Original Article

Amplicon Sequencing Reveals Bacterial Diversity of Indoor Air Microbiome in Hospital Buildings

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ABSTRACT: *Airborne transmission of pathogens in hospitals poses a significant risk for healthcare-associated infections. Traditionally, assessing microorganisms in hospital air relies on culture methods, limiting the identification process. Advances in genome sequencing technology now allow for more accurate and unbiased identification of microbial content. This study employed amplicon sequencing to analyse the bacterial community in the indoor air microbiome of Sultan Ahmad Shah Medical Centre (SASMEC). Dust samples from 12 randomly selected air-handling unit (AHU) rooms were collected, and genomic DNA was extracted and sequenced targeting the V3 region of 16S rRNA. The Illumina NovaSeq 6000 system performed short-read amplicon sequencing. The results revealed diverse bacterial communities in AHU supply and return units, with Proteobacteria, Firmicutes, and Actinobacteria being the most common phyla. Dominant bacterial species included Methylobacterium spp., Nesterenkonia spp., Rubrobacter A bracarensis, Flavobacteriaceae spp., and Salinisphaera spp., with Methylobacterium spp. posing concerns due to its association with opportunistic infections. The study highlights the importance of using next-generation sequencing and culture-independent methods to monitor indoor air microbiomes. This provides crucial insights for managing biological contaminants, including airborne transmission, and enhances infectious disease surveillance in healthcare settings.*

Keywords: *Air Microbiome, Amplicon Sequencing, Bacteria, Culture-Independent Method, Hospital, Malaysia*

1.0 INTRODUCTION

The potential transmission of pathogenic microorganisms through air within hospitals has emerged as an increasing concern in the healthcare industry. Airborne pathogens can lead to healthcare-associated infections and diseases, thus exposing patients and healthcare personnel to various health risks. Previous studies have provided insights into the presence of various microorganisms, such as bacteria (Afeera et al., 2023), fungi, and viruses, in hospital environments (Mat Yassim et al., 2021; Ummu Afeera; Zainulabid et al., 2022; Ummu Afeera Zainulabid et al., 2021). Epidemiological studies have shown that exposure to pathogenic microbes increases morbidity and mortality rates in healthcare settings. Exposure to bacterial phyla, including Proteobacteria, Firmicutes, and Chloroflexi, can cause various respiratory infections, such as pneumonia and bronchitis, among hospital occupants (Fakunle et al., 2020). Studies have also shown that the presence of pathogenic bacteria in indoor air may contribute to secondary bacterial infections among immunocompromised patients, thus prolonging hospital stays and increasing death rates (Lee et al., 2021).

Over the years, conventional culture methods have been predominantly used to investigate microorganisms in the air, which, although useful, have limitations in terms of specificity, sensitivity, and comprehensiveness. In this technique, microbial growth depends solely on laboratory culture or media, which may introduce bias since not all microorganisms can be cultured or grown under such conditions (Mucci et al., 2020). This technique is also labourious and time-consuming, as the microbial culture needs to be incubated for 24 to 48 h before the identification process can begin. In addition, some microbes may have special growth requirements, such as high or low temperatures, and some may take longer to grow than others. This has led to the overgrowth of fast-growing microorganisms in the culture medium compared to slow-growing bacteria, resulting in biased and inconclusive findings regarding microbial profiling in indoor air (Adams et al., 2016).

Advances in genome sequencing have led to a paradigm shift in the study of airborne microbes. This new technology eliminates the requirement for a series of culture processes, thus offering more precise and unbiased results in a short analysis time for the identification of microbial communities in indoor air. This technique involves amplicon sequencing, polymerase chain reaction (PCR), and high-throughput sequencing, which enables the complete analysis of genetic material directly extracted from environmental samples (Ökten et al., 2020). The extracted bacterial DNA is subjected to amplicon sequencing, library preparation, and genomic sequencing. These technologies generate vast amounts of sequencing data that can be processed using bioinformatics to reconstruct the diversity and structure of indoor microbial communities (Ghosh et al., 2018).

