

Metagenomics profiling of the lower respiratory tract microbiome and relevant respiratory pathogens in pediatric intensive care unit patients: a pilot exploratory study in Egypt

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Background: Lower respiratory tract infections (LRTIs) are a leading cause of mortality in children. These infections disrupt the equilibrium of lower respiratory tract (LRT) microbiota, allowing respiratory pathogens to dominate. The conventional culture method has limitations in describing complex microbiomes and may fail in the detection of respiratory pathogens. In the present study, we sought to use the advanced technology of 16S metagenomics next-generation sequencing (16SmNGS) to characterize the LRT microbiome among children with LRTIs and to identify the underlying respiratory pathogens that commonly evade detection by traditional culture.

Methods: Twenty LRT specimens from hospitalized children with LRTIs were analyzed using 16SmNGS, as well as standard microbiological culture.

Results: The 16SmNGS taxonomical analysis revealed the highest relative abundances for *Streptococcus* (27.7%) and *Escherichia* (13.3%) genera, which belong to the phyla of Firmicutes (45.4%) and Proteobacteria (45.3%), respectively. *Streptococcus pneumoniae* (45%), *Escherichia coli* (45%), *Pseudomonas aeruginosa* (15%), *Staphylococcus aureus* (10%), *Acinetobacter baumannii* (5%), and *Haemophilus influenzae* (5%) were the primary respiratory pathogens. Conventional culture failed to detect growth in 100%, 77.7%, and 55.5% of 16SmNGS-positive specimens for *H. influenza*, *S. pneumoniae*, and *E. coli*, respectively.

Conclusions: The 16SmNGS technique revealed a predominance of *Streptococcus* and *Escherichia* genera belonging to the phyla of Firmicutes and Proteobacteria in pediatric LRTIs. In this exploratory study, 16SmNGS was able to enhance the identification of significant respiratory pathogens, particularly those difficult to isolate in culture. However, to rule out contamination by flora, it is advisable not to interpret metagenomics results independently from culture, clinical, and radiological data. In addition, further clinical correlations are desired to reach appropriate clinical decisions.

Key Words: hospitalized children; intensive care unit; metagenomics, microbiome; respiratory tract

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INTRODUCTION

At present, infectious diseases pose the primary threat to human health [1]. The rapid progression and severe course of acute lower respiratory tract (LRT) infections (LRTIs) result in unfavorable clinical outcomes, making them one of the most severe infectious illnesses [2-4]. Pediatric patients with compromised health status, especially those admitted to intensive care units (ICUs), are highly susceptible to infections [5]. LRTIs are ranked among the top 10 causes of death worldwide, with the majority of deaths occurring in developing countries and among young individuals [6]. Indeed, the World Health Organization recently reported that LRTI is the primary cause of death among children under the age of 5 years in Egypt [7].

Contracting respiratory pneumonia may occur due to a wide range of etiological pathogens [8]. Bacterial causes are of non-inferior significance and may occur secondary to viral infections, where viruses are considered the most prevalent causes of respiratory tract infections [8]. Microbiological culture to isolate causative bacteria remains the traditional method for diagnosis; however, this approach is a poorly sensitive, time-consuming method that is undermined by several challenges [9], including difficulty in isolating multiple pathogens in combined infections or fastidious pathogens that require special growth requirements, such as *Streptococcus pneumoniae* and *Haemophilus* spp. [10,11]. Moreover, prior empirical intake of antimicrobial agents can attenuate the ability of bacteria to grow in culture [11]. Meanwhile, the emergence of nucleic acid-based molecular techniques has enhanced the laboratory capacity for precise and prompt diagnosis of infections, yet a significant number of existing molecular diagnostics include panels consisting of a predetermined narrow range of microbial targets [11]. Therefore, developing more robust and comprehensive advanced molecular diagnostics is crucial for more accurate and efficient diagnosis of infections [11].

