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Original Article

Molecular sequencing and phylogenetic analysis of bioaerosols in hospital wards with different ventilation conditions

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Abstract

Introduction: Bioaerosols are one of major sources of hospital-acquired infections (HAI's) that can pose serious health implications to the patients, health care workers and visitors in the hospitals across the world.

Methodology: In this study, the molecular identification and phylogenetic analysis of bioaerosols collected from Orthopedic Wards (OW) and Orthopedic Emergency Rooms (OER) of six hospitals in Lahore, Pakistan was done to investigate their diversity and genetic relatedness. Moreover, the role of different ventilation practices (i.e., centrally air-conditioned and non-central air-conditioned) in determining bioaerosols load was evaluated by using both culture and non-culture based (Flow cytometry) approaches.

Results: The molecular characterization based on 16S rRNA gene and phylogenetic analysis of frequently recovered bacterial isolates showed 97-99% similarity to diverse sources i.e., air, soil and clinical strains isolated from various countries. The centrally air-conditioned hospitals had significantly lower levels of bioaerosols at most of the sites as compared to non- central air-conditioned hospitals.

Conclusions: The molecular characterization and phylogenetic analysis based on 16S rRNA gene sequences can be effective tool in identifying nature and evolution of bioaerosols, and can improve infection control and surveillance in hospitals. The observed levels of bioaerosols suggest hospitals equipped with central air conditioners have considerably more air hygiene compared to non-central air conditioning systems. These findings are imperative for informing policies on planning and implementation of infection control strategies in hospitals in resource limited settings.

Key words: Bioaerosols; flow cytometry; hospitals; Pakistan; air quality; ventilation.

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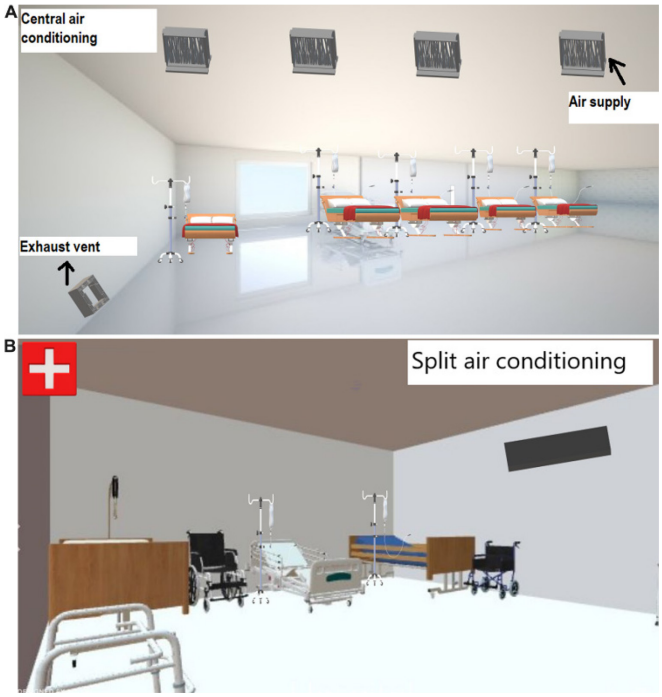
Introduction

The hospitals are particularly susceptible to the infection because of the people carrying infectious agent and immunocompromised patients share the same environment [1]. For an infection to spread, three modes of transmission can be involved: droplet, airborne and contact [2]. Airborne transmission of infection is considered to be a major route and is critical to control hospital infections. The exposure to bioaerosols in health care facilities can cause respiratory diseases, decrease lung function, allergic reactions and infectious diseases which can be an occupational safety and health concern [3]. Various factors i.e., ventilation, number of occupants, indoor activities, building design and use, air cleaning devices and maintenance affect the bioaerosols concentration [4]. Moreover, the environmental variables such as relative humidity and temperature also influence the survival of microorganisms [5]. Serious clinical and non-clinical infections have been associated with inadequate ventilation. Increased ventilation has been proposed to be an effective environmental control in reducing airborne infections and maintaining good air hygiene in enclosed environments. Mechanical and natural ventilation can be employed to control bioaerosols, however, both types have their own shortcomings [6]. The effective environmental monitoring can provide assistance to control infection rates at hospitals, however the shortage of advance methods to monitor the changes in airborne pathogens limits the control and management of hospital infections. In recent times, the 16S rRNA sequencing techniques have been proven to be more useful for the diversity analysis of airborne bacterial genera in the

