Iranian Journal of Microbiology

Volume 15 Number 6 (December 2023) 750-758

ORIGINAL ARTICLE

Comparison of culture and PCR-DGGE methods to evaluate the airways of cystic fibrosis patients and determination of their antibiotic resistance profile

Somayeh Moazami Goudarzi[1,2†], Yasamin Shahpouri Arani[1†], Ahya Abdi Ali[1*], Parisa Mohammadi[1], Nassim Ghorbanmehr[3], Mohammadreza Modaresi[2,4*], Mahtab Ghorban Movahed[1], Tooba Ghazanfari[5]

[1]Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran
[2]Cystic Fibrosis Research Center, Iran CF Foundation (ICFF), Tehran, Iran
[3]Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran
[4]Pediatric Pulmonary Disease and Sleep Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran
[5]Immunoregulation Research Center, Shahed University, Tehran, Iran

Received: March 2023**, Accepted:** October 2023

ABSTRACT

Background and Objectives: Respiratory infections are the most serious condition in cystic fibrosis (CF) patients; therefore, a thorough comprehension of the diversity and dominant microbial species in CF airways has a crucial role in treatment. Our objective was to determine the antibiotic resistance profile of CF airways microbiota and compare culture methods and PCR-DGGE to evaluate bacterial diversity.

Materials and Methods: Pharyngeal swabs from 121 CF patients were collected. The samples were then cultured, identified and antibiotic resistance testing was performed. Thirty samples were subjected to further molecular surveys. DNA contents of these samples were extracted and amplified using nested-PCR technique and their bacterial diversity was assessed by DGGE. The DGGE patterns were visualized and certain bands were excised and purified. Next, the DNA was amplified by another round of PCR and sent out for sequencing.

Results: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were the most prevalent species isolated using culture methods. *S. aureus* was the most common bacteria among 6 years and younger patients; while, *P. aeruginosa* had more prevalence among older ones. The PCR-DGGE results showed more diversity than culture methods, particularly in younger patients who exhibited more bacterial diversity than the older groups. Sequencing results unveiled the presence of certain bacterial species including *Haemophilus parainfluenzae* and *Stenotrophomonas maltophilia* which were completely missed in culture.

Conclusion: Even though culture-dependent methods are cost-effective, PCR-DGGE appeared to be more efficient to determine bacterial diversity. PCR-DGGE detects less abundant species, though their viability could not be determined using this method.

Keywords: Cystic fibrosis; Respiratory tract infections; Antibiotic resistance; Denaturing gradient gel electrophoresis; Microbiota

*Corresponding authors: Ahya Abdi Ali, PhD, Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran. Tel: +98-21-88044040 Fax: +98-21-88058912 Email: abdialya@alzahra.ac.ir

Mohammadreza Modaresi, MD, Cystic Fibrosis Research Center, Iran CF Foundation (ICFF), Tehran, Iran; Pediatric Pulmonary Disease and Sleep Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran. Tel: +98-9131134710 Fax: +98-21-66930024 Email: modaresipul@gmail.com

†These authors contributed equally to this work.

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal hereditary disorder among Caucasians. Mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) encoding gene gives rise to the physiopathology of CF. Impaired CFTR or lack of it leads to the emergence of unusually thick and sticky mucus, which accumulates in the patient's airways and disables mucociliary clearance in the lungs. Hence, microenvironments in CF respiratory tract are not the same as that of healthy individuals and are going through drastic changes throughout a patient's life which eventually results in the favorable condition for pathogens to reside while inefficient immune responses further damage their lung (1). Mainly, bacterial lung infections reduce life expectancy in most CF patient. UK Cystic Fibrosis Registry reported that the median predicted survival age of CF between 2014 and 2018 was 47.3 years and also 73.1% of the recorded causes of death from 2016 to 2018 were reported as respiratory/cardio-respiratory (2).

Recent studies have drawn attention to the decreased diversity of the CF airway microbial community which is related to a patient's age, worsened lung disease, and antibiotic usage (3-5). The most significant bacterial pathogens of these communities are considered to be *Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae,* and *Burkholderia cepacia* complex, with *P. aeruginosa* reported as the most common pathogen in CF lung disease (1). Improved treatment approaches for these classical CF lung pathogens have prompted the appearance of novel airway pathogens including, *Stenotrophomonas maltophilia, Mycobacterium abscessus, Achromobacter xylosoxidans, Ralstonia spp., Pandorea* spp., and members of *Streptococcus milleri* group (6). Furthermore, less common bacteria like *Rothia mucilaginosa, Gemella haemolysans* and several anaerobic species, such as *Prevotella* spp, *Veillonella* spp., and *Fusobacterium* spp., are increasingly being recognized (6, 7).

A vital challenge in CF is to determine bacterial members of the respiratory microbiota, but it mostly consisted of a small group of bacterial species, the majority of which could be readily isolated by conventional aerobic culture-based approaches (6, 8). There are two major types of methods for evaluating microbial diversity, culture-dependent and culture-independent methods (9).

