Microbiome Profiles of Nebulizers in Hospital Use

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Abstract

Background: Nebulizers are used to provide treatment to respiratory patients. Concerns over nosocomial infection risks from contaminated nebulizers raise the critical need to identify all microbial populations in nebulizers used by patients. However, conventional culture-dependent techniques are inadequate with the ability to identify specific microbial populations only. Therefore, the aims of this study were to acquire complete profiles of microbiomes in nebulizers used by in-patients with culture-independent high-throughput sequencing and identify sources of microbial contaminants for the development of effective practices to reduce microbial contamination in nebulizer devices.

Methods: This study was conducted at the University of Tennessee Medical Center in Knoxville, TN, USA. Nebulizers were collected between May 2018 and October 2018 from inpatients admitted to the floors for pneumonia or chronic obstructive pulmonary disease (COPD) exacerbations. Nebulizers were sampled for 16S rRNA gene-based amplicon sequencing to profile nebulizer microbiomes and perform phylogenetic analysis. A Bayesian community-wide culture-independent microbial source tracking technique was used to quantify the contribution of human-associated microbiota as potential sources of nebulizer contamination.

Results: Culture-independent sequencing detected diverse microbial populations in nebulizers, represented by 18 abundant genera. *Stenotrophomonas* was identified as the most abundant genus, accounting for 12.4% of the nebulizer microbiome, followed by *Rhizobium*, *Staphylococcus*, *Streptococcus*, and *Ralstonia*. Phylogenetic analysis revealed the presence of multiple phylotypes with close relationship to potential pathogens. Contributing up to 15% to nebulizer microbiomes, human-associated microbiota was not identified as the primary sources of nebulizer contamination.

Conclusion: Culture-independent sequencing was demonstrated to be capable of acquiring comprehensive profiles of microbiomes in nebulizers used by in-patients. Phylogenetic analysis identified differences in pathogenicity between closely related phylotypes. Microbiome profile-enabled community-wide culture-independent microbial source tracking suggested greater importance of environmental sources than human sources as contributors to nebulizer microbiomes, providing important insight for the development of effective strategies for the monitoring and control of nebulizer devices to mitigate infection risks in the hospital.

Introduction

The spread of nosocomial infections during hospital stays is a major challenge facing modern healthcare.[1] Respiratory infections represent an important class of nosocomial infections[2], which is especially problematic for patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) who employ nebulizers on a consistent basis.

Respiratory patients frequently require the use of nebulizer devices to deliver fine particle aerosols deep in the respiratory tract.[3] Nebulizer devices, if contaminated with pathogens, could deliver aerosolized medication along with pathogens to the distal areas of the respiratory system, putting patients at elevated risk of infection.[4] The pathogens in used nebulizers could be attributed to the deposition of sputum and pulmonary aerosols from the patient during nebulizer therapy. Additionally, nebulizers are constantly exposed to the ambient environment during usage and storage. Thus, when the ambient environment is contaminated by pathogens, aerosols and particulates could serve as potential sources of microbial contamination in nebulizers. Moreover, microbial contaminants could be introduced into the nebulizers if improper procedures are used in the cleaning of used nebulizers. Therefore, microbial contamination of

nebulizers has been investigated among respiratory patients.[5-7] Pathogens identified in nebulizers include *Burkholderia cepacia*[8], *Pseudomonas aeruginosa*[9], and *Stenotrophomonas maltophilia*.[10] Previous studies also identified other populations of potential pathogens in nebulizer devices, including *Streptococcus*[11], *Staphylococcus*[12, 13], *Rothia*[14], and *Ralstonia*.[15]

Notably, previous studies have relied on culture-based techniques to identify specific populations of pathogen contaminating nebulizer devices.[7, 16] It is known that the large majority of microorganisms are not amenable to laboratory cultivation.[17] Even for microorganisms that can be cultured in the laboratory, certain populations could remain viable but unculturable.[18] Thus, it is highly likely that a significant portion of the microbial contaminants would be undetectable by culture-based techniques. More importantly, given the diversity of pathogens potentially present in nebulizer devices used by patients[7, 9, 10], culture-based techniques are unlikely to capture the majority of microbial contaminants. This challenge could be overcome with the application of culture-independent sequencing-based techniques well suited for the comprehensive profiling of microbiomes[19, 20], defined as the entire microbial community in a habitat.[21] Therefore, it is hypothesized that culture-independent sequencing-based techniques would provide the capacity to profile microbial contaminants in used nebulizers at the microbiome-scale.