The emergence of 16S metagenomics next-generation sequencing (16SmNGS) has revolutionized the diagnostic field by enabling rapid, sensitive, and accurate microbial identification with the highest degrees of discrimination and confidence, thereby overcoming the drawbacks of conventional methods [12]. The extensive and comprehensive genomic data generated by mNGS has improved our knowledge of the microbial composition in different human body systems and

KEY MESSAGES

- The 16S metagenomics next-generation sequencing (16SmNGS) technique provides a more comprehensive view of the lower respiratory tract (LRT) microbiome and improves pathogen detection in pediatric LRT infections.
- 16SmNGS revealed a taxonomical predominance of *Streptococcus* and *Escherichia* genera in LRT infections and improved the detection of significant respiratory pathogens compared to conventional culture.
- To rule out potential flora contamination, metagenomics results should not be interpreted in isolation from culture results, clinical data, and radiological findings.

their implicated involvement in various health pathologies [12]. Currently, NGS has become widely used as a potent diagnostic tool in clinical microbiology for the identification of infectious diseases, where it provides the advantages of high sensitivity and simultaneous detection of several microbial pathogens [13,14].

Therefore, our objective was to characterize the LRT microbiome in pediatric patients with acute respiratory illness and to highlight the capability of 16SmNGS in capturing respiratory pathogens that are often overlooked by conventional culture.

MATERIALS AND METHODS

The study was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Cairo University (REC: N-39-2024), in accordance with the Declaration of Helsinki guidelines. Written informed consent was obtained from the parents or guardians of the participants.

The study was conducted on LRT samples collected from patients admitted to the pediatric ICU (PICU) of a tertiary-care pediatric hospital over a 3-month period. Patients considered eligible for this study included children with an initial diagnosis consistent with an acute LRTI of any severity, with symptoms such as new onset of cough, dyspnea, fever, increased sputum production, tachypnea or difficulty in breathing and/or radiological findings indicative of pulmonary infection, as shown in Figure 1. The study excluded patients who were admitted to the ICU for non-infectious medical disorders or respiratory lesions (e.g., congenital lung anomalies) or those

with additional infections in other body systems. LRT samples were sent to the microbiology laboratory for microbiological culture and further molecular testing via 16S mNGS.

Specimen Collection and Transport

LRT samples were collected in the form of sputum, endotracheal aspirate (ETA), or respiratory sample via non-bronchoscopic bronchoalveolar lavage (mini-BAL). All samples were collected under stringent precautions to ensure optimal representation of the LRT and to minimize the risk of upper airway contamination. In ventilated patients, ETA was chosen for LRT sample collection because the endotracheal tube (ETT) avoids the upper airway, facilitating direct access to the lower airways and reducing the likelihood of oropharyngeal contamination. Sample collection was performed using a sterile, single-use suction catheter (mucus trap catheter) through the ETT, adhering to complete aseptic procedures. Suction was not applied during advancement of the catheter until resistance was encountered; it was then applied intermittently during withdrawal of the catheter to ensure collection of secretions from the distal airways rather than from the ETT lumen. Finally, the catheter part was removed, and the sterile container was tightly closed and transported to the laboratory. To collect a respiratory sample via mini-BAL, a specialized

BAL catheter connected to the mucus trap was advanced past the ETT and into the distal airway, thereby avoiding contact with the colonized ETT lumen. Sterile saline was instilled in aliquots and subsequently aspirated back. The initial aspirate was discarded to eliminate contaminants from ETT and central airways, while subsequent aliquots were pooled [15]. In older cooperative children, early-morning sputum induction was attempted in the morning after mouth rinse, through inhalation of a nebulized hypertonic saline solution following chest physiotherapy to mobilize deep respiratory secretions; expectorated sputum was then collected in a sterile container [15]. To ensure the quality of LRT samples, Gram smears were performed in the microbiology laboratory, where a sample was deemed valid when it displayed fewer than 10 squamous epithelial cells per low-power field. All collected samples were immediately transported from the PICU to the microbiology laboratory within 30 minutes for microbiological culture and archived for further molecular testing.

In the laboratory, samples were aliquoted aseptically into three cryovials. The first aliquot underwent routine microbiological procedures, and the two other aliquots were stored in a freezer at -80°C . Of these, the second aliquot was used for the molecular testing, with thawing completed once prior to the DNA-extraction step, while the third aliquot was further stored for reference.