This study aimed to analyse bacterial communities in the indoor air of the Sultan Ahmad Shah Medical Centre (SASMEC) using genomic sequencing technology. Dust samples were collected from an air-handling unit (AHU) room that was randomly chosen from various wards and clinics within the medical centre. Many patients in wards and clinics are immunocompromised owing to weakened immune systems, making it essential to mitigate the risk and reduce the prevalence of hospital-acquired infections. The AHU acts as a critical component of ventilation systems in various environments, particularly in regulating and circulating air. The collection of dust samples from AHUs serves as a strategic means of capturing a diverse array of microbial communities present in indoor air. Dust is often associated with particles and microorganisms in the air, making it a representative medium for acquiring an indoor microbiome. To the best of our knowledge, the use of dust samples from AHUs for bacterial identification through amplicon and next-generation sequencing (NGS) represents a pioneering approach in microbial indoor air quality research in Malaysia. Previous studies on microbial exposure in AHUs were conducted using active sampling with culture-dependent methods. Using amplicon sequencing, specifically targeting the V3 region of the 16S rRNA gene, and NGS, we enabled a comprehensive analysis of the bacterial composition within dust samples. The identification of bacterial communities directly from bacterial genomic materials extracted from dust samples circumvents the limitations associated with traditional culture methods, thus providing a more accurate and comprehensive profile of the bacterial diversity present in indoor air. Employing the integration of amplicon sequencing and NGS with dust samples from the AHU may become an alternative option for better understanding the sources of microbial contamination and the dynamics of indoor microbial communities to improve indoor air quality and minimise risks to human health.

2.0 MATERIALS AND METHODS

2.1 Sample collection

Prior to sample collection, a walk-through building inspection was conducted to identify and select the sampling locations. Clinics and wards that utilised air conditioning were included in the sample collection, and those using mechanical ventilation were excluded. To fulfill the inclusion criteria for sample analysis, samples had to be collected from both the indoor air and AHU rooms. Sampling was conducted during office hours from 08:00 to 17:00 (Sarah Fatihah Tamsi et al., 2022). Before sample collection, the AHUs were shut down, and with the help of the hospitals' technical staff, dust samples from the AHU

room were collected using sterile forceps. The samples were placed into sterile 15-mL tubes filled with 5 mL of phosphate-buffered saline (PBS). Ten samples were collected from both the supply and return vents in the selected AHUs. Samples were labelled AS_1–AS_5, indicating AHU supply air numbers 1 through 5, and AR_1–AR_5, representing AHU return air numbers 1 through 5. This labelling system was requested by the hospital management to avoid disclosing the names of specific sampling locations. All the collected samples were transported to the laboratory at room temperature for further analysis.

2.2 DNA extraction, library preparation, and sequencing

The dust samples in 15-mL tubes were subjected to homogenisation using a vortex for 5 min before proceeding with the bacterial DNA extraction procedures. Bacterial DNA was extracted using the QIAamp PowerFeacal Kit (QIAGEN, Hilden, Germany), with some modifications (Dean et al., 2022). First, dust samples were placed in a PowerBead ProTube and mixed with 400 µL of CD 1 solution provided by the manufacturer in the extraction kit. The tubes were incubated in a water bath at 65°C for at least 1 h. Subsequently, 400 µL of CD 1 solution was added to each tube and incubated for 15 min for homogenisation before centrifugation. After homogenisation, the remaining procedure was performed according to the manufacturer's protocol. The quality and quantity of the extracted DNA were examined by 1% gel electrophoresis and a Qubit 4 fluorometer (Thermo Fisher Scientific, Invitrogen, USA). The samples were then stored at −20°C for downstream application.

A library for bacterial characterisation was prepared using a polymerase chain reaction (PCR) approach targeting the 16S rRNA V3 region with the primer sequences listed in Table 1. PCR mixtures were prepared by mixing Promega GoTaq Green Mastermix with forward and reverse primers, as well as the DNA template. One tube without the DNA template served as the negative control. The mixtures were then subjected to amplification in an Eppendorf 5331 MasterCycle Gradient Thermal Cycler (Eppendorf, Germany), according to the protocol shown in Table 2. Using 2% gel electrophoresis, the PCR products were visualised and purified using SPRI beads. An index PCR was performed on the purified product to incorporate Illumina-specific barcodes and the remaining Illumina adapter sequences, as shown in Table 3. Finally, an Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) was used to sequence the amplicon libraries.