In this study, jet nebulizers used by in-patients (most of whom had COPD/pneumonia) were investigated. The application of 16S rRNA gene-based amplicon library sequencing provided a comprehensive profile of microbial communities established in nebulizer devices following patient use, presenting much needed insight for the development of strategies to mitigate risks arising from potential microbial contamination of medical devices.

Materials and Methods

Sample collection

Jet nebulizers (Model 210, Westmed, Tucson, AZ, USA) were collected between May and October 2018 from adult subjects admitted to the University of Tennessee Medical Center (UTMC) hospital, Knoxville, TN, USA with a respiratory condition other than CF, that required nebulized treatments (Table 1). Subjects were included in this study if they were admitted for a medical illness requiring routine use of a nebulizer, over the age of 18 years old, and able to understand and cooperate with the study assessments. Subjects were excluded if they had a known HIV infection, a drug-resistant TB infection, or other transmissible diseases without a known treatment. In this study, all patients used nebulizers with a mouthpiece. None of the patients had a tracheostomy. Nebulizers used by subjects were collected by certified respiratory therapists and placed in sterile plastic bags for further analysis. No samples were taken directly from the subjects.

The devices were collected during a subject's hospital stay according to the following scheme so that comparisons could be made between nebulizers used by the same patient but with different durations (Supplementary Fig. 1): an unused nebulizer was provided to the subject upon admission. After being used by the subject for 24 hours, the used nebulizer was collected for analysis and designated as the 24-hr sample. A second unused nebulizer was subsequently provided to the same subject. After being used by the subject for 48 hours, the 2nd nebulizer was collected for analysis and designated as the 48-hr sample. If the subject remained hospitalized, a third unused nebulizer was provided to the subject. After being used by the subject for 72 hours, the 3rd nebulizer was collected for analysis and designated as the 72-hr sample. Since the availability of 72-hr samples was limited to subjects with a hospital stay of at least six days, only

one 72-hr nebulizer sample was collected during the study period. The University of Tennessee Graduate School of Medicine Institutional Review Board (UTGSM IRB) approved the study procedures, including consent by hospitalized subjects requiring the use of a nebulized therapy, as described in UTGSM IRB Protocol 4279.

Sample processing

The nebulizers, sealed in sterile plastic bags, were kept on ice in an insulated container and transported to the laboratory for analysis. The nebulizers were processed immediately upon receipt in the laboratory for both 16S rRNA gene-based amplicon sequencing to profile the microbial populations and heterotrophic plate count (HPC) analysis, which was used as a measure of the abundance of heterotrophic bacterial populations in the nebulizers. Sterile polyester-tipped swabs moistened with sterile phosphate buffered saline (PBS) were used to collect microbial biomass from nebulizer parts as previously described.[22] The swab samples were preserved at -80°C for subsequent DNA extraction and amplicon sequencing.

For HPC analysis, nebulizers were aseptically dissected to allow the parts of the devices to fit in 50-mL centrifuge tubes for vortexing in PBS as previously described.[23] The resulting suspensions represented microbial biomass removed from the nebulizer parts, which were plated onto agar plates for HPC analysis according to standard protocols as previously described.[24] HPC analysis was conducted for individual parts of the nebulizer assembly (Fig. 1) in order to identify the parts with the highest level of microbial contamination.