indoor environment as compared to the traditional culture-based techniques [7]. Additionally, the phylogenetic analysis can be an effective tool for the microbial characterization to control circulating bacterial flora in health care facilities [8]. The traditional surveillance methods of infection use culture-based diagnostic features that can only identify viable microorganisms. Therefore, it does not cover broad range of airborne pathogens and could not reflect the actual load of bioaerosols both qualitatively and quantitatively. Recent technologies have led to the large-scale testing done with a single collection that complements and even sometimes replace traditional methods [9]. Among various modern non-culture methods, the flow cytometry (FCM) can improve the bioaerosols characterization by improving the viability and total cell count of microorganisms [10]. FCM has been widely used in the environmental, microbiological and biomedical samples from aquatic environments to quantify phytoplankton, eukaryotic cell populations and yeast or bacterial cells. Recent studies are exploring the capability of FCM to fingerprint microbial communities and quantify single cells in the environmental samples. However, literature available on use of FCM to explore bioaerosols in the indoor and ambient environment appears to be limited. It is necessary to develop alternative techniques for the detection and quantification of bioaerosols specifically in the health care facilities to comprehend the nature and magnitude of bioaerosols, where the rapid detection can help to control airborne infections and inform regulatory policies.

Figure 1. Illustrative diagram of a hospital room. (a) Centrally air conditioned (b) Non-centrally air conditioned.



The health care delivery system of Pakistan primarily consists of public and private sectors and most of the healthcare facilities are in the urban areas of the country. The incidence of Hospital-Acquired Infections (HAI) Whilst the risk associated with airborne disease transmission are acknowledged, there are fewer studies available on the nature and magnitude of airborne microbial and impact of different environmental controls to manage air hygiene is understudied [11,12]. A range of factors including ventilation, building design, management and usage can greatly affect the bioaerosols levels in hospitals. These factors vary across the globe due to socioeconomic and climatic conditions [13]. In Pakistan, infection control strategies and their applications are highly variable across the health settings. Therefore, it is necessary to investigate the nature and magnitude of bioaerosols loads in hospitals, particularly focusing on evaluating the impact of the existing air cleanliness control practices. This study intends to investigate the microbial diversity and levels using molecular techniques and biochemical testing in the differentially ventilated orthopedic wards and emergency rooms of public and private sector hospitals of Lahore, Pakistan. Moreover, the genetic relatedness of these isolates was assessed by phylogenetic analysis. Total and differential counts of airborne bacteria and fungi were determined using culture-dependent methods. In addition, a comparison was conducted between culture-based and non-culture-based (FCM) methods.

Methodology

Sampling sites

The current study was conducted in six hospitals of Lahore, Pakistan and were classified into two groups (Group I and II) on the basis of ventilation system as defined by Jung *et al*. [14]. The group I contained two private hospitals that were centrally-air conditioned by Air Handling Units (AHU) whereas the group II contained four public hospitals were non-centrally air conditioned by split type. Figure 1 shows a typical hospital room equipped with central air conditioning and non-central air conditioning. The orthopedic wards (OW) and emergency rooms (OER) from each hospital were selected as monitoring sites based on our previous findings [15] as they are reported to have high risk of infection in the orthopedic treatment rooms [16]. Four sampling campaigns were organized during January to December 2017 at an interval of three months.

Bioaerosols sampling, quantification and identification

Filtration method was used for indoor and outdoor air sampling at each hospital. A volumetric pump sampler operating at air flow rate of 26 L/min with a polycarbonate filter (0.45 µm pore size, 47 mm diameter) was operated for 15 min. The samples were collected in triplicates for both culture-based and non-culture based (FCM) analysis at each site. The indoor samples were taken around 12:00 pm at a height of at 1.5 m above the ground level in the middle of room. The outdoor samples were collected simultaneously 50 m away from the main building. The filters were subsequently transferred to 20 mL falcon tubes filled with 5 ml of phosphate buffer saline (PBS). These PBS tubes besides other sampling equipment were sterilized by autoclaving at 121 °C and 15 psi pressure for 40 minutes. Serial dilutions were made for both indoors and outdoors samples, using 1 ml of the stock PBS solution added to another tube containing 9 mL of PBS, and dilutions were prepared up to 10^{-5}. Nutrient agar (NA) and Sabouraud dextrose agar (SDA) plates were prepared and inoculated with 100 µL from 10^{-2} and 10^{-4} dilutions, respectively. Afterwards, the NA plates were incubated at 37 °C for 24 hours, while SDA plates were incubated at 28 °C for 6 to 7 days.