Table 1: Primer Sequences for PCR Targeting the Bacterial 16S rRNA Gene (Ayob et al., 2023; Hasain et al., 2022)

Primer	Sequence
341F	**CCTACGGGNGGCWGCAG**
518R	**ATTACCGCGGCTGCTGG**

Table 2: Protocol for Amplification of Bacterial DNA (Siew et al., 2022)

Protocol	Temperature	Duration
Initial denaturation	**95°C**	**2 min**
Denaturation	**95°C**	
Annealing	**50°C**	**10 s (30 cycles)**
Extension	**72°C**	
Final extension	**72°C**	**30 s**

Table 3: Protocol for Index PCR (Ummu Afeera Zainulabid et al., 2021)

Protocol	Temperature	Duration
Initial denaturation	**95°C**	**2 min**
Denaturation	**95°C**	
Annealing	**50°C**	**10 s (8 cycles)**
Extension	**72°C**	
Final extension	**72°C**	**30 s**

2.3 Amplicon sequencing and statistical analysis

Amplicon data were generated by overlapping raw paired-end reads with Fastp (Chen et al., 2018), which were then trimmed to remove primers using Cutadapt v4.4. (Martin, 2011). The overlapping readings were imported into QIIME2, where they were denoised, and table counts were constructed using DADA2 (Callahan et al., 2016). Amplicon sequence variants (ASVs) were classified using the qiime2-feature classifier, which was trained on the GTDB 16s rRNA V3 region database (r207 release). The taxonomic categorisation and ASV table were manually formatted before being uploaded to the MicrobiomeAnalyst web server for data visualisation.

MicrobiomeAnalyst 2.0 was used for the statistical analysis of microbiome data to profile the bacterial population identified in indoor air (Siew et al., 2023). The data were filtered using a zero minimum count and a 10% interquartile range. The taxonomic composition of the samples was determined using bar plots at the phylum, genus, and species levels. Certain phyla were included in the phylum abundance if they reported an abundance of 0.5% or more in at least 25% of all samples (Siew et al., 2022). Permutational multivariate analysis of variance (PERMANOVA) was applied for diversity analysis, and the Bray–Curtis dissimilarity index was used to measure significant differences between groups. For the alpha diversity analysis, Chao1 was used to explore bacterial diversity within a group, with p-values <0.05 considered significant.

3.0 RESULTS

This study primarily focused on profiling the bacterial community in indoor air at a hospital using a culture-independent method and genomic sequencing. Ten dust samples were collected using passive sampling, and high-throughput amplicon sequencing was used to characterise the bacterial composition in the V3 region of the 16S rRNA gene. These findings revealed a wide range of bacterial composition in each sample. Fig. 1–3 show the profiles of bacterial community abundance in the dust samples at the phylum, genus, and species levels, respectively.

Figure 1: Relative Abundance of the Top Bacterial Phyla Isolated from the Return (AR) and Supply (AS) Vents in the Air Handling Unit (AHU) Room

