Clinical Cancer Investigation Journal-ISSN: 2278-0513

Detection of Airway Microbiome in Iranian Cystic Fibrosis Patients and the assessment of their bacterial resistance

Abstract

In people with Cystic Fibrosis (CF), lung infections are the leading cause of mortality. As a result of CF, the human body is predisposed to microbial colonization of its airways due to an inherited systemic metabolic condition. Microorganisms that are harmful to Iranian CF patients are rarely studied. This study was conducted to identify airway microbiota in oropharyngeal swabs of Iranian CF patients and determine their antibiotic resistance. A total of 64 Oropharyngeal swab samples were investigated using PCR and culture methods. Testing for antibiotic susceptibility was then conducted on the confirmed isolates. Finally, the samples were prepared to be sequenced. Among the 80 bacterial isolates in oropharyngeal swabs, 39 (48.75%) were *Staphylococcus aureus*. This was followed by *Pseudomonas aeruginosa* 20 (25%), *Streptococcus pyogenes* 5 (6.25%), *Streptococcus* group C or G 5 (6.25%), *Enterococcus faecalis* (45%), and *Citrobacter freundii* 2 (2.5%). In the study, bacteria resistant to oxacillin (17.78%), trimethoprim-sulfamethoxazole (26.67%), vancomycin (8.89%), and novobiocin (6.67%) were found. In addition to aztreonam, ceftazidime, meropenem, and ciprofloxacin, Gram-negative bacteria were resistant to tobramycin (7%) and ciprofloxacin (18%). The most common isolates in the oropharyngeal CF population are *S. aureus* and *P. aeruginosa*. However, the life expectancy of CF patients may be improved by avoiding the colonization of these bacteria.

Keywords: *Cystic fibrosis (CF), Oropharyngeal swabs, antibiotic resistance, Pseudomonas aeruginosa, Staphylococcus aureus.*

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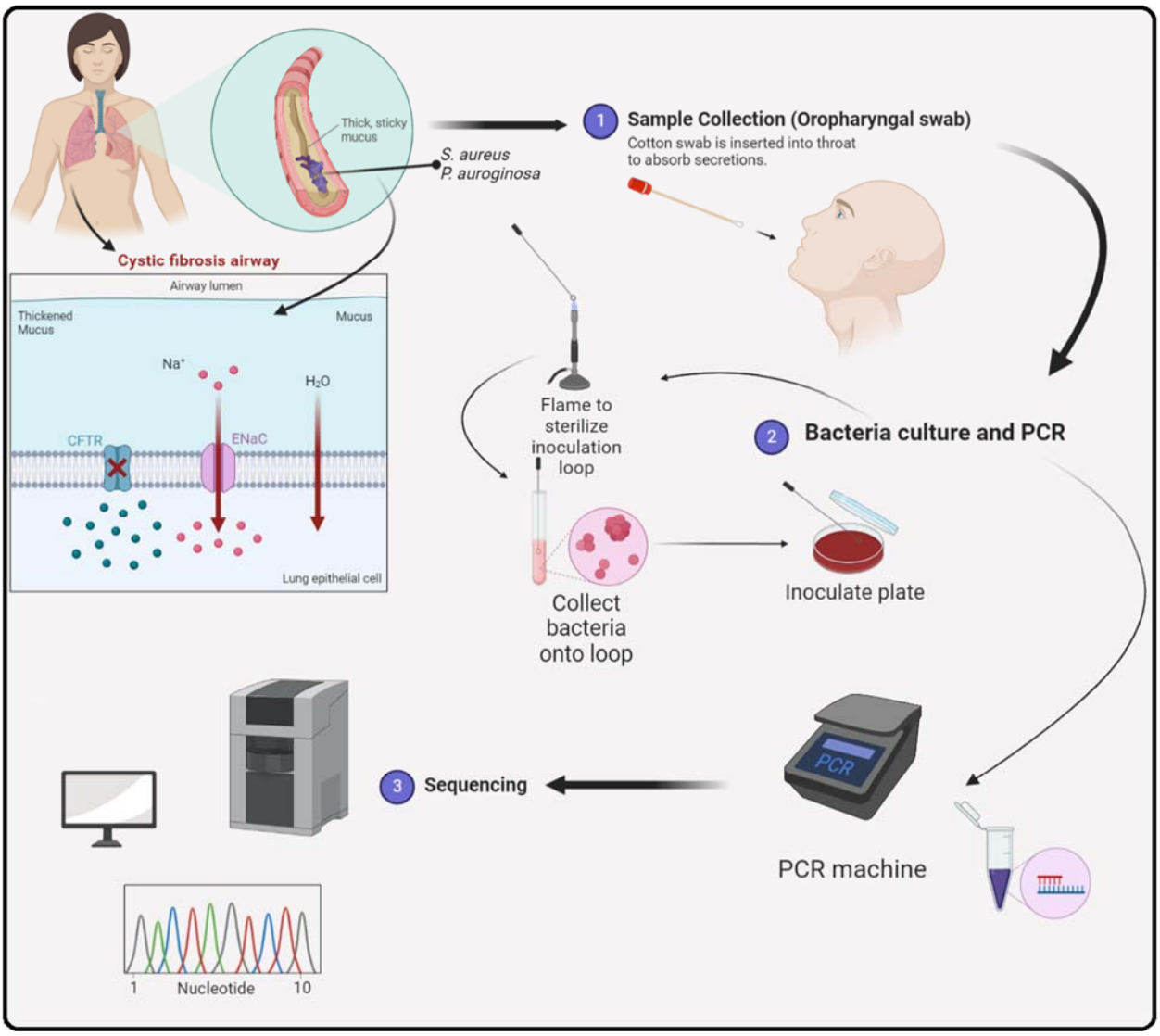