Bacterial quantification and identification

The airborne-bacterial colonies in triplicate samples were counted using colony counter for each sample and expressed as average number of colonies per cubic meter (CFU/m^3) using the following formula [17]:

$$\frac{CFU}{m^3 of air} = \frac{number of colonies \times Aliquot dilution factor}{Vol of the air sampled/m\,3}$$

The morphological features of the obtained colonies such as colony size, shape, elevation, margins and texture were recorded and the primary growth cultures were further purified on blood agar to get bacterial pure cultures. The different colonies were further classified morphologically and biochemically using Bergey's manual [18]. The morphological tests included gram staining, endospore staining and motility detection by hang-drop method, while oxidase, catalase, coagulase, mannitol-salt agar, and DNase tests were performed to characterize microbial isolates biochemically. Clustered gram-positive cocci that were catalase-positive, mannitol fermenter, and coagulase-positive were identified as *Staphylococcus aureus* [19].

Molecular identification by 16S rRNA partial gene sequencing and phylogenetic analysis

The most frequently observed bacterial colonies were further identified up to species level by PCR and sequencing partially the 16S rRNA gene. The gene amplification by PCR was done using thermal cycler (PTC-200, Hampton, USA), using Roche PCR master mix kit (Sigma Aldrich, Taufkirchen, Germany). The 1521bp fragment form the bacterial genome was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 492R (5'-TACGGYTACCTTGTTACGACTT-3'). The DNA sequencing was done using Applied Biosystem (ABI model 3700, California, USA) sequencer.

The extraction of amplified DNA from agarose gels was done using MEDOX-BIOTUM Gel extraction kit (Biotum, Melbroune Australia) following the manufacturer's instructions. The DNA sequencing was done using Applied Biosystem sequencer (ABI model 3700, California, USA).

For the phylogenetic analysis, 16S rRNA partial sequences of the bacterial isolates were compared with the available sequences in National Center of Biotechnology Information (NCBI) database, using nucleotide BLAST [20]. The alignment of isolated sequences and their closest relatives were conducted by MEGA6 software. Moreover, screening of the sequences for the manual alignment was done and a pylogenetic tree was contructed by maximum-likelihood method, with a pairwise gap removal using MEGA6. In order to examine the statistical significance of braching pattern, the interior branch test was conducted using 500 bootstrap replication. Then, evolutionary patterns and homology were analysed for the similarity with other isolates from NCBI database. Sequences of environmental and clinical samples from database were added into the phylogenetic analysis to find similarity.

Fungal quantification and identification

Fungal colonies were counted using the same formula as above. Then fungal isolates were identified morphologically on the basis of colonial characteristics (color, texture, size and pigmentation), lactophenol cotton blue staining and riddle's slide culture method [21].

Flow cytometry analysis (FCM)

Flow cytometry analysis (FCM) was conducted to quantify total cell concentrations using BD Accuri TM C6 flow cytometer (BD Bioscience, San Jose, USA). The filters were vortexed at 2 rpm for 2 minutes before fluorescent staining. Further on, serial dillutions up to 10-5 were made using Evian 0.22 µm strained mineral

water (Evian, Evian-les-Bains, France). The flourescent staning and cytometry analysis method described by Whitton *et al*. [22] was used in the current study.

