. This suggests that *E. faecium* is better at surviving than *E. faecalis* [25]. Our result showed that *E. faecium* was not dominant in the floor and handwashing sink samples of the environments of PICU. *K. pneumoniae* is a Gram-negative, encapsulated, non-motile organism that lives in both the environment and humans. It may

result in meningitis, which kills 20% of children, pneumonia, bloodstream infections, wound or surgical site infections, etc. During this one-year research, *K. pneumoniae* infections were discovered in 15% of positive culture results (54/360) in this PICU [26]. Our analysis of the environments of the PICU showed that *K. pneumoniae* was dominant in the floor and handwashing sink samples.

Our data showed that *Staphylococcus aureus* was dominant on the surfaces of the mechanical ventilator and the floor around patients. *Staphylococcus aureus*, commonly linked to HAIs, can survive on abiotic surfaces for extended periods. A prior study showed Methicillin-sensitive *Staphylococcus aureus* (MSSA) strains could survive for over 25 days in hospital settings compared to Methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Surface characteristics significantly impact bacterial survival, with *S. aureus* surviving over eight weeks on various surfaces when immersed in sterilized water, urine, blood, or saliva [27].

E. coli, a significant foodborne pathogen, causes hemolytic-uremic syndrome in children and hospitalized bloodstream infections. In a moist environment, *E. coli* survived for over 28 days, except on 20°C metal surfaces, where it was inactivated after 7 days. Persistence on floors and mattresses exceeded 8 weeks, but viability decreased after four weeks on synthetic fibre and eight weeks on cotton [25].

Pseudomonas aeruginosa is an aerobic, Gram-negative bacterium that causes severe infections in children with compromised immune systems and severe nosocomial infections. It is a prototypical opportunistic pathogen, with rare illness incidence in individuals without preexisting risk factors. *Pseudomonas aeruginosa* is more likely to cause infections when the host's defences are weak, such as neutropenia, mucositis, immunosuppression, or reduced mucociliary transport [28]. It is crucial to exercise vigilance, as our investigation revealed the prevalence of *Pseudomonas aeruginosa* at the sink in the PICU.

Our data from March 2022 shows that at the PICU of General Hospital Yogyakarta, Indonesia, *Acinetobacter baumannii* was the most common species of bacteria discovered in handwashing sinks, on the floor around patients, and in the ventilator screens and tubes. Previous research has shown that bacteremia and meningitis are children's most common symptoms of invasive *Acinetobacter* infections [29].

The intricate interplay between pathogens and the environment is underscored by the impact of both the quantity and quality of nutrients on bacterial virulence. Environmental

nutrients play a significant role as triggers for the expression of virulence genes, shaping the interaction between environmentally transmitted opportunistic pathogens and their hosts [30].

In this study, the presence of the virulome-comprising genes involved in adherence, biofilm formation, and nutritional/metabolic factors extends the survival of ESKAPE pathogens in the environment, posing challenges in preventing their transmission and HAIs outbreaks. Notably, ESKAPE pathogens, endowed with virulence factors linked to metabolism and nutrient factors, augment the virulence of this crucial human pathogen [31]. Effective infection control in the PICU involves preventing pathogen persistence through strict cleaning protocols, frequent hand hygiene, and proper use of personal protective equipment. Education and training for staff, and air quality control measures are crucial [32]. Isolation, monitoring, and continuous monitoring of patients with MDR pathogens are also essential. Staying updated on research and innovations in infection control further enhances these measures. Regularly updating a tailored plan is essential for healthcare facilities [33].

Earlier studies have been limited and not specific in the PICU room. To analyse our environment hospital samples, we used shotgun metagenomics, which has the benefit of quantifying thousands of genes from culturable as well as nonculturable taxa simultaneously. However, the study's limitations are that it was only performed in a hospital in the city of Yogyakarta; the results could not be generalized to other hospitals in Indonesia or other low- and middle-income country settings.

5. Conclusions

ESKAPE pathogens are well known for their role in the onset of a variety of life-threatening clinical infections. Despite their prevalence in nosocomial settings, studies have reported on the isolation of ESKAPE pathogens from various environmental reservoirs, such as floors, sinks, and ventilators. Our approach using shotgun metagenomics sequencing has advantages in studying microbiome profiles in specific environments, especially PICU health care settings, and can capture the dynamic of the microbial population in a specific temporal study.

Our results study can provide empirical evidence of the presence of ESKAPE pathogen microbiome, resistome, and virulome genes in the environment for researchers in molecular microbiology and infection control. A suggested recommendation for researchers in this field is to consider the highly relevant application of a shotgun metagenomics approach as a research method.

The conclusions of this study can also be used to evaluate the risks of ESKAPE bacteria in hospital environments, especially the PICU, because ESKAPE pathogens can endure and persist in a dynamic population for as long as 30 days, retaining both antibiotic-resistant and virulence factor genes. Because ESKAPE pathogen isolates have been found in the environment and the number of HAIs is rising, strict surveillance and disinfection procedures must also be used in the intensive care unit to stop and control infections.

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Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

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