Figure 1- Graphical Abstract

Introduction

1. Introduction

Cystic fibrosis (CF), an autosomal recessive illness with a life-limiting outcome, primarily affects people of European ancestry. In the United States, the majority of CF patients are diagnosed within their sixth month of life. There are around 30,000 people in the nation who have CF. In Canada, there are roughly 4000 CF sufferers. At the same time, 1 in every 25 people of European heritage and one in every 30 Caucasian Americans have a CF mutation. CF has many aspects, but the primary cause of morbidity and early death is increasing damage to the lungs and airways, which increases the likelihood of bacterial infections. More recent research has illustrated that the CF airway microbiome is inhabited primarily by a polymicrobial community that includes aerobic, aerotolerant, and obligate anaerobe bacteria, as well as yeast and viruses. Expanded culturing techniques and culture-independent molecular methods characterize the composition and behavior of polymicrobial communities found in CF patients.[1, 2].

The leading cause of mortality in CF patients is respiratory failure brought on by recurrent and chronic lung infections caused by various pathogenic microorganisms. *Haemophilus influenzae* first colonizes the CF airways, then by *Staphylococcus aureus*. One of the primary CF infections affecting up to three-fourths of adults is *pseudomonas aeruginosa* [3]. Due to improved airway clearance and more effective treatment for conventional CF pathogens, new airway pathogens have appeared. These include *Stenotrophomonas maltophilia*, *Mycobacterium abscessus*, *Achromobacter*, *Burkholderia cepacia*, *Streptococcus spp*, *Aspergillus fumigatus*, and *obligate anaerobes*, most commonly *prevotella*. [1, 4]

Bacteria discovered using independent culture methods included *Lysobacter*, *Pandoraea,* *Veillonella*, *Porphyromonas*, *Propionibacterium*, *Actinomyces*, *Prevotella oris*, *Prevotella histicola*, *Prevotella melaninogenica*,

Fusobacterium spp, *Rothia, Streptococcus anginosus*, *Streptococcus milleri*, *Peptostreptococcus micros*, *Gemella, Granulicatella*, *Neisseria*, *Atopobium*, *Pandoraea*, *Ralstonia*, *Achromobacter ruhlandii* [1, 5, 6]. Moreover, *recently, Malassezia and Kluyveromyces have been among the yeasts detected in CF patients* [5, 7, 8]. In CF patients, an opportunistic bacterium called *B. cepacia* is a multiresistant and contagious bacterium that continuously deteriorates their clinical condition. The *B. Cepacia* complex (BCC) consists of eight different genomovars with different levels of virulence and transmission from patient to patient. It is necessary to develop a reliable early detective test to detect trace amounts of this bacteria in clinical samples. This is because the prevalence of this bacteria in CF patients is low. Consequently, conventional microbiological diagnostics of BCC based on culture and subsequent biochemical identification are not suitable for this purpose due to the possibility of misidentification. [9]. Additionally, research has been conducted on how Haemophilus influenza might contribute to CF.