To quantify the microbial concentration SYBR Green (SG) dye (10000X stock by InvitrogenTM, Waltham,USA) diluted with dimethyl sulfoxide (DMSO) was used to get a working concentration of 100X. For the FCM analysis, a 96 wells reaction plate was prepared using 200 µL of samples in PBS and 2 µL SG dye. Afterwards, the plate was placed in a closed microplate thermoshaker (GrantBioTM, PHPM series, Chelmsford,UK) and incubated at 250 rpm for 5 minutes at 37 ℃. Afterwards, the analysis was done with a BD Accuri TM C6 flow cytometer (BD Bioscience, San Jose, USA). A sample volume of 50 µL was used to further analysis by Accuri C6 software. The total cell count in the air samples were then calculated as:

$$\frac{Numberof Cells}{m^3 of air} = \frac{numberof cell \times AliquotDilutionfactor}{Volof theair\ sampled/m\,3}$$

Statistical analysis

The data sets were evaluated using IBM statistics SPSS 21 version. Kolmogrov –smirnov and Levene tests were performed. Data were non-parametric and were normalized and further evaluated for parametric test. An independent t-test was used to compare mean levels of microbial counts and FCM between two groups.

Results

Sequencing analysis

The colonies were first identified using morphological features and biochemical tests. Among them, most frequently observed colonies were further identified up to species levels by PCR (Supplementary Figure 1), sequencing the partial 16S rRNA amplified region and were blast analyzed (Supplementary Figure 2). The genotypic characterization of frequently observed colonies characterized gram-positive rods into *Bacillus cereus*, *Bacillus subtilis* and *Clostridium perfringens*, while the gram-negative rods into *Escherichia coli* and *Klebsiella pneumoniae*. The gram-positive cocci were characterized into *Staphylococcus aureus* and *Micrococcus luteus.* The sequences of study isolates were submitted to GenBank and accession number were allotted. The partial 16S rRNA gene nucleotide sequences of each bacterial isolates were compared to globally available 16S rRNA gene sequences of diverse isolates of clinical and environmental origin. The bootstrap consensus tree inferred from 500 replicates is taken to present the evolutionary relatedness of the studied isolates with the representative control strains from the NCBI GenBank. In the phylogram, the isolates were added from possible sources such as soil, water, air, skin and clinical nature. The nucleotide blast indicated that the environmental strains showed 97-99% sequence homology to the NCBI database sequences.

Bacterial count and characterization

The mean bacterial count on the Nutrient agar (NA) in the OW and OER was found to vary with the type of ventilation system. In the OW, the mean bacterial count was significantly low ($1.38\times10^4 \pm 7.70\times10^3$) in group I, compared to group II ($3.76\times10^4 \pm 2.47\times10^4$), $p < 0.05$ (Table 1). Similarly, in the OER, the bacterial count was significantly lower ($1.90\times10^4 \pm 1\times10^4$) in group I, compared to ($4.81\times10^4 \pm 2.47\times10^4$) group II, $p < 0.05$ (Table 1). In Outdoors (OD), the mean bacterial count was significantly higher in group I ($6.15\times10^6 \pm 2.47\times10^3$) hospitals as compared to group II hospitals ($7.08\times10^5 \pm 4.03\times10^4$), $p < 0.05$ (Table 2). It is inconsistent to the trend observed in the indoor sites, suggesting other sources in the outdoors.

Table 1. Indoor airborne microbial and FCM counts in hospital groups I and II.

Microbial load assessment technique	Microbial load		*p*-value
	Group I (Centrally air-conditioned) Avg ± SD	Group II (Non-centrally air-conditioned) Avg ± SD	
Orthopedic Wards (OW)			
NA (CFU/m^3)	$1.38\times10^4 \pm 7.70\times10^3$	$3.76\times10^4 \pm 2.47\times10^4$	< 0.05
SDA(CFU/m^3)	$2.83\times10^3 \pm 1.46\times10^3$	$3.25\times10^3 \pm 1.43\times10^3$	0.08
FCM(Cells/m^3)	$2.81\times10^6 \pm 2.94\times10^6$	$1.58\times10^8 \pm 2.18\times10^7$	< 0.05
Orthopedic Emergency Rooms (OER)			
NA(CFU/m^3)	$1.90\times10^4 \pm 1\times10^4$	$4.81\times10^4 \pm 2.49\times10^4$	< 0.05
SDA (CFU/m^3)	$8.55\times10^2 \pm 7.79\times10^2$	$1.14\times10^3 \pm 9.36\times10^2$	< 0.05
FCM (Cells/m^3)	$2.19\times10^7 \pm 2.10\times10^7$	$1.01\times10^7 \pm 9.27\times10^6$	< 0.05

NA: Nutrient agar; SDA; Sabouraud dextrose agar; FCM: Flow cytometry.