High-throughput sequencing

Sequencing of 16S rRNA gene amplicon libraries were conducted following previously described protocols for in-depth microbial community analysis.[24-26] To extract DNA from swab samples, the FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA) was used following the manufacturer's instructions. DNA extracts were then subjected to polymerase chain reaction (PCR) amplification using 515F and 806R as the forward/reverse primers with unique 12-base specific barcodes.[24-26] These primers target the V4 region of the 16S rRNA genes. Clean-up of PCR products was performed with the ChargeSwitch Nucleic Acid Purification Technology (Invitrogen, Carlsbad, CA, USA) to remove contaminants including primer dimers. Purified PCR products were pooled and the subsequent amplicon library concentration was measured using the KAPA Illumina Library Quantification Kit (Kapa Biosystems, Wilmington, MA, USA). Paired-end sequencing of the amplicon library was conducted with the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Unused nebulizers were tested as negative controls and processed along with other nebulizers using the same protocols for DNA extraction, PCR amplification, and clean-up of PCR products. The lack of detection of PCR products from the negative controls prevented the inclusion of negative controls in the pooled amplicon libraries for sequencing. As a result, no sequence data were available from the negative controls.

Data analysis

Raw sequence reads were initially processed with QIIME2 version 2020.6 for quality filtering.[27] The SILVA database (release 138) was used for general taxonomic classification of sequences via the QIIME2 platform.[28, 29] Sequences were clustered into operational taxonomic units (OTUs) with QIIME2 using the de novo clustering protocol with the similarity threshold set

at 97%. A phylogenetic tree was constructed of the OTUs with an average relative abundance greater than 2.5% with MEGA X[30-32] using the neighbor-joining algorithm with 1,000 bootstrap re-samplings.[30-32] Taxonomic context of selected OTUs was represented by closest relatives to these OTUs, which were identified by SeqMatch as the best sequence match in the Ribosomal Database Project (RDP) database with validly published taxonomy nomenclature.[33] The R package ggplot2 was used to visualize all data, including non-sequence data.[34] Raw sequence reads were deposited at the sequence read archive database (SRA) of NCBI with the accession numbers SRX10150882 and SRX10151646-SRX10151656.

Source identification

The sources of microbial contamination were analyzed using the SourceTracker software as previously described.[35] The three sources analyzed were human fecal, oral, and skin microbiota with data from the Earth Microbiome Project and the European Nucleotide Archive, which included 467 human gut and fecal samples (accession number ERR1866468-ERR1867190, ERR1867465-ERR1867524, and ERR1868423-ERR1868675), 992 human skin samples (accession number ERR1867196-ERR1867464 and ERR1867837-ERR1868161), and 509 human oral samples (accession number ERR1868164-ERR1868674 and ERR1868679-ERR1868749). These datasets were selected as the sequences were obtained with the same sequencing primers that were used in this study.

Results

Microbial contamination of jet nebulizers

The extent of microbial contamination in nebulizers collected in this study was determined by HPC analysis of microbial abundance. Unexpectedly, extended use did not necessarily lead to higher levels of microbial contamination, as no statistically significant differences were found between the 24-hr and 48-hr samples taken from any part of the nebulizer assembly (Fig. 2).

HPC analysis of unused nebulizers was unable to detect any microbial growth, suggesting very low levels of microbial contamination in unused nebulizers. HPC analysis further revealed the lack of statistically significant differences between the mouthpiece, T-piece, and corrugated tubing of the nebulizers (Fig. 2).

Composition of microbiomes in nebulizers

The prevalence of microbial presence in used nebulizers (Fig. 2) makes it all the more important to determine the identify of these microbial populations and evaluate their potential health impacts. Accordingly, the identities of all microbial populations in used nebulizers were profiled using 16S rRNA gene-based amplicon library sequencing.

It should be noted that DNA extraction from unused nebulizers followed by PCR amplification did not yield detectable amounts of DNA for sequencing-based analysis, indicating the lack of microbial contamination in unused nebulizers. These results further demonstrated that no contaminants were introduced by the testing protocols used in this study.

Composition at phylum level

Proteobacteria was identified as the most abundant population at the phylum level, accounting for 50.6±22.1% of the microbiomes in nebulizers on average (Fig. 3). Comprising 24.1±21.0% of the nebulizer microbiomes, *Firmicutes* represented the second most abundant phylum in used nebulizers, followed by *Actinobacteria*, *Acidobacteria*, and *Chloroflexi*, with relative abundance averaging 8.4±4.0%, 3.8±4.6%, and 2.3±2.5%, respectively.