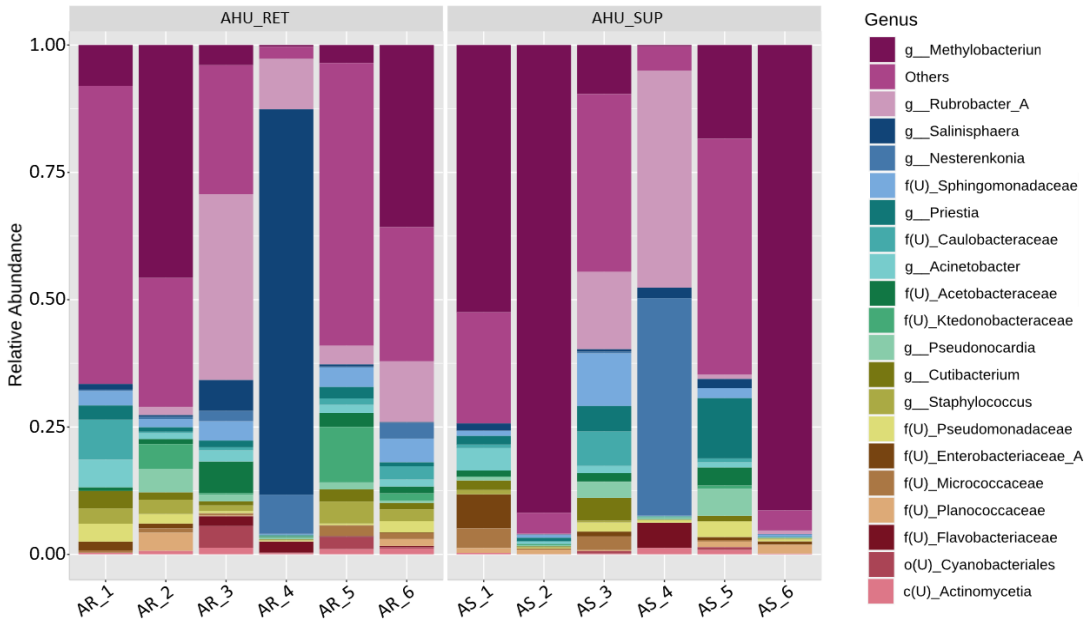

Figure 2: Relative Abundance of the Top Bacterial Genera Isolated from the Return (AR) and Supply (AS) Vents in the AHU Room

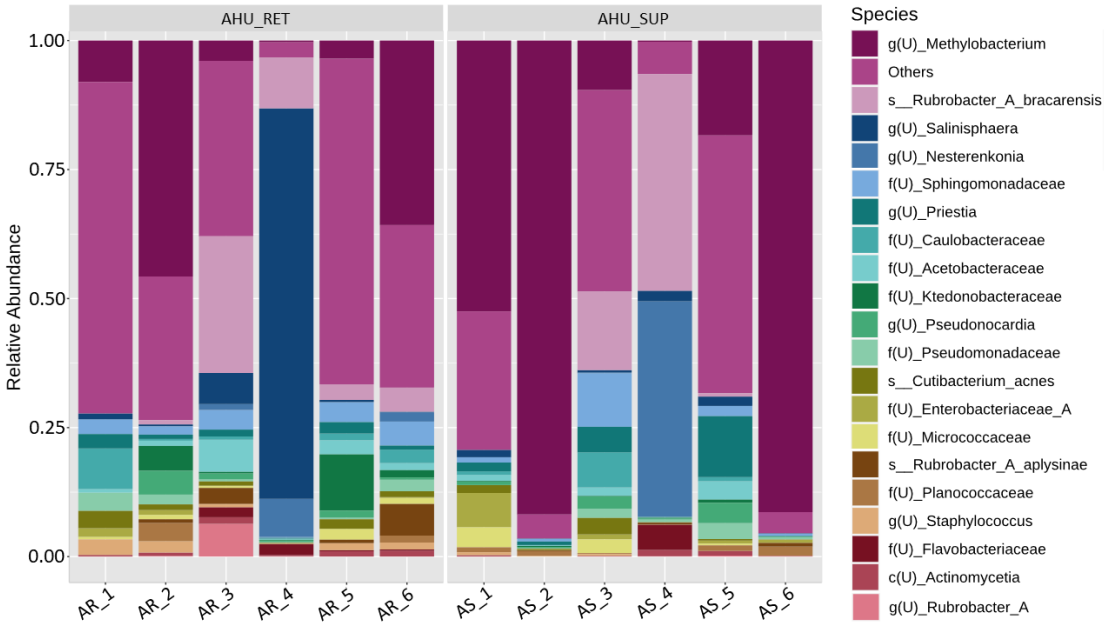

Figure 3: Relative Abundance of the Top Bacterial Species Isolated from the Return (AR) and Supply (AS) Vents in the AHU Room