Table 2. Outdoor airborne microbial and FCM counts in group I and II.

Microbial load assessment technique	Microbial load		*p*-value
	Group I (Centrally air conditioned) Avg ± SD	Group II (Non-centrally air conditioned) Avg ± SD	
NACFU/m^3)	$6.15 \times 10^6 \pm 2.47 \times 10^3$	$7.08 \times 10^5 \pm 4.03 \times 10^4$	< 0.05
SDA (CFU/m^3)	$1.29 \times 10^3 \pm 7.82 \times 10^2$	$1.45 \times 10^3 \pm 2.50 \times 10^2$	0.09
FCM (Cells/m^3)	$3.23 \times 10^7 \pm 2.12 \times 10^6$	$2.12 \times 10^8 \pm 4.08 \times 10^7$	< 0.05

NA: Nutrient agar; SDA: Sabouraud dextrose agar; FCM: Flow cytometry.

The bacterial characterization on the basis of phenotypic and genotypic characteristics showed the higher percentages of *Staphylococcus aureus* and *Micrococcus* in the group I and II OW. Moreover, higher percentages of *Micrococcus* and coagulase-negative *Staphylococci* (CoNS) were found in group I OER as compared to *Micrococcus* and *Bacillus* in group II, respectively (Figure 2). The frequently recovered bacterial isolates in outdoors were CoNS and *Bacillus* in group I and *Micrococcus* and CoNS in group II hospitals (Figure 2).

Fungal count and characterization

The air samples were also quantitatively and qualitatively analyzed for fungi using SDA (Table 1). In the OW, the mean count was lower in group I ($2.83 \times 10^3 \pm 1.46 \times 10^3$) as compared to group II ($3.25 \times 10^3 \pm 1.43 \times 10^3$) hospitals but were not significantly different $p > 0.05$. In the OER, the mean count was significantly lower in group I ($8.55 \times 10^2 \pm 7.79 \times 10^2$) as compared to group II ($1.14 \times 10^3 \pm 9.36 \times 10^2$) , $p < 0.05$. For outdoors (OD), the fungal counts were comparatively low ($1.29 \times 10^3 \pm 7.82 \times 10^2$) in group I compared to group II hospitals ($1.45 \times 10^3 \pm 2.50 \times 10^2$) but were not statistically different $p > 0.05$ (Table 2).

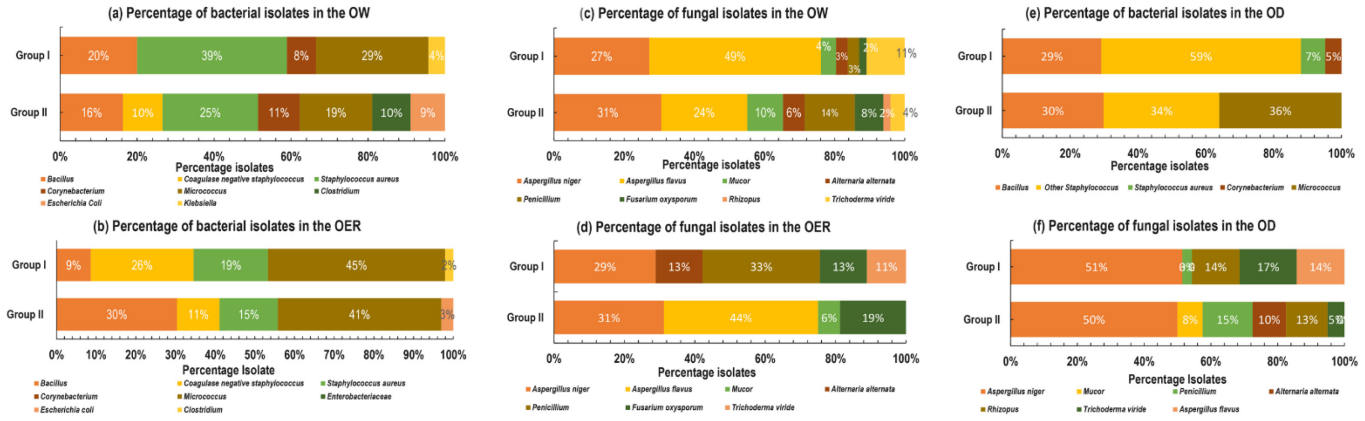
The macroscopic examination of fungal species showed the highest percentages of *Aspergillus flavus* and *A. niger* in the group I and II OW (Figure 2). While, in the OER, higher percentages of *Penicillium* and *A. niger* were found in group I as compared to *A. flavus* and *A. niger* in group II, respectively. In outdoors (OD) frequently recovered fungal isolates were *A. niger* and *Trichoderma viride* in group I and *A. niger* and *Penicillium* in group II hospitals.