Using culture-independent methods of microbial community analysis has unveiled a more diverse community of micro-organisms, including even unexpected anaerobic members while interspecies interactions in these communities could even affect antibiotic effectiveness. As a result of these interactions, changes in metabolic pathways, virulence factors or quorum sensing signals production, and formation of biofilm might happen to some members which could alter their viability and their susceptibility to certain antibiotics (3).

Genetic fingerprinting techniques are powerful tools to be utilized for investigating microbial diversity and structure of a community including those in CF airways. Denaturing gradient gel electrophoresis (DGGE) is a fingerprinting method that has been employed in inspections of oral and respiratory microbial communities of the different diseases frequently (10). Theoretically, this method could separate double-strand segments of DNA or RNA of the same size with at least 1 different base pair which might be a case of substitution, insertion, or deletion, so it is able to detect different alleles or mutations of a particular gene (11).

In order to illustrate the diversity in a microbial community, first the 16S rRNA gene is amplified using PCR and then investigated through DGGE technique. In DGGE, electrophoresis is performed in a polyacrylamide gel which contains a linear gradient of urea and formamide that act as denaturants. While PCR products migrate in gel, they will face more concentrations of denaturants, and at some points, they are going to be denatured partially or completely (10). The additional GC-clamp tail in one of the PCR primers has been recommended to avoid total dissociation of double-stranded DNA in gel and permits separation of fragments from different bacterial species that present in one sample (12). However, the GC-clamp might alter the annealing temperature of primers and defect their ability to anneal with templates; hereafter, inefficiency in the detection of less abundant species could occur in more complex communities. To overcome this problem, the usage of nested-PCR has been recommended. Besides being a cost-effective, easy to perform, and reliable technique, bands in a DGGE gel might be excised and sequenced for further investigation, unlike other profiling methods (13).

In this study, we performed conventional culturing methods to survey airway pathogens and antimicro-

bial susceptibility tests were carried out to determine their resistance pattern in pharyngeal samples of CF patients. Since our culturing results did not exhibit the expected diversity, some samples were chosen for deeper analysis by PCR-DGGE and further sequencing to evaluate the accuracy of these cultures and to gain a perspective towards the species that are usually missed in these conventional techniques.

MATERIALS AND METHODS

CF patients. In this study, 121 patients from the Iranian CF center in the children's medical center in Tehran participated in this project between November 2018 and August 2019. Among participants, 56.19% were male and 43.8% were females.

Culture methods. Respiratory specimen samples (pharyngeal swabs) were routinely cultured onto several solid media, including Chocolate agar, Sheep's Blood agar, Mannitol Salt agar, and MacConkey agar. Additionally, colonial morphology, Gram staining, and other standard biochemical tests were used for the identification of each bacterial species. All isolates were kept at -20°C in tryptic soy broth (Merck, Germany) containing 10% dimethyl sulfoxide (Merck, Germany) (v/v) (14). After that, the residual specimen was stored at -80°C in 0.5 ml aliquots. Culture results for each specimen were obtained from the clinical microbiology laboratory database (7).

Antibacterial susceptibility testing. Antibacterial susceptibility profiles of the isolates were determined by disk diffusion according to the Clinical and Laboratory Standards Institute (15). The used antibiotic disks (Padtan Teb, Iran) were co-trimoxazole (1.25/23.75 µg), gentamicin (10 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), imipenem (10 µg) amikacin (30 µg), azithromycin (15 µg), clindamycin (2 µg), erythromycin (15 µg), cefixime (5 µg), cefoxitin (30 µg), cefalotin (30 µg), oxaciline (1 µg), fosfomycin (200 µg), cefaclor (30 µg), ceftriaxone (30 µg), vancomycin (30 µg), ampicillin (10 µg), nalidixic acid (30 µg), penicillin (10 µg), levofloxacin (5 µg), tobramycin (10 µg), and norfloxacin (10 µg). All tests were repeated 3 times and their average inhibition zones were reported. *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 were used as controls.

PCR-DGGE and 16S rRNA sequencing. In this step, 30 samples were chosen for further molecular investigations. A phenol-chloroform technique with modifications was used to obtain bacterial DNA (16). Then, 500 µl of cold lysis buffer (NaCl 1.5 M, Tris base 100 mM, EDTA 100 mM), 13 µl of SDS (25%), and 100 mg of glass beads (0.5 mm) were added to 200 µl of thawed pharyngeal samples. The tubes were vortexed for 90 seconds, 700 µl of chloroform-isoamyl alcohol (24:1 v/v) was added and the tubes were centrifuged at 16000× g for 10 min. Next, the supernatant was transferred to another sterile tube and one volume of isopropanol was added to precipitate DNA overnight at -20°C. The tubes were again centrifuged at 20000× g for 15 min and the supernatant was removed. Then the pellet was washed by adding 300 µl of 70% ethanol followed by centrifugation at 20000× g for 12 min. At this step, the supernatant was removed and the pellet was dissolved in 30 µl of sterile TE buffer and stored at -20°C. The same procedure was used to extract DNA from pure cultures of *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and, *Enterobacter aerogenes* isolated from patients.