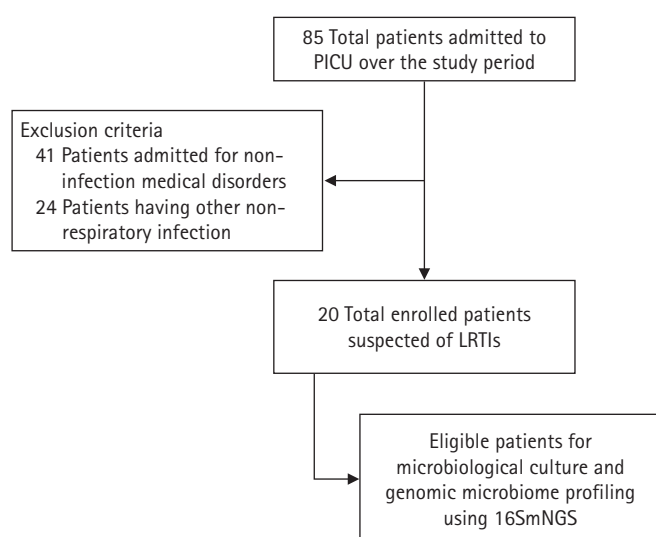


Figure 1. Schematic flowchart for the enrollment of patients eligible for microbiological culture and genomic microbiome profiling using 16S metagenomics next-generation sequencing. PICU: pediatric intensive care unit; LRTI: lower respiratory tract infection; 16S mNGS: 16S metagenomics next-generation sequencing.

Microbiological Culture of LRT Samples

The LRT samples were sent to the microbiology laboratory, as described, where they were cultured on blood agar, chocolate agar, and MacConkey agar media (Oxoid) for overnight incubation at 37°C . The recovered bacterial isolates were identified using conventional microbiological identification methods such as Gram stain and biochemical reactions according to the standard microbiological procedures [16]. Following the cultivation of samples, the residual quantity of samples was stored at -70°C for further molecular analysis.

16S mNGS and Bioinformatics Analysis

Initially, bacterial DNA was extracted from each sample using the QIAamp DNA Mini kit (Qiagen), following the instructions provided by the manufacturer. The extraction control for each specimen was a negative buffer control. Polymerase chain reaction (PCR) amplification of the bacterial 16S gene V1-V2

region was used to determine the presence or absence of DNA during library preparation. The preparation of PCR reactions was carried out using a laminar flow PCR workstation. Subsequently, 12.5 µL of Platinum PCR supermix (Invitrogen) and 0.5 µL of 10 µM of each primer and 11.5 µL of template were added to final reaction volume of 25 µL. DNA amplification was carried out using the following cycle conditions: 95 °C for 5 minutes, then 35 cycles at 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds. PCR products were run on a 2% agarose gel and visualized using ethidium bromide. Final library products were purified using 0.9× of Agencourt AMPure beads (Beckman Coulter), according to the manufacturer's instructions. Following this, the library products were eluted in low Tris-EDTA and quantified using the Qubit dsDNA HS Assay kit (Invitrogen) [17,18]. Safe stops were applied within the allowed time period as directed by the Illumina protocol [17]. The raw sequence data generated from the Illumina MiSeq platform (Illumina) were subjected to quality filtering, processing, and analysis through the EzBioCloud 16S-based Microbiome Taxonomic Profiling pipeline (EzBiome Inc.; <https://www.ezbiocloud.net>). This pipeline conducts an extensive quality-control process, operational taxonomic unit selection, and taxonomic assignment using the EzBioCloud 16S database, which was updated in 2019 [19].

The metagenomic analysis results were compiled according to the findings from phylogenetic mapping. The relative abundance (RA), for each organism was calculated based on the number of reads mapped for each species as a percentage of the root read value, with the species with the largest percentage of reads classified as the species of highest RA. Microbial reads from the mNGS library were reported only after successfully passing all quality-control filters and after ensuring that the identified microbe was not displayed among the negative controls.

Statistical Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences version 20 (IBM Corp.). Data are presented as frequencies (n) and percentages (%). The compared differences were measured using the chi-square test. $P < 0.05$ was considered statistically significant. Cohen's kappa (κ) index was calculated for assessing agreement between results of the NGS assay and conventional culture results.