Overall, 11 bacterial phyla, including 21 bacterial genera and 21 bacterial species, were identified in the dust samples. The most abundant bacterial phyla that dominated the AHU supply and return units were Proteobacteria, Firmicutes, and Actinobacteria (Fig. 1). More than 90% of Proteobacteria were found in AHU supply vents AS_2 and AS_6, and over 50% in most AHU return vents, except AR_3 and AR_5, which were dominated by Actinobacteria. Firmicutes were also present in all samples, with 20% in AHU supply vents AS_3 and AS_5. Further examination at the genus level revealed different dominant bacteria across the samples (Fig. 2). The supply vents were primarily dominated by *Methylobacterium* spp., except for AS_4, which was dominated by *Nesterenkonia* spp. (43%). In contrast, in the return vents, *Methylobacterium* spp. and *Rubrobacter* spp. were present in all samples, with high levels of *Salinisphaera* spp. found in AR_4 (76%). As for bacterial species, *Cutibacterium acnes*, *Salinisphaera* spp., *Priestia* spp., *Methylobacterium* spp., *Sphingomonadacae* spp., *Psedomonadacae* spp., and *Caulobacterecae* spp. were present in all collected dust samples with varying levels of abundance (Fig. 3). *Salinisphaera* spp. and *Methylobacterium* spp. dominated the AHU return vents at 31% and 18%, respectively, whereas the supply vents were dominated by *Methylobacterium* spp. and *Nesterenkonia* spp. at 41% and 20%, respectively.

Further analysis of alpha and beta diversity was conducted to examine the diversity of the bacterial air microbiome community in the supply and return vents of the AHUs. The total species diversity of the samples can be referred to as alpha diversity, which is defined as the similarity or distance discovered across sample groups. On the other hand, beta diversity is a metric that may be used to compare the composition or feature similarity of samples. Fig. 4 shows significant results for alpha diversity but not for beta diversity ($p = 0.031$ and $p = 0.259$, respectively).

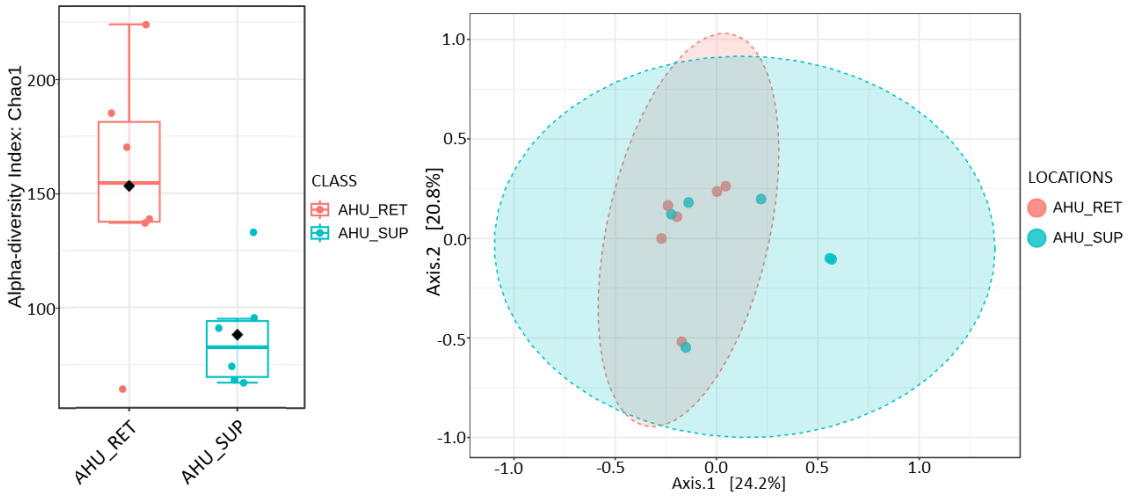

Figure 4: Results for Diversity Analysis. (A) Significant Alpha Diversity Results (p = 0.031) Using the Shannon Diversity Index. (B) Non-Significant Beta Diversity Results (p = 0.259) Shown as PCoA Plots Using Bray–Curtis Dissimilarity.