FCM analysis

The air samples analyzed by FCM showed higher microbial count compared to NA and SDA (Table 1). In the OW, FCM counts were $2.81 \times 10^6 \pm 2.94 \times 10^6$ and $1.58 \times 10^8 \pm 2.18 \times 10^7$ in group I and II hospitals, respectively. In the OER, the FCM counts were $2.19 \times 10^7 \pm 2.10 \times 10^7$ and $1.01 \times 10^7 \pm 9.27 \times 10^6$ in group I and II hospitals, respectively. Moreover, the FCM counts in both OW and OER were significantly lower in group I, compared to group II hospitals, $p < 0.05$.

The FCM counts varied significantly in the outdoor air samples of group I ($3.23 \times 10^7 \pm 2.12 \times 10^6$) and group II ($2.12 \times 10^8 \pm 4.08 \times 10^7$) hospitals, $p < 0.05$ (Table 2). The overall FCM counts were higher than the microbial counts obtained from culture-based technique.

Figure 2. Percentage microbial isolates in group I and II hospitals. Bacterial isolates in the wards (OW) and emergency rooms (OER) (a and b), fungal isolates in the wards (OW) and emergency rooms (OER) (c and d), and bacterial and fungal isolates in the outdoor (OD) sites (e and f).



Discussion

In this study, eight different genera of bacteria and fungi were recovered from indoor sites of group I and II (OW and OER) sites. However, five bacterial and seven fungal genera were isolated from the OD sites. Phenotypic characterization of bacterial isolates by biochemical test indicated gram positive cocci to be the most dominant genera; *Staphylococcus* and *Micrococcus* in the OW and OER of both group I and II hospitals, respectively. This in line with various studies [23,24] reported high prevalence of *Staphylococcus, Micrococcus* and *Bacillus* in different health care settings. Comparing to the other studies conducted in Pakistan, Memon *et al*. [25] also reported highest prevalence of *S. aureus* in different wards, which is one the most notorious pathogen reported to be a source nosocomial infection in the immune-compromised patients. Moreover, Asif *et al*. [12] observed high prevalence of *S. haemolyticus* at different sites of a HVAC supported hospital. *E.coli, S. aureus* and *K. pneumoniae* is also reported to be most prevalent in three hospitals of Pakistan [26]. Most of these studies suggested that frequent disinfection and cleaning practices may help to reduce airborne microbial loads. Following gram positive cocci, the gram-positive rods showed high prevalence in the OW and OER of both group I and II hospitals. The frequently isolated gram-positive rods include *Bacillus*, *Corynebacterium* and *Clostridium*. Finally, gram negative rods were the least recovered, including *E. coli* and *Klebsiella*.