It should be noted that populations associated with *Acidobacteria* and *Chloroflexi* are frequently found to inhabit niches in aquatic and soil environments.[36-38] Thus, it is surprising that microorganisms typically present in the environment such as *Acidobacteria* and *Chloroflexi* were identified as two of the most abundant phyla in used nebulizers (Fig. 3). The other three dominant contributors to nebulizer microbiomes, i.e. *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, have constituents with diverse functions and habitats.[39-42] A more careful examination at finer taxonomic resolution is warranted to evaluate the potential significance of these microbial contaminants in pathogenicity and infection.

Composition at genus level

At the genus level, *Stenotrophomonas* was identified as the dominant constituent of the nebulizer microbiome, accounting for 12.4% of the microbial abundance on average (Fig. 4). Representing 11.9% of the nebulizer microbiome, *Rhizobium* was found to be the second most abundant genus in used nebulizers, followed by *Staphylococcus*, *Streptococcus*, and *Ralstonia*, with relative abundance averaging 10.7%, 4.0%, and 3.6%, respectively (Fig. 4). Of these five most abundant genera, three (i.e. *Stenotrophomonas*, *Rhizobium*, and *Ralstonia*) belong to *Proteobacteria*, the most predominant phylum (Fig. 3). The other two genera with high abundance (i.e. *Staphylococcus* and *Streptococcus*) are associated with *Firmicutes*, the second

most abundant phylum identified in the nebulizers (Fig. 3). Thus, the distribution of microbial populations at the genus level is consistent with the pattern observed at the phylum level.

Notably, members of *Staphylococcus* and *Streptococcus* are known to have humans as hosts.[43, 44] In contrast, populations of *Rhizobium* are soil bacteria specialized in nitrogen fixation.[45, 46] The genera of *Stenotrophomonas* and *Ralstonia*, however, have members identified in both natural environments and human hosts.[15, 47, 48] Nonetheless, these observations point to possibilities that both the patient and environment could be sources contributing to nebulizer contamination.

Phylogenetic analysis of abundant phylotypes

Further analysis revealed the presence of multiple phylotypes in the same genus identified in used nebulizers. Among the 7 genera with relative abundance greater than 2.5%, both *Streptococcus* and *Staphylococcus* were represented by 4 OTUs, followed by *Pseudomonas* and *Burkholderia*, having 3 and 2 OTUs, respectively (Fig. 5). The other three prevalent genera, i.e. *Stenotrophomonas*, *Rhizobium*, and *Ralstonia*, each only had one OTU identified across all nebulizers (Fig. 5). Five of the bacterial genera represented in Fig. 5, i.e. *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Burkholderia*, and *Stenotrophomonas*, have strains found to be potential pathogens. However, the phylogenetic diversity observed in some of the microbial genera suggests the presence of closely related but potentially functionally divergent populations, which needs to be further evaluated.

Streptococcus

Of the four OTUs comprising the genus *Streptococcus*, OTU3888 was the most prevalent (Fig. 5). Phylogenetic analysis found OTU3888 to be most closely related to *Streptococcus parasanguinis* (Fig. 6), a common oral bacterium.[49] Similarly, OTU3513 was shown to be most closely related to *Streptococcus sobrinus* (Fig. 6), another common oral bacterium.[50] The other major *Streptococcus* OTUs, OTU2060 and OTU2888, were related to zoonotic pathogens *Streptococcus equi* and *Streptococcus suis*.[51, 52]

Staphylococcus

The genus of *Staphylococcus* had four phylotypes detected in the nebulizers; however, only one phylotype, OTU0963, was prevalent. In fact, OTU0963 was the dominant phylotype accounting for more than 56% of the microbial sequences in nebulizers used by patient D (Fig. 5). The other three *Staphylococcus* phylotypes were not present as significant populations as none had relative abundance greater than 0.1% in any nebulizer. Phylogenetic analysis of OTU0963 indicates that this phylotype is most closely related to *Staphylococcus aureus* (Fig. 6), which is a common constituent of the human microbiota and recognized as opportunistic pathogens in certain infections.[12, 53]

Pseudomonas

Microbial populations identified as *Pseudomonas* comprised of three phylotypes (Fig. 5). OTU1152 was found to be most closely related to *Pseudomonas borealis* (Fig. 6), while OTU0935 was closely associated with *Pseudomonas viridiflava* (Fig. 6). The ecological niches of both *P. borealis* and *P. viridiflava* have been linked exclusively to natural habitats.[54, 55] In contrast, another *Pseudomonas* phylotype identified with significant presence in nebulizers, i.e.