In undeveloped samples, polymerase chain reaction (PCR) is a practical approach for tracking these bacteria. The recA gene and the 16S rRNA gene are the two target genes most frequently employed for BCC studies. Analyzing recA gene polymorphisms makes it possible to distinguish BCC from other closely related bacteria and classify it according to its genomovars. [9]. Hib pili, expressed on the bacterial surface, facilitate the initial attachment of Haemophilus influenzae type B to the human nasopharynx. A crucial role in the pathogenicity of this bacteria is played by this pilus encoded by the Hib gene. As such, it can be used to identify and vaccinate subjects as a proprietary surface antigen.[10]. A study is currently being conducted to determine whether the CF microbiota plays a role in airway illness. A new outlook will be provided as a result of the management of CF illness. It is unknown whether harmful microorganisms are prevalent in Iranian children and adolescents with CF. This study investigated the antibiotic susceptibility of Iranian CF patients' oropharyngeal swabs and identified the airway microbiome.

2. Material and methods

2.1 Study population

Throughout 2017 and 2018, 64 oropharyngeal swabs were obtained from children and adults sent to Tehran University's Children's Medical Center (aged 7 months to 20 years). The study was approved by the Institutional Review Board of Tehran University's Children's Medical Center. All ethical aspects of this project have complied with the principles laid down in the Declaration of Helsinki.

2.2 Culture and isolation

CF respiratory samples were cultured according to standard procedures in the microbiology lab. In total, three types of agar medium were used (Chocolate Agar, MacConkey Agar, and MSA). A 48-hour aerobic incubation was conducted at 37 °C with two mediums. On the other hand, incubation of the Chocolate agar medium was carried out in an environment containing 5% CO_2. Following accepted practices, the growth of bacteria in each species was semi-quantitatively assessed. Further detection of non-tuberculous Mycobacterium and Haemophilus species was accomplished by acid-fast and Gram staining of early samples.

2.3 Antimicrobial susceptibility testing.

Most isolates were isolated using the disk diffusion technique under the Clinical and Laboratory Standards Institute (CLSI) [11]. Gram-negative isolates were treated with antibiotic disks (Padtan teb, Iran) containing azithromycin, ceftazidime, ciprofloxacin, meropenem, and tobramycin, and gram-positive isolates were treated with trimethoprim-sulfamethoxazole, oxacillin, novobiocin, and vancomycin. In each determination, *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were used as controls. A standard chart was used to determine whether isolates were susceptible or resistant based on the diameter of the inhibition zone in mm.

2.4 Identification with PCR.

Amplifying the 16s rRNA region was performed to identify and differentiate bacteria. A reaction mixture containing 2 µL of the extracted DNA, 12 µL of Taq DNA Polymerase Master Mix White (Amplicon), 0.3 µL of each the 27F (5′-AGAGTTTGATCMTGGCTCAG-3′), and 1525R (5′AAGGAGGTGWTCCARCC-3′) primers [12].

2.5 Preparation of template DNA from CF patient sputum samples

This stage involved the study of 26 primitive samples. Due to this intention, 100 µl samples were resuspended in 50 µl of TE buffer. During a snap-freezing procedure on ice for 3 minutes, bacteria were fractured by heating for 15 minutes at 100°C. A new microfuge was used to collect the superficial liquid containing DNA after centrifugation at 12,000 g for 10 minutes.

2.5 PCR analysis

A DNA thermal cycler was used for the PCR study (Eppendorf). According to McDowell's study [13], the primers BCR1 and BCR2, which target the 5 and 3 ends of the recA gene locus, were used to amplify the *B. cepacia* complex recA gene (1,040 bp). Primer3 and the blasting software Chromas were also used to create the primers for the *H. influenzae* Hib gene (249 bp) (**Table 1**).

Bacteria	Primers
B. cepacia	LEFT PRIMER 5'TGACCGCCGAGAAGAGCAA3'
	RIGHT PRIMER 5'CTCTTCTTCGTCCATCGCCTC3'
H. influenzae	LEFT PRIMER 5' ACAAGGTGGCATCGAAGGTA 3'
	RIGHT PRIMER 5' GCGCCAATACCATAGCTTCC 3'

Table 1-the primers were used in PCR

PCR was performed with a total volume of 25 µl. Samples contained 12 µl of Master mix, 9 µl of deionized sterile water, 1 µl of each Forward and Reverse primer (1:10), and 9 µl of DNA. To detect *B. cepacia*, the PCR was performed according to the following guidelines. The reaction mixture was first denatured for 5 minutes at 95°C, then through 35 cycles of denatured for 1 minute at 96°C, annealed for 1 minute at 56°C, extended for 60 seconds at 72°C, and finally extended for 10 minutes at 72°C. In addition, to confirm the presence of *H. influenzae*, The reaction mixture was first heated for 10 minutes at 94°C, and then through 25 cycles of denatured for 1 minute at 94°C, annealed for 1 minute at 59°C, extended for 60 seconds at 72°C, and finally extended for 10 minutes at 72°C. PCR-amplified products were routinely analyzed by electrophoresis in 1% (wt./vol) agarose gels containing TBE buffer (0/5 X) and Green viewer color (10 %).