RESULTS

In the present study, 20 LRT clinical samples were obtained from pediatric patients admitted to the PICU of a tertiary care hospital for microbiological culture and 16S-mNGS.

Demographic and Clinical Characterization of the Patients

The study included 20 specimens from 11 boys (55%) and nine girls (45%) aged between 6 months and 7 years. The predominant symptoms reported were cough (70%), fever (50%), and increased sputum production (55%). Six patients (30%) experienced respiratory distress and required mechanical ventilation. Radiographic findings in all patients were indicative of pulmonary infection (Table 1).

Results of Microbiological Culture

A total of 11 (55%) specimens were culture-positive, with types and frequencies of identified bacteria displayed in Table 2. Culture-positive specimens showed pure and mixed bacterial growth in 8/11 (72.7%) and 3/11 (27.2%) specimens, respectively, with a statistically significant difference ($P = 0.043$). *Pseudomonas* spp. was the most frequently isolated bacteria (15%), followed by *Escherichia coli* spp. (10%), among all cultured specimens ($n = 20$). Culture-negative specimens accounted for 45% of total specimens.

Table 1. Demographic and clinical characterization of the patients

Variable	No. (%)
Sex	
Male	11 (55)
Female	9 (45)
Age (yr)	
1/2–<1	6 (30)
1–<4	10 (50)
4–7	4 (20)
Clinical feature	
Cough	14 (70)
Increased sputum production	11 (55)
Fever	10 (50)
Respiratory distress	6 (30)
Radiographic findings	20 (100)
Mechanical ventilator	
On	6 (30)
Off	14 (70)