OTU4190 (Fig. 5), was most closely related to *Pseudomonas aeruginosa* (Fig. 6), a well-documented opportunistic respiratory pathogen.[56]

Burkholderia

The genus *Burkholderia* was identified as part of the nebulizer microbiome with two phylotypes. However, only one phylotype, OTU2020, was detected as prevalent (Fig. 5). Phylogenetic analysis revealed that OTU2020 was most closely related to *Burkholderia cepacia* (Fig. 6), a well-known respiratory pathogen that has been isolated from nebulizers in a previous study.[8] More importantly, OTU2020 was detected in nebulizers used by every patient in this study (Fig. 5), thereby indicating the potential infection risk with this microbial contaminant.

Stenotrophomonas

Sequences of *Stenotrophomonas* retrieved from used nebulizers belong to a single phylotype, OTU2948 (Fig. 5). Notably, this phylotype was among the most frequently detected microbial populations in nebulizers, representing the most abundant microbial population in the majority of nebulizers tested in this study (Fig. 5). Phylogenetic analysis links OTU2948 most closely to *Stenotrophomonas maltophilia* (Fig. 6), which has been recognized as an emerging opportunistic pathogen particularly for respiratory illnesses[57] and shown to contaminate nebulizers in previous studies.[10] It should be noted that *S. maltophilia* has also been identified in diverse natural habitats as non-human pathogens.[58]

Ralstonia

Sequences of *Ralstonia* were detected in all nebulizers, accounting for as much as 13.9% of the nebulizer microbiome (Fig. 5). Phylogenetic analysis classified all sequences of *Ralstonia* in used nebulizers into one single phylotype, OTU4012, which is most closely linked to *Ralstonia syzygii* (Fig. 6). *R. syzygii* is well known for its association with plant and plant diseases.[48]

Rhizobium

Sequences of *Rhizobium* retrieved from used nebulizers formed a single phylotype, OTU0709 (Fig. 5), which was phylogenetically linked to *Rhizobium leguminosarum* (Fig. 6). Populations of *R. leguminosarum* have been reported to specialize exclusively in nitrogen fixation in close association with plant roots[46], suggesting *Rhizobium* in used nebulizers were likely derived from soil.

The occurrence of *Rhizobium* in nebulizers was characterized by substantial variations. For example, *Rhizobium* represented the most abundant population in nebulizers used by one patient, accounting for more than 52% of the sequences retrieved; however, *Rhizobium* was not detectable in some other nebulizers (Fig. 5). These observations appeared to be consistent with patterns of accidental contamination from the environment, which needs further examination.

Source identification of nebulizer microbiomes

Analysis of microbiomes in used nebulizers identified microbial populations potentially associated with both human and environmental sources (Fig. 6). Infection risks from exposure to microbial contaminants from human sources are of particular concern as some of these microbial populations can be potential pathogens. SourceTracker, a Bayesian community-wide culture-independent microbial source tracking technique[35], was used to quantify the contribution of

skin, gut, and oral microbiota as three sources of human-associated microorganisms to the microbiomes in nebulizers. It was found that human gut, skin, and oral sources were not the primary contributors to nebulizer microbiomes, consistently accounting for less than 15% of the microbiomes identified in used nebulizers (Fig. 7). The only exception was nebulizers used by patient F, where over 50% of the microbial abundance could be attributed to human skin, indicative of the occurrence of considerable differences in nebulizer contamination between subjects that needs to be further investigated in future studies. The observation that human oral microbiota was not a major source of nebulizer microbiomes, with the highest contribution at 14±20% (Fig. 7), was an unexpected finding in this study. In contrast, the large majority of the microbiomes in nebulizers, i.e. 82% on average, had unidentified sources. Given that microbial populations dominant in used nebulizers, such as *Stenotrophomonas* and *Rhizobium* (Fig. 4), have been frequently identified in indoor environments[24], it is possible that the indoor microbiome had significant impacts on the microbiomes in used nebulizers.