Seven of the strains were ribotyped and their sequences were compared with the reference sequences by NCBI BLAST. The strains showed 97-99% similarity with the reference sequences in NCBI Genbank. The phylogenetic analysis showed that the *B. cereus* study isolate showed high similarity with the previously reported airborne pathogen isolated in the laboratory from Singapore and China. The *B. subtilis* study isolated showed 98% similarity to the *B. subtilis* isolated from paint polluted soil from Pakistan. This can be understanding as the scraping of old paint may result in mixing of paint flakes in the soil, if indoors can land on floor and later can become suspended in air. The persistent presence of *B. cereus* and *B. subtilis* in the hospital air can be well associated to their growth characteristics; can sustain into broad range of temperature, pH and desiccation due to endospore formation [27]. In case of studied *S. aureus* isolate, 97% similarity was found to *S. aureus* isolated from the patient's wound in India. *S. aureus* is one of the most frequently isolated organism from the severe wounds, which requires careful management due to its association with nosocomial infections and acquired antibiotic resistance [28]. *M. luteus study* isolate showed 97% similarity to the soil isolates submitted from India. *M. luteus* have diverse sources, which may produce pathology in immune-compromised patients [29]. The soil or dust coming from the shoes, ventilation systems may cause resuspension of bioaerosols in the air [2]. The *C. perfringens* isolate was similar to the isolates recovered from the air of clean rooms of space aircraft in USA. For the gram-negative isolates, the *E. coli* and *K. pneumoniae* study isolate were more similar to the previously reported soil isolate submitted from India, as compared to clinical isolates. *E. coli* has been reported as the most common nosocomial pathogen causing urinary tract infection (UTIs) in the Europe followed by the *K. pneumoniae* with serious health implication [30]. Moreover, in Pakistan, the highest number of UTIs in the hospitalized patients are reported to be caused by *E. coli* [31]. Overall, the isolates showed higher similarity to isolates recovered from soil, air and clinical origin.

Although the genotypic characterization of the fungi could not be done due resource limitations, the phenotypic characterization indicated *A. flavus* and *A. niger* to be the most prevalent in the OW and *Penicillium* and *A. flavus* in OER of group I and II hospitals. The presence of *Aspergillus* can be explained by frequent construction activities in and around hospital premises. Moreover, the highest prevalence 49% in the group I OW was also probably due to the improper maintenance of air ducts as *Aspergillus* can grow around air duct leakages [32]. The increase prevalence of *Aspergillus* can cause a serious nosocomial infection aspergillosis in hospitalized patients.

In the current study, the mean range of indoors total bacteria and fungi was 1.38×10^4 to 4.81×10^4 and 8.55×10^2 to 3.25×10^3 respectively. The microbial concentration varied with the type of ventilation and this was found to be in agreement with the observations of Jung *et al*. [14], who described the range of bacteria and fungi as 3.19×10^2 to 1.37×10^3 and 2.42×10^2 to 1.73×10^3, respectively. According to the authors, the levels of bioaerosols were higher in non-central air conditioning as compared to central-air conditioning spaces. Similar observations were made in the current study, where group II had higher bioaerosols emissions as compared to group I hospitals. The group I had AHU ventilation, in which the outdoor air was periodically introduced to indoor as compared to group II hospitals which had single split air conditioner indoor unit without introduction of fresh air. The filtration system,

directional flow and increased air change per hour, and introduction of fresh air to indoor can reduce bioaerosols concentration [33]. The present study is also in agreement with a recent review by Stockwell *et al*. [34] reporting less levels of bioaerosols in mechanically ventilated inpatient rooms as compared to the naturally ventilated room in hospitals. Moreover, they also reported that gram-positive bacteria mostly prevail in health care facilities which was also observed in current study. However, a study from Pakistan [12], has reported comparatively lower airborne levels of bacteria and fungi in a Heating, ventilation and Air Conditioning (HVAC) supported hospital ranging from 2.21×10^2 to 1.65×10^3 and 4.1×10^1 to 1.93×10^2 cfu/m^3 respectively. This was probably due to different bioaerosols sampling procedure, duration and filter sampler, and sampling conditions [35]. Moreover, the construction activities were frequently observed in the group I and II hospitals which may also resulted increased bioaerosols concentration. According to many studies [36,37] the bioaerosols concentration in health care facilities can be controlled with the use of sophisticated mechanical ventilation providing the maintenance activities are not compromised. Although in this study the bacterial and fungal levels were less in group I compared to group II hospitals, were still far higher than the recommended levels of 1×10^3 and 5×10^2 cfu/m^3 respectively by World Health Organization (WHO) [38], reflecting the compromised microbial air quality in hospitals.

In this study, the microbial counts recorded by culture-based methods were less as compared to the FCM method and this is consistent with other studies [39,40]. This confirms the limitations of culture-based method for the quantitative characterization of bioaerosols. The culture-base methods are laborious, time consuming and offer underestimation of microbial load [41]. Moreover, all viable bioaerosols may not be recovered by culture-based method due to specific use of cultivation media and incubation temperature [42]. In the current study, a relatively low bacterial count was observed in hospital indoors as compared to outdoor sites that is consistent with the finding by Jung *et al*. [14] and Sudharsanam *et al*. [43]. However, the fungal count was higher in the indoors that was likely due to various factors such as ventilation system, increase humidity and release of droplets during various medical procedures, that can favor fungal growth. The microbial diversity was found to be rich indoors as compared to outdoors, probably due to more diverse sources indoors.