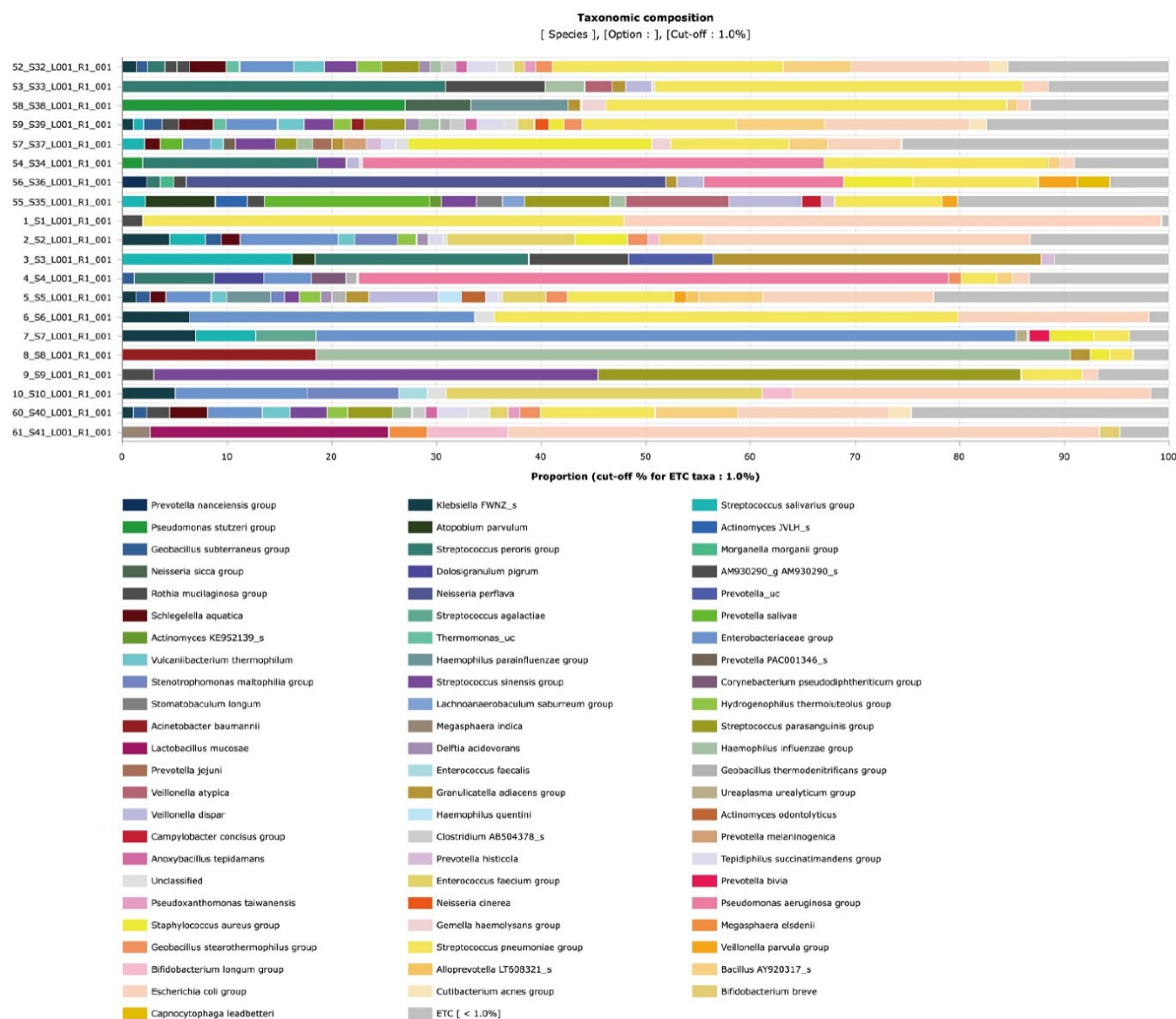


Figure 3. Taxonomical composition of microbes at the “species” level. The taxa of microbial species and their proportions within the ecological taxonomic analysis taxa are displayed in a stacked bar chart. Each color-coded horizontal bar represents a specific bacterial species, with its proportion within the sample. The horizontal axis of the chart labelled as proportion (cutoff % for et cetera [ETC taxa]; other minor taxa: 1.0%) indicates the percentage of each bacterial species present in the sample, while the vertical axis represents the list of analyzed samples.

not detect some organisms due to several factors [12,20]. The advanced technology of NGS metagenomics has demonstrated its value in directly identifying microbial pathogens from clinical samples and revealing the composition of the microbiome in various human systems [10]. The present study used 16S-mNGS to analyze the LRT microbiome and identify the pertinent respiratory pathogens in PICU patients with LRTIs. The demographic data of the gathered respiratory samples indicated a gender distribution in favor of boys (55%) aged

1–4 years (50%), consistent with previous studies examining LRTI distributions [12,21]. Our study revealed that all patients exhibited radiological findings indicating LRTI. Our clinical findings revealed that cough and sputum production were the primary symptoms, while fever and respiratory distress were reported in 50% and 30% of the patients included in this study, respectively. Furthermore, these clinical characteristics were most commonly documented in several studies on LRTIs [12,22]. However, other studies reported a greater prevalence

Table 3. Comparative results of conventional culture and NGS of analyzed specimens