Discussion

Microbial contamination of nebulizers is a significant concern for respiratory patients, as pathogens could be introduced into the respiratory tract leading to elevated risks for infection. The identification of pathogens in used nebulizers has relied on culture-dependent techniques targeting specific pathogenic populations associated with microbial taxa including *Pseudomonas*[7, 16], *Staphylococcus*[7, 16], *Burkholderia*[8], and *Stenotrophomonas*.[10] However, culture-dependent techniques are not suitable for the acquisition of complete profiles of microbial contaminants in nebulizers due to the following limitations: 1) inability to identify the large majority of microbial contaminants which are known to be recalcitrant to laboratory

cultivation[17]; 2) inability to detect significant portions of the microbial contaminants that are viable but unculturable[18]; and 3) inability to target each population of diverse microbial contaminants with population-specific media and cultivation conditions.[7, 9, 10]

In this study, the application of culture-independent 16S rRNA gene amplicon library-based sequencing enabled the profiling of all microbial populations present in used nebulizer (Fig. 3 and 4). In addition to microbial taxa identified previously with culture-dependent techniques such as *Pseudomonas*, *Staphylococcus*, *Burkholderia*, and *Stenotrophomonas*, the culture-independent sequencing technique revealed the presence of diverse microbial populations including *Achromobacter*, *Blautia*, and *Sphingomonas* (Fig. 4). More importantly, the culture-independent sequencing technique was able to distinguish closely related phylotypes. For example, four phylotypes of *Streptococcus* were identified in this study (Fig. 5), with each having a unique pattern of occurrence in used nebulizers. The capability to distinguish closely related microbial populations also enabled fine-resolution phylogenetic analysis to evaluate potential differences in pathogenicity between closely related phylotypes, which is illustrated by the differentiation of pathogenic and non-pathogenic populations of *Pseudomonas* identified in the nebulizers (Fig. 6).

The ability to acquire complete profiles of microbial populations in nebulizers also made it possible to apply Bayesian community-wide culture-independent microbial source tracking technique[35], which is much more robust than microbial source tracking tools relying on specific indicator organisms. The deficiencies of indicator-dependent microbial source tracking tools are best illustrated with *E. coli* as the indicator for fecal sources.[59] Using profiles of microbiomes in nebulizers from culture-independent sequencing, it was found that human sources, which included gut, oral, and skin microbiota, were not significant contributors to microbiomes present

in nebulizers (Fig. 7). Instead, microbial populations typically present in the environment were found to account for a considerable fraction of the microbial contaminants in nebulizers. These results are consistent with findings from HPC analysis, a traditional culture-dependent technique. It was observed that longer duration of use did not necessarily lead to higher levels of microbial contamination, as no statistically significant differences were found between the 24-hr and 48-hr samples taken from any part of the nebulizer assembly (Fig. 2). The duration of use could be considered as a direct measure of contamination by the patient. The lack of significant influence by the duration of use on microbial contamination points to the possibility that the primary source of nebulizer contamination might not be attributable only to the subjects. HPC analysis further revealed the lack of statistically significant differences between the mouthpiece, T-piece, and corrugated tubing of the nebulizers (Fig. 2). It is reasonable to assume that different parts of the nebulizer would have varying levels of interaction with the patient, hence contamination. The similarity in the level of microbial contamination between nebulizer parts, however, supports the finding that microbial contaminants in the nebulizer might also originate from sources other than the subjects, which is consistent with the findings from the Bayesian community-wide culture-independent microbial source tracking technique used in this study.