2.6 DNA sequence analysis

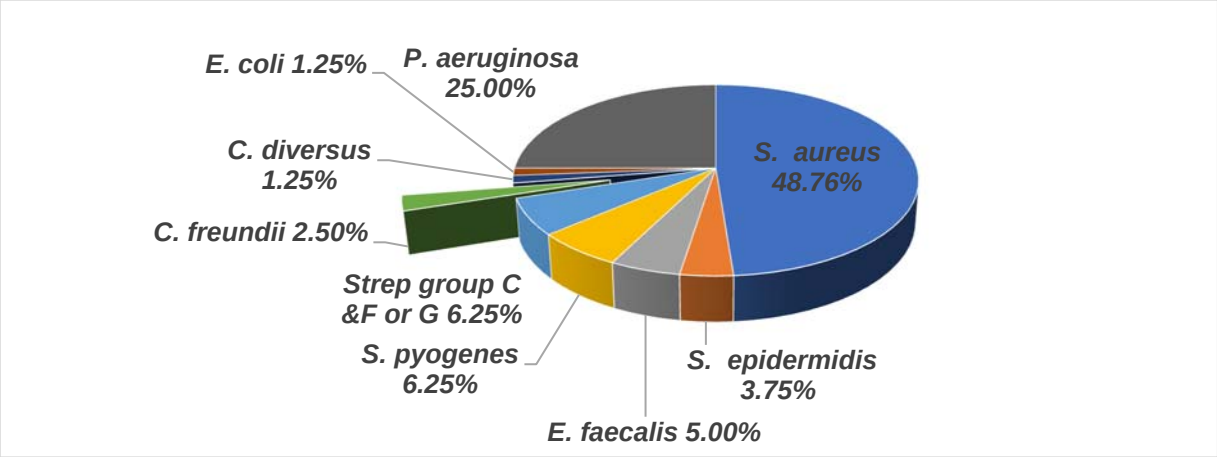
PCR amplicons were compared with the positive control sample and checked for the presence of the bacteria in primitive samples. The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit was used to sequence the 16s rRNA region of the PCR products.

2.7 Statistical analysis

All tests were carried out in triplicate and at least three times independently. All statistical analyses were carried out using SPSS 21.0 and one-way ANOVA. If the two-tailed *p*-value was < 0.05, the data was deemed substantially different.

3. RESULTS

Out of the 64 Oropharyngeal swab samples, 80 isolates with different Morphology were identified by conventional culture-based techniques and universal PCR. The results of identification via these methods are clustered in 6 groups; gram and positive catalase coccus (*S. aureus*, *S. epidermidis*), gram and catalase negative coccus (*Enterococcus faecalis, Streptococcus. pyogenes, and Streptococcus* group *C &F* or *G*), fermentative gram-negative bacillus (*Escherichia coli*, *Citrobacter freundii* and *Citrobacter diversus*), non-fermentative gram-negative bacillus (*P. aeruginosa*), suspicious isolates into yeast and normal flora including *Neisseria sp*. And γ hemolysis *Streptococci.*



Out of the 80 bacterial isolates detected in oropharyngeal swabs, 39 (48.75%) were *S. aureus*, followed by *P. aeruginosa* 20 (25%), *S. pyogenes* 5 (6.25%), *Streptococcus* group C or G 5 (6.25%), *E. faecalis* 4 (5%), *C. diversus* 1 (1.23%), E. *coli* 1 (1.25%), *C. freundii* 2 (2.5%). The percentages in **Figure 2** are due to the 80 identified isolates.

3.1 Antimicrobial susceptibility testing.

Following CLSI recommendations, the Kirby-Bauer disk diffusion method was used to determine the susceptibility

profiles of most of the isolates. A total of 9 common antibiotics have been detected, including aztreonam, ceftazidime, ciprofloxacin, meropenem, tobramycin, trimethoprim-sulfamethoxazole, oxacillin, novobiocin, and vancomycin. **Figure 3** provides the results of the antibiotic susceptibility test.