Specimen No.	Culture result	Metagenomic result ^{a)} (RA%)
1	Inhibited growth	<i>Escherichia coli</i> (51%) <i>Streptococcus pneumoniae</i> (45.9%)
2	Mixed growth >3 organisms (Gram-positive and Gram-negative organisms)	<i>Escherichia coli</i> (31%) <i>Enterobacteriaceae</i> (9%) <i>Enterococcus Faecium</i> (12%) <i>Staphylococcus aureus</i> (5%)
3	Inhibited growth	<i>Streptococcus peroris</i> (20.4%) <i>Granulicatella adiacens</i> (31.03%) <i>Streptococcus salivarius</i> (16.2%)
4	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i> (56.4%)
5	Inhibited growth	<i>Escherichia coli</i> (16%) <i>Streptococcus pneumoniae</i> (10%)
6	Mixed growth > 3 organisms (Gram-positive and Gram-negative organisms)	<i>Streptococcus pneumoniae</i> (44%) <i>Enterobacteriaceae</i> (27%) <i>Escherichia coli</i> (18%)
7	<i>Klebsiella</i> spp.	<i>Enterobacteriaceae</i> (66.8%)
8	<i>Acinetobacter</i>	<i>Haemophilus influenzae</i> (72%) <i>Acinetobacter baumannii</i> (18%)
9	Inhibited growth	<i>Streptococcus sinensis</i> (42.3%) <i>Streptococcus parasanguinis</i> (40.4%)
10	<i>Escherichia coli</i>	<i>Escherichia coli</i> (30%)
11	Inhibited growth	<i>Streptococcus pneumoniae</i> (22%) <i>Escherichia coli</i> (13%)
12	Inhibited growth	<i>Streptococcus pneumoniae</i> (35%) <i>Streptococcus peroris</i> group (30%)
13	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i> (44%)
14	Mixed growth (>3 Gram-positive organisms)	<i>Prevotella salivae</i> (15.7%) <i>Streptococcus pneumoniae</i> (10.27%) <i>Veillonella atypica</i> (9.8%) <i>Veillonella dispar</i> (6.95%)
15	<i>Pseudomonas aeruginosa</i>	<i>Neisseria perflava</i> (44%) <i>Pseudomonas aeruginosa</i> (13%)
16	<i>Staphylococcus aureus</i>	<i>Staphylococcus aureus</i> (23%)
17	Inhibited growth	<i>Streptococcus pneumoniae</i> (38%) <i>Pseudomonas stutzeri</i> (27%)
18	Inhibited growth	<i>Streptococcus pneumoniae</i> (14%) <i>Escherichia coli</i> (13%)
19	Inhibited growth	<i>Escherichia coli</i> (14%) <i>Streptococcus pneumoniae</i> (13%)
20	<i>Escherichia coli</i>	<i>Escherichia coli</i> (56%) <i>Lactobacillus mucosae</i> (22%)

NGS: next-generation sequencing; RA: relative abundance.

a) Detected species with the highest RA.

Table 4. Analytical comparison between culture and NGS in terms of known respiratory pathogens with the highest RA in analyzed specimens

Key respiratory potential pathogens	Analyzed specimens (n=20)			
	16SmNGS, sequencing	Culture		Kappa index
		Positive ^{a)}	Negative	
<i>Streptococcus pneumoniae</i>	Positive (n=9)	2	7	0.23
	Negative (n=11)	0	11	
<i>Staphylococcus aureus</i>	Positive (n=2)	2	0	1
	Negative (n=18)	0	18	
<i>Pseudomonas aeruginosa</i>	Positive (n=3)	3	0	1
	Negative (n=17)	0	17	
<i>Escherichia coli</i>	Positive (n=9)	4	5	0.46
	Negative (n=11)	0	11	
<i>Haemophilus influenzae</i>	Positive (n=1)	0	1	0
	Negative (n=19)	0	19	
Other <i>Enterobacteriaceae</i> (<i>Klebsiella</i> spp.)	Positive (n=3)	3	0	1
	Negative (n=17)	0	17	
<i>Acinetobacter baumannii</i>	Positive (n=1)	1	0	1
	Negative (n=19)	0	19	

Positive: present detection of the outlined respiratory pathogen; Negative: absent detection of the outlined pathogen.

NGS: next-generation sequencing; RA: relative abundance; 16SmNGS; 16S metagenomics next-generation sequencing.

a) The isolated microbe is isolated as a single growth or among mixed microbes in culture. Kappa index was used to measure the categorical agreement between 16mNGS and culture results.

of both fever and shortness of breath compared to our study. This disparity may be attributed to variations in the study population and age group [22].

In our study, the microbiological culture showed overall growth in 55% of specimens, with nearly half of the specimens experiencing absent growth (45%). Consistent with previous research, which reported microbial growth in only 59.42% and 78.16% of cultured LRT specimens, this finding highlights the limited effectiveness of conventional microbiological culture, which has long been regarded as the gold-standard method for diagnosing infections [12,21]. Most of the culture-positive specimens in our study demonstrated single pure microbial growth, while 27.7% showed mixed growth of at least three bacteria in culture. This type of specimen is known to exhibit mixed microbial growth in respiratory cultures, which can be attributed to microbial colonization, shed flora, or polymicrobial infection. These findings have been corroborated by prior research that documented the occurrence of combined microbial growth in cultures of LRT specimens at rates of 18.4% and

38.8%, respectively [21,23]. We observed *Pseudomonas* and *E. coli* Gram-negative bacteria as the most frequent isolates in cultured specimens with pure growth. These findings are consistent with the respiratory pathogens documented in several previous studies [24,25]. In addition, these results align with established microbial etiological patterns of infections in hospitals and ICUs [26].