Conclusions

The effective infection control management in hospitals needs knowledge on dynamics of bioaerosols. The 16S rRNA-based PCR assay and sequencing of airborne bacterial isolates and phylogenetic analysis showed taxonomical relationship to international isolates available in NCBI Genbank. Ventilation can play a vital role in managing air hygiene in health care facilities, particularly in context of ongoing COVID-19 pandemic, and our results showed bioaerosols concentration varies with the type of ventilation. Moreover, indoor hospitals had less concentration of bacteria, but higher levels of fungi as compared to the outdoors. Gram-positive bacteria were frequently recovered from both indoors and outdoors hospitals. Overall, in all hospitals microbial levels were higher than the ones recommended by WHO. Pakistan had first infection control guidelines during 2006, but is still struggling with the compliance of guidelines. Various factors such as ventilation, indoor activities, along with management practices affects the bioaerosols concentration. The higher FCM counts compared to culture-based techniques clearly demonstrate the limitations of culture-based methods to inform about magnitude and nature of bioaerosols. This study provide snapshot of bioaerosols in healthcare settings of Pakistan and can help policy regulators to understand underlying factors enhancing the risk of airborne infection and develop mitigation strategies. Moreover, the genotypic characterization and phylogenetic analysis of airborne microbial strains can provide better understanding about the epidemiology and help to improve strategies to monitor and control bioaerosols.

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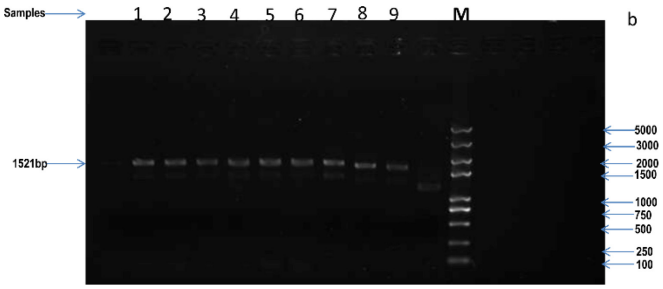
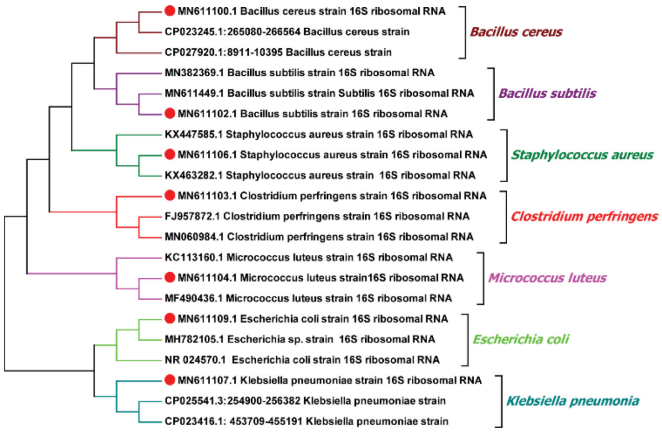
Annex – Supplementary Items

Supplementary Figure 1. Analysis of partially amplified 16S rRNA gene.



Arrows show the band of 16S gene bp that amplified by PCR, Marker (M): 5000bp, DNA ladder, Lanes 1-9 bands of 16S gene.

Supplementary Figure 2. Phylogenetic tree showing genetic relationships between bacterial isolates recovered in current study (red circles) and of closely related sequences from NCBI Genbank, based on 16S rDNA.



Evolutionary relationships of taxa neighbor-joining method was used to infer the evolutionary history1.The optimal tree with sum of branch length =0.67430023 is displayed. Maximum Composite Likelihood method was used to calculate evolutionary distances2 and are displayed in the units of numbers of base substitutions per site. The analysis involves 21 nucleotide sequences. The positions having gaps and missing data were excluded and therefor 1344 positions were considered in the final data set. MEGA6 was used to conduct the evolutionary analysis