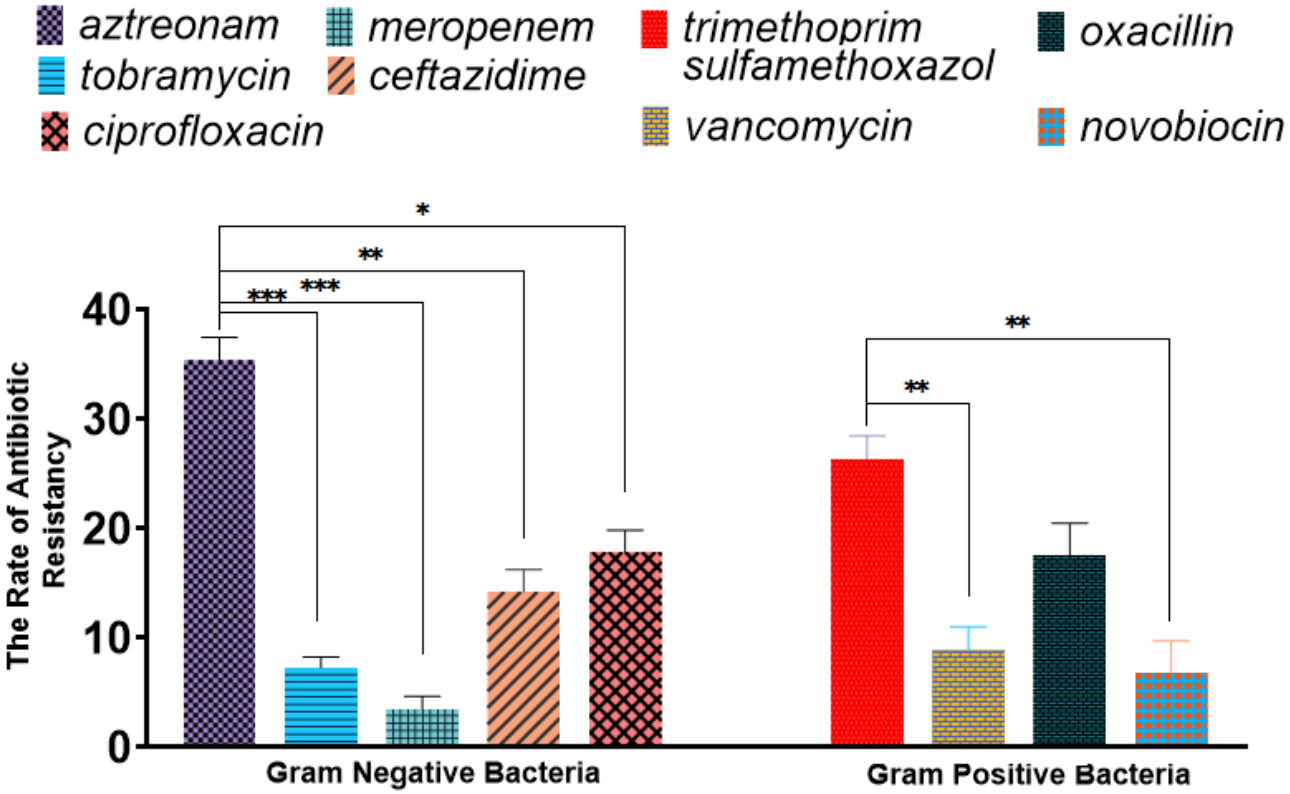


Figure 3. Percentage of antibiotic resistancy in identified isolates

As a result of the test, Gram-positive bacteria demonstrated resistance to oxacillin (17.78%), trimethoprim-sulfamethoxazole (26.67%), vancomycin (8.89%), and novobiocin (6.67%). Additionally, Gram-negative bacteria also exhibited resistance to aztreonam (36%), ceftazidime (14%), meropenem (4%), ciprofloxacin (18%), and tobramycin (7%).

One of the *S.aureus* isolates was resistant to all antibiotic agents except cefoxitin. The *S.aureus* isolates revealed the most resistance to trimethoprim-sulfamethoxazole (15.38%), followed by oxacillin (7.69 %).

3.2 Molecular Investigation

This investigation focused on identifying *H. influenza* and *B. cepacia* species. *B. cepacia* and *H. influenzae* were not found in any of the primitive samples, as shown in **Figures 2 and 3**.