In previous decades, the LRT of healthy individuals was believed to be free of bacteria. However, the development of culture-independent advanced NGS technologies has provided evidence of the presence of microbiota in the healthy LRT. This microbiota encompasses various phyla such as Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria [27-29]. These microbiota are most likely migrating from the upper airways and oropharynx [27]. As reported in the literature, the structure of the respiratory microbiome can be altered due to several factors, such as age, diet, smoking habits, and underlying chronic respiratory illness [28]. In our patients with LRTIs, 16SmNGS revealed a prevalence of Firmicutes over the phyla Proteobacteria and Bacteroidetes. This can be attributed to overrepresentation of the *Streptococcus* genus, including *S. pneumoniae*, which is classified under the phylum Firmicutes. Furthermore, NGS revealed that *E. coli*, *Pseudomonas*, and *Enterobacteriaceae* (phylum: Proteobacteria) were the most commonly identified respiratory pathogens with the highest RA. These results are consistent with the primary identified genera of lung microbiota in prior research, which include *Streptococcus*, *Enterobacteriaceae*, and *Pseudomonas* [30,31]. *S. aureus* was detected in two patients, and *A. baumannii* and *H. influenzae* were each detected once by NGS, although the latter did not grow in culture. This finding is supported by previous NGS data that identified these bacteria as significant pathogens associated with LRTIs [32,33]. The 16SmNGS results of numerous specimens that exhibited hindered or mixed growth in culture revealed the presence of genera such as *Prevotella*, *Veillonella*, *Staphylococcus*, and *Enterobacteriaceae*. These genera have been recognized as components of the oral microbiome [28], suggesting potential colonization or contamination by upper airway flora. Several studies have highlighted the role of the upper airway in shaping the structure of the lower airway microbiome due to the unrestricted contact between upper and lower airways and possible aspiration of secretions from the oropharynx [34].

In addition to the characterization of LRT microbiota, the use of 16SmNGS provided an additional advantage in identifying fastidious potential respiratory pathogens that did not grow in culture. The results of our study indicate that, in 100%, 77.7%, and 55.5% of 16SmNGS-positive specimens, conventional culture failed to detect the growth of *H. influenzae*, *S. pneumoniae*, and *E. coli*, which underscores the superiority of NGS over traditional microbiological culture methods. Notably, these results corroborate previous research that validated the successful identification of respiratory pathogens resistant to growth in traditional culture (up to a rate of 94.49%) in patients with LRTIs using NGS technology [30,35]. It is worth noting also that mNGS reported multiple top-ranking microbes in some samples of our study, contrasting with findings of single or absent growth in culture, suggesting that potential polymicrobial infections may have been overlooked by the standard culture technique. This may highlight the outperformance of mNGS relative to culturing in detecting polymicrobial infections by identifying poorly cultivable microbes that are often difficult to grow in culture due to their fastidious nature or prior antibiotic intake. Furthermore, some samples in our study revealed the presence of mixed anaerobic bacteria not previously detected in culture, including *Prevotella*, *Veillonella*, and *Lactobacillus*, using 16SmNGS. Several reports have agreed on the superiority of 16SmNGS in identifying the microbial etiology of infections with multiple anaerobes, such as lung abscess, aspiration pneumonia, and atypical pneumonia [36-38]. Additionally, 16SmNGS can play a significant role in investigating the microbial etiology of infections among immunocompromised patients or patients under prolonged antibiotic intake by effectively identifying atypical microbial pathogens [11,13,21,37,39,40].