Additionally, these results were consistent with the OTUs identified in the nebulizers with high abundance. Potential contamination from human sources was suggested by several OTUs that were closely related to the normal human microbiota (Fig. 6), including OTUs 3888 (*Streptococcus parasanguinis*, a common oral bacterium)[50], 3513 (*Streptococcus sobrinus*, a common oral bacterium)[51], and 0963 (*Staphylococcus aureus*, a common skin bacterium).[44] However, more OTUs were found to be closely related to non-human sources (Fig. 6), including OTUs 2060 (*Streptococcus equi*)[53], 2888 (*Streptococcus suis*)[52], 1152 (*Pseudomonas*

borealis)[55], 0935 (*Pseudomonas viridiflava*)[56], 2948 (*Stenotrophomonas maltophilia*)[48, 59], 4012 (*Ralstonia syzygii*)[49], and 0709 (*Rhizobium leguminosarum*).[47] These observations support the interpretation of microbial source tracking and HPC results that non-human sources might be important contributors of microbial contaminants to the nebulizers.

Limitations

While high-throughput sequencing as a culture-independent technique has the unique ability to acquire comprehensive profiles of the entire microbiome present in the nebulizer, it relies on the presence of microbial DNA for analysis. Since genomic DNA can persist for a period of time subsequent to the death of the bacterial cell, results from high-throughput sequencing may include dead cells. This is not desirable if only live cells are of interest. This technical limitation could be mitigated in part with the inclusion of specific culture tests that enumerate live cells only following the identification of bacterial taxa of interest by high-throughput sequencing.

It should also be noted that the profiles of microbiomes provided by culture-independent high-throughput sequencing is based on relative abundance, which is not a measure of the bacterial cell counts that are typically provided by conventional cell cultures. One strategy to address this limitation is to perform culture-independent techniques specifically designed for the quantitation of target microbial taxa, such as real-time quantitative polymerase chain reaction (RT-qPCR) assays. Another strategy to mitigate this limitation is to combine the sequencing technique with other methods capable of enumerating the overall microbial abundance, which could be achieved with both culture-dependent and culture-independent assays, such as HPC and RT-qPCR. Subsequently, the abundance of individual taxa could be derived based on the relative abundance determined by high-throughput sequencing. This strategy is capable of quantifying

the abundance of all individual bacterial taxa present in the nebulizer by overcoming the inability of culture-dependent techniques to target each microbial taxon in a complex mixture of microbial contaminants with taxon-specific media and cultivation conditions.

Additionally, results from culture-independent sequencing techniques as well as other molecular methods could be complicated by contaminants introduced from supplies and processes used in the laboratory protocols.[60] While the protocols adopted in this study did not directly demonstrate the significance of these contaminants, specific efforts are needed in full-scale studies to identify and mitigate potential interferences from artifacts introduced in the laboratory.

It should be noted that the 16S rRNA gene amplicon sequencing technique evaluated in this study relies on the 16S rRNA gene as the biomarker for taxonomic annotation. Despite the extensive use of 16S rRNA gene sequences for taxonomic studies, these biomarkers might not be adequate for accurate identification of microbial populations at fine taxonomic resolutions. Techniques capable of providing full-length microbial genome sequences, such as metagenome or whole genome sequencing, are recommended as more superior technical alternatives when possible.

Conclusion

In this study, culture-independent sequencing was demonstrated to be capable of acquiring complete profiles of microbiomes present in nebulizers used by in-patients. In addition to microbial taxa identified previously with culture-dependent techniques such as *Pseudomonas* and *Staphylococcus*, the culture-independent sequencing technique revealed the presence of diverse microbial populations including *Blautia* and *Sphingomonas*. Phylogenetic analysis of

microbiome profiles distinguished closely related microbial populations to enable assessment of potential differences in pathogenicity between closely related phylotypes. Bayesian community-wide culture-independent microbial source tracking further revealed that non-human sources likely contributed more to the microbiomes in nebulizers. Findings from this study have important implications on developing effective strategies for the monitoring and control of nebulizer devices to improve patient care in the hospital.

Author Contribution Statement

QH and RD conceived and designed research. QH acquired funding. CSE and JW conducted sampling. CSS and LC conducted laboratory testing and analysis. CSS and LC analyzed data. CSS, QH and RD wrote the manuscript. CSS, LC and JW revised the manuscript. All authors read and approved the manuscript.

Author Disclosure Statement

The authors declare they have no competing financial interests.

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