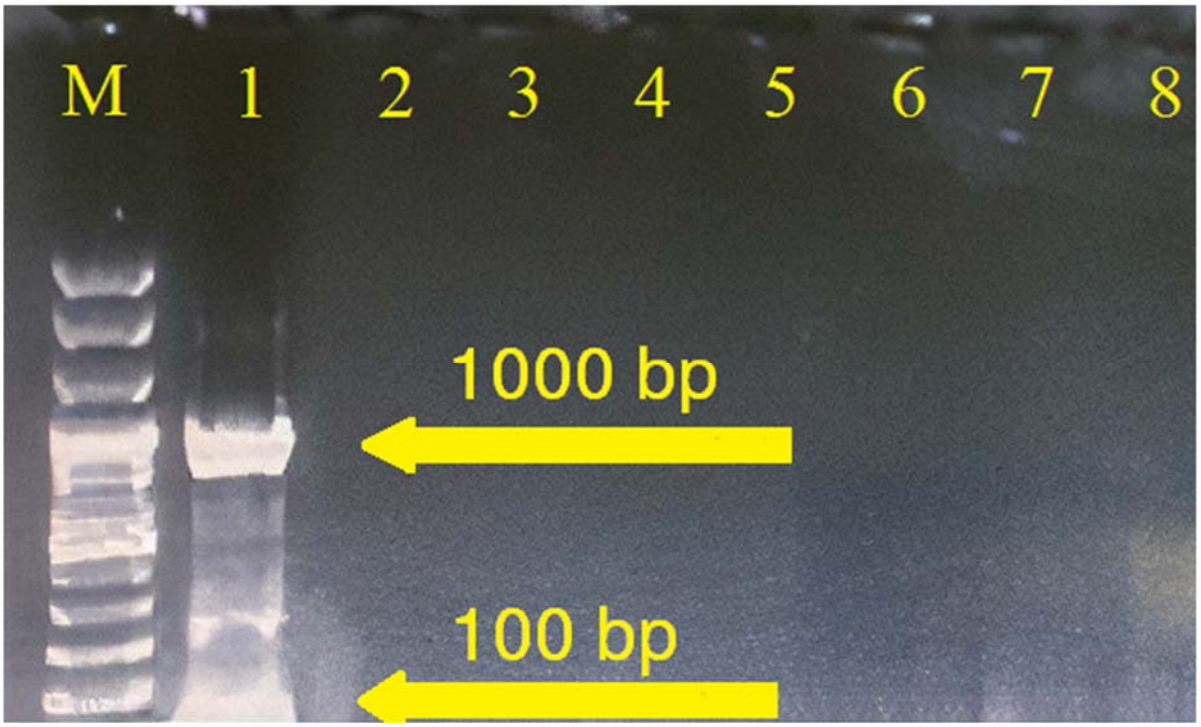


Figure 4-PCR amplification profile of B. cepacia. from DNA isolated from Oropharyngeal swab samples with BCR1 and BCR2 primers. Lane M: 1500 bp DNA marker, Lane 1: Positive control (1040 bp amplicon size), Lane 13: Negative control, and Lanes 2-12 Negative sample

Figure 5- PCR amplification profile of *H. influenzae*. from DNA isolated from Oropharyngeal swab samples with *Hib* gene primers. Lane M: 1500 bp DNA marker, Lane 14: Negative control, Lane 5: Positive control (249 bp amplicon size), and other Lanes negative samples.

After gathering the sequencing data shown in Figure 4, using FSTA and Chromas BLAST program, the unknown bacterium was identified as *E. coli* by universal PCR.