The mNGS technology has the potential to profoundly influence clinical decision-making in LRTIs, especially in culture-negative cases, by expanding the detection spectrum of atypical and poorly cultivable pathogens and by capturing multiple microbes, thus facilitating more optimized therapeutic guidance and enhanced patient outcomes [21]. Several studies in the literature have highlighted the advantages of 16SmNGS, revealing the outstanding potential of this technology that surpasses the traditional culture and currently available molecular techniques [14,23,41]. Available mNGS technology has managed to address the shortcomings of tra-

ditional culture methods by enhancing pathogen detection, especially among uncultivable microbes and instances of low culture yields resulting from the initiation of empirical therapy, while also reducing assay time [42]. However, routine use of NGS in the laboratory diagnostic field is currently hindered by a variety of obstacles, including occasional detection of non-viable organisms or residual DNA that do not represent true active infection, along with other limitations such as high costs, limited accessibility in low-resource settings, and the need for special equipment and technical expertise [42]. Further wide-scale research is required to study the cost-effectiveness of adopting this technology in routine clinical settings. Another challenging issue inherent with mNGS is determining the clinical relevance of microbial pathogens in specimens collected from human body sites that contain microbial flora to distinguish between actual infection, colonization, and flora contamination. The extensive spectrum of bacteria detected by 16SmNGS raises uncertainty regarding the authenticity of the identified bacteria as infectious pathogens [43,44]. There are some suggested criteria and emerging frameworks in the literature for interpreting 16S metagenomics of LRTIs. Some studies propose thresholds, decision rules, or combined metrics that may classify cases in terms of infection as definite, probable, possible and unlikely infections; however, these are not yet universally standardized. In the absence of standardized criteria, clinical judgement remains essential, and metagenomics results shall not be interpreted in isolation from culture results, clinical data, and radiological findings to rule out potential flora contamination [21,30,42].

In our study, we attempted to address true infection through the following endeavors. Initially, all enrolled patients had clinical and radiological findings suggestive of LRTI. Secondly, we applied conservative stringent precautions during sample collection to ensure proper representation of LRT, while also minimizing the risk of upper airway colonization or contamination. Third, microbial reads from the mNGS library were included only after successfully passing all quality-control filters and after ensuring that the identified microbe was not displayed in the negative controls. Finally, the identification of potential true pathogens was pursued by considering the predominant well-established clinically relevant taxa with the highest RA in mNGS reporting.

Our study was challenged by limitations, including a small

sample size, that warrant further complementary research efforts for establishing comprehensive evidence. Moreover, our study was challenged by incomplete data on patient inflammatory markers and clinical outcomes, which hindered our ability to establish correlations with mNGS results. Furthermore, it is important to highlight that NGS results should be considered in relation to microbial RA, along with microbiological cultures and clinical and radiological findings, to achieve a reliable diagnosis and rule out colonizing or contaminating bacteria. Multidisciplinary cooperation between laboratorians and clinicians is recommended to improve the accurate comprehension and interpretation of metagenomics results.

The taxonomical analysis of the LRT microbiome in PICU children with LRTI revealed an overrepresentation of *Streptococcus* and *Enterobacteriaceae* genera that belong to the phyla of Firmicutes and Proteobacteria, respectively. The use of 16SmNGS identified *S. pneumoniae*, *E. coli*, *P. aeruginosa*, *S. aureus*, *A. baumannii*, and *H. influenzae* as the primary microbial drivers of LRTI. Our study highlighted the outperformance of 16SmNGS over conventional culture in the detection of significant respiratory pathogens such as *S. pneumoniae* and *H. influenzae*, as well as its capability to identify anaerobic bacteria that failed to grow in regular culture, displaying absent to moderate agreement with 16SmNGS results. Metagenomics may serve as an advanced diagnostic tool that enhances pathogen detection and prompt disease management. However, it is crucial to interpret these findings with regard to culture data as well as clinical and radiological data to ensure accurate diagnosis.

CONFLICT OF INTEREST

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

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AUTHOR CONTRIBUTIONS

Conceptualization: MSS, HFA, AAE. Methodology: MSS, NSS, AAE. Formal analysis: MSS, NSS, AMA. Data curation: MSS, NSS, HFA, AMA. Writing-original draft: NSS, AMA. Writing-review & editing: NSS. All authors read and agreed to the published version of the manuscript.

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