4.0 DISCUSSION

In Malaysia, studies on the monitoring of microbiological contaminants in indoor air have been conducted using culture-dependent methods and have reported total bacterial and fungal counts (Aziz et al., 2018; Sarah Fatihah Tamsi et al., 2022). However, a drawback of using culture-dependent methods is that not all bacteria present indoors can be cultivated or cultured, thus leading to inconclusive findings on the profiling of the indoor bacterial community. Advancements in NGS have led to evolutionary changes in air microbiome monitoring. Bacterial genomic material or DNA can be extracted and analysed directly from environmental samples without the need for culture and growth in the laboratory (McCombie et al., 2019). The shift from conventional culture methods to NGS eliminates the limitations associated with traditional techniques and offers a more accurate and comprehensive understanding of microbial communities in hospital air within a shorter timeframe (Ökten et al.,

2020). This methodological advancement is crucial in the context of sudden outbreaks and healthcare-associated infections, where airborne pathogen transmission is a significant concern.

Analysis of the indoor air microbiome in the SASMEC showed diverse bacterial communities in both the supply and return vents of the AHUs. Identification of Proteobacteria, Firmicutes, and Actinobacteria as the predominant bacterial phyla underscores the complexity of the microbial ecosystem in hospital air. The isolation of Proteobacteria and Firmicutes from ventilation systems is of concern because these bacterial phyla may contribute to various respiratory infections, including pneumonia and bronchitis, among hospital occupants (Fakunle et al., 2020). The identification of *Methylobacterium* spp., *Nesterenkonia* spp., *Rubrobacter A bracarensis*, *Flavobacteriaceae* spp., and *Salinisphaera* spp. as the top five bacterial species could have implications for transmission dynamics and potential sources of pathogens in healthcare environments. The high abundance of *Methylobacterium* spp. is particularly alarming, as it can lead to colonisation and bacterial infections in immunocompromised patients, even though it has a low virulence factor. Typically, these species cause mild clinical symptoms, including fever, bacteraemia, peritonitis, and pneumonia (Szwetkowski & Falkinham, 2020). In addition, the presence of gram-negative bacteria, such as *Nesterenkonia* spp. and *Salinisphaera* spp., raises concerns about potential colonisation and asymptomatic bacteraemia, emphasising the need for targeted interventions to reduce the risk of infection (Chander et al., 2017).

In investigating bacterial diversity within the supply and return vents of AHUs in hospital buildings, this study obtained distinct results from the alpha and beta diversity analyses. Alpha diversity analysis, which assesses diversity within specific sample groups, revealed significant differences in species richness or evenness between the supply and return vents (Mahno et al., 2023). In contrast, beta diversity analysis, which examines microbial composition differences between samples, showed no significant dissimilarities, suggesting a high degree of similarity in bacterial communities between the two sites of investigation (Dean Tay et al., 2021). The absence of diversity distinctions implies homogeneity in the indoor air microbiome within the AHU components (Li et al., 2016). To further interpret these results, factors such as effective air filtration, consistent ventilation cleaning, and other environmental variables influencing these findings should be considered. Despite these limitations, this study highlights a rapid and comprehensive culture-independent method for sampling and analysing bacterial diversity and communities present in AHUs in hospitals.

5.0 CONCLUSION

In conclusion, the analysis of the indoor air microbiome presented in this study revealed a diverse array of bacterial communities within hospital AHUs, with Proteobacteria, Firmicutes, and Actinobacteria emerging as the dominant phyla. The identification of *Methylobacterium* spp., *Nesterenkonia* spp., *Rubrobacter* A *bracarensis*, unclassified *Flavobacteriaceae* spp., and *Salinisphaera* spp. as the most common bacterial species in dust samples raises concerns, particularly regarding the high abundance of *Methylobacterium* spp., which is known for its association with opportunistic infections, posing a heightened risk for immunocompromised individuals. The presence of *Nesterenkonia* spp. and *Salinisphaera* spp. further underscores their potential for colonisation and asymptomatic bacteraemia. This study underscores the significance of employing NGS and culture-independent methods as vital tools for assessing indoor air microbiomes, offering valuable insights for monitoring biological contaminants in built environments. By enhancing our understanding of the microbial landscape, these findings will contribute to the refinement of strategies for controlling airborne transmission and infectious disease surveillance among patients and healthcare workers, ultimately fostering healthier and safer indoor environments.

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