Figure 6-Genomic sequence of unknown isolate

4. Discussion.

Mucosal secretions may cause chronic and recurrent respiratory infections in the lungs of people with CF, an inherited genetic condition. These patients are susceptible to a variety of opportunistic pathogens. Several bacteria contribute to chronic infections, including *Pseudomonas*, *Staphylococcus*, *Burkholderia*, and *Haemophilus*. The diagnosis of this illness is typically attributed to a group of bacterial infections, which are acquired through an age-dependent process. Healthy people may only be exposed to CF-causing agents like *S. aureus*. Some opportunistic infections are nonpathogenic in healthy hosts, such as *Aspergillus* and non-tuberculous *mycobacteria*. There are three stages of infection involving these pathogens: 1) early infections, primarily caused by *S. aureus* and *H. influenzae*; 2) infection with *P. aeruginosa*, and 3) acquisition of these pathogens. Among the pathogens that have emerged recently are *B. cepacia*, *S. maltophilia*, *A. xylosoxidans*, *Aspergillus*, and non-tuberculous *mycobacteria*, as well as others[14] . The median life expectancy of someone with CF has increased from early childhood in the 1950s to more than 40 years. As a result of management strategies such as increasing mucociliary clearance and treating infections aggressively, life expectancy has gradually increased.[1, 15, 16]. Appropriate antibiotic therapy is essential for treating CF lung disease caused by respiratory system bacterial infections. CF patients should have their respiratory secretions frequently isolated and identified to determine their infection pathogens' antibiotic susceptibility profiles since bacteria may become resistant to antibiotics. [17]. Many studies have been conducted around the world to identify and characterize the microbiome of CF patients taking into account the role played by bacterial pathogenicity in the development of the disease process into an acute phase and its association with morbidity and mortality. Despite this, little is known about the prevalence of pathogenic microbes among Iranian CF children, especially among the youngest patients. In a study published in 2018, Sharifi *et al.* investigated the microscopic properties of upper respiratory tract pathogens among cystic fibrosis patients. In this study, *S. aureus* was found to be the most commonly encountered bacterium in CF patients, followed by *P. aeruginosa* with 21 (21%). In infants younger than one year, *Enterococci* and *Klebsiella pneumonia* were the most often isolated pathogens. Other age groups were most likely to encounter *S. aureus* and *P. aeruginosa*. All pathogens had a higher sensitivity to Ceftriaxone, Amikacin, and Ceftazidime. The strongest sensitivity to Cefoxitin, Clindamycin, and Linezolid was observed with *S. aureus*. In contrast, the strongest sensitivity to Amikacin, Ceftazidime, and Ceftriaxone was observed with *P. aeruginosa* [18]. An investigation of the microbiology of airway illness among CF patients was conducted by Antonietta Lambiase *et al.* in 2006. The investigation included 300 patients infected with P. aeruginosa over three years, 7% with *B. cepacia* complex, 11% with *S. maltophilia*, and 7% with *Alcaligenes xylosoxidans*. It was determined that 460 isolates were multidrug resistant. Moreover, the scientists proved again that *P. aeruginosa* and the *B. cepacia* complex were multiresistant. [19]. In research by Panickar and David (2015), 182 CF children's outpatient and lavage samples were examined, and 5 (3%) *Burkholderia*, 17 (19%), *Exophiala*, 32 (18%), *Rhodotorula*, 18 (10%), *Ralstonia*, and 6 (3%) *Pandoraea* were found. This research demonstrated a rise in *Achromobacter* and *Pandoraea* bacteria in children with CF [20]. In respiratory samples from children with cystic fibrosis, Anzaudo MM. and colleagues (2005) examined the Isolated pathogen microorganisms. Their findings indicated that *S. aureus* (38.7%), *P. aeruginosa* (37.4%), and *Haemophilus spp*. (15.3%) were the most commonly isolated microbes. *S. aureus* bacteria exhibited substantial levels of resistance to erythromycin (35.0%), clindamicine (29.4%), and methicillin (25.9%). The majority (31.0%) of *P. aeruginosa* strains were gentamicin-resistant. Beta-lactamases were the cause of the 23.0% incidence of ampicillin-resistant *Haemophilus spp.*, while a significant rate of trimethoprim-sulfamethoxazole resistance was found in this bacterium (59.0%) [21].

Christine Rumpf stated in 2021 that *S. aureus* is the most isolated pathogen from the airways of CF patients, who are often infected by a dominant *S. aureus* clone for lengthy periods. To survive, the bacteria must adapt to the adverse environment of the airways, where it must contend with host defense, antibiotic treatment, and competition from coinfecting infections[22]. In one study At the Danish CF Center, patients with chronic *Pseudomonas aeruginosa* lung infection were treated 3–4 times a year (from 1976) with a 2-week intravenous antipseudomonal course, including preferentially an aminoglycoside and a β-lactam antibiotic. Over 18 years, the development of antibiotic resistance in *P. aeruginosa* strains was studied by testing the in vitro efficacy of carbenicillin, piperacillin, ceftazidime, tobramycin, and ciprofloxacin against *P. aeruginosa* strains collected in 1973 (51 strains), 1980 (80 strains), 1985 (58 strains), and 1991 (51 strains) (100 strains). Using nitrocefin, all strains were screened and semi-quantitatively tested for -lactamase activity. The MIC values of *P. aeruginosa* strains against piperacillin and ceftazidime increased significantly ($p < 0.005$). However, no significant association was seen between the MIC and the number of antipseudomonal antibiotic courses. During the study period, the fraction of resistant *in vivo* chosen *P. aeruginosa* strains, thought to be stably derepressed producers of chromosomal β-lactamase, grew dramatically. The findings support the hypothesis that lactamase synthesis is a major mechanism of antibiotic resistance in *P. aeruginosa* [23]. In the current study, 64 oropharyngeal swabs were collected from children and adults (aged ≥ 7 months to < 20 years) referred to the Children's Medical Center of Tehran University. Out of the 80 bacterial isolates detected in oropharyngeal swabs, 39 (48.75%) were *S. aureus*, followed by *P. aeruginosa* 20(25%), *S. pyogenes* 5(6.25%), *Streptococcus* group C or G 5(6.25%), *E. faecalis* 4(5%), *C. diversus* 1(1.23%), *E. coli.* 1(1.25%), *C. freundii* 2(2.5%).

Based on the results of the antibiogram test, trimethoprim-sulfamethoxazole (26.67%) has the most effective, followed by oxacillin (17.78%), vancomycin (8.89%), and novobiocin (6.67%) against gram-positive bacteria. Also, aztreonam (35/71%) was the most antimicrobial agent in this study, followed by ciprofloxacin (17/86%) and ceftazidime (14/29%), tobramycin (7/4%), and meropenem (3/57%) against gram-negative isolates. It is important to note that no *B. cepacia* or *H. influenzae* had been detected in the samples collected. Like the observation of other investigations, our results showed that *S. aureus* and *P. aeruginosa* have the most prevalent among CF patients. Most of the P. aeruginosa isolates were mucoid, suggesting that the patients in our research are in the chronic stage of infection. Children with CF of the pancreas had their respiratory tract colonized by *Enterobacteriaceae* in 1975. Their immune system responded by producing antibodies. In this research, 49 of the 72 individuals with CF had *Enterobacteriaceae*, which included *E. coli*, *Klebsiella*, and *Enterobacter*, in their respiratory tracts. Twenty-nine patients had two to four genera of colonization recorded. Of these 49 individuals, *S. aureus* was discovered in 44 of them. The distribution of *E. coli* serogroups was comparable to that of patients with uTIs.

In contrast, individuals with Enterobacteriaceae colonization and no *P. aeruginosa* had a milder form of the illness. The antibody response against the O antigens in patients with *Enterobacteriaceae* was examined in 29 children. It was associated with patients with severe diseases [24]. In this investigation, we found *E. coli*, *C. freundii*, and *C. diversus* in patients under 10. Given that *E. coli*, *C. diversus*, and *C. freundii* are all typical fecal flora, it is evident that these patients do not practice good hygiene.

To learn more about the relationship between the presence of these microorganisms and the worsening of a CF patient's clinical parameters, it is necessary to investigate further the function yeast and non-tuberculous mycobacteria play in CF infection. In Ziehl-Neelsen staining, we had not found any non-Tubercolus *mycobacteria*. Additionally, 6/25% of the samples included a worrisome yeast isolate.

The main limitation of this study was the expensive materials and equipment and also there were few participants in sampling. Due to the small number of volunteer patients for sampling, we missed the chance of tracing some bacteria that were acquired at certain ages, such as *Haemophilus.* Moreover, there was no difference between those who were in the acute or chronic infection stage. Some isolates have got new and particular biochemical specificity due to the inappropriate use of antibiotics among the patients participating in the sampling which made the diagnosis process difficult. In addition, in isolates from CF patients, in the laboratory, the antibiotic resistance might be different from that in vivo.

5. Conclusions

According to our research, S. aureus and P. aeruginosa are the most prevalent bacteria isolated from the oropharyngeal CF population. Utilizing methods to stop these germs from colonizing will lengthen CF sufferers' lives. According to the function of microorganisms in CF infections, CF patients' microbiomes must be identified, characterized, and tested for antimicrobial susceptibility to create a treatment plan. Diagnostic microbiology labs have a hurdle in correctly identifying this bacterial species. This article claims that combining molecular and traditional culture methods simultaneously is the most effective method for analyzing the microbiome of CF patients.

Declaration:

Funding

This work was supported financially by the Pasteur Institute of Iran.

Competing interests

The authors declare that they have no competing interests.

Acknowledgment

none

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