In the next step, 16S rRNA gene was amplified using universal primers (27f and 1492r). PCR reactions were performed in a final volume of 25 µl, containing 12.5 µl of ready to use PCR master mix, 2×(SinaClon, Iran), 5 µM of each primer, and 100 ng of DNA templates. Following conditions were used for amplification: 95°C for 10 min for initial denaturation, 30 cycles of 94°C for 1 min, 55°C for 45 s and 72°C for 2 min, and a final extension at 72°C for 10 min. After that, V3-V4 region of 16S rRNA gene was amplified in nested-PCR by using primers 341f (5´-GC-clamp-ACTCCTACGGGAGGCAGCAG-3´) containing a 40 bp GC-clamp, and 782r (5'-GCGTGGACTACCAGGGTATC-3') (17). Each 25 µl PCR reaction mixture contained 12.5 µl of ready to use PCR master mix, 2× (SinaClon, Iran), 3 µM of each primer, and 0.5 µl of the diluted previous PCR product (1:10) as DNA template. The second PCR was carried out with the following conditions, 95°C for 4 min, 35 cycles of 95°C for, 65°C for 45 s and 72°C for 1, and a final extension at 72°C for 10. The products of the latter PCR were separated by using D-Code system (Bio-Rad, USA) on polyacrylamide gel (8%) with a 35% to 75% linear denaturing gradient. The amplified DNA of isolates was loaded on each gel alongside the samples (18, 19). The electrophoresis was done at 60 V and 60°C for 12.5 hours in 1× TAE

buffer, as it is described elsewhere, with modifications made to adjust to the samples (20, 21). Then, the gels were stained using DNA gel stain (Pishgam Biotech Co., Iran) and manually documented by the inGenius3 system (Syngene, USA). Some bands were excised from DGGE gel and their DNA was extracted (Pishgam Biotech Co., Iran). After that, purified DNA was reamplified using 341f and 782r primers without GC-clamp with the previously mentioned program. The PCR products were sent for further sequencing (Pishgam Biotech Co., Iran). Finally, the sequencing results were compared to the GenBank database using BLAST search (NCBI).

Statistics analysis. Statistical analyses were achieved using nonparametric analysis and two-tailed Mann–Whitney U test in SPSS 19 for comparison of prevalence of *P. aeruginosa* and *S. aureus* between patients less than 6 and more than 6 years old as well as for comparison of the frequency of bacterial diversity between patients less than 6 and more than 6 years old.

Acquired sequences were submitted in GenBank database and are available under accession numbers OK103998-104004.

RESULTS

CF patients. Of 121 CF patients who participated in this project, 41.32% were newborns to 6 years old (14% of which are less than 1 year), followed by 6 to 12 (31.4%) and 12 to 18 (14%). The lowest rates of patients (12.4%) were older than 18 years old.

Culture methods. The results of the culture-based method revealed that among CF patients participating in this project, *S. aureus* (38.8%), *P. aeruginosa* (26.44%), and *K. pneumoniae* (12.39%) are the most prevalent species. Other less prevalent species are shown in Table 1. Moreover, The other less common isolated bacterial or fungal species were *C. albicans, E. coli, Acinetobacter* sp., *Citrobacter* sp., and *Enterobacter* sp.. The frequency of each isolate is shown in Table 1. As shown, in the first 6 years of life, *S. aureus* was the prevalent bacteria, whereas, in older patients, *P. aeruginosa* was more common ($P<0.05$).

Antibacterial susceptibility testing. For each bacterial isolate, the antibiotic susceptibility testing was done whose results are shown in Table 1. Only 3.3% of isolates were multi drug resistance (MDR). MDR was defined as resistance to at least one antibiotic within three or more different antibiotic classes (e.g., beta-lactam, aminoglycoside, and fluoroquinolone) (22).

PCR- DGGE and 16S rRNA sequencing. Profile of DGGE presented a relatively different pattern and number of bands for each CF patient (Fig. 1). An average of 5.1 ± 2.2 bands per sample was observed in DGGE gel, and a maximum of 10 bands was recorded for one sample, while the maximum number of bacterial species recovered by cultivation techniques was 2 isolates per sample. The highest diversity was observed in the younger group with the age under 6 years than the older one ($p<0.05$). The presence of certain bacterial genera in samples was evaluated by comparison of the migration pattern of clinically isolated pathogens with the samples. The results showed that the corresponding band to *P. aeruginosa* was observed in 22 of 30 samples, while only 10 of them were recovered in culture. Furthermore, *S. aureus* was recovered by culture in 10 of these 30 samples; while, only 4 of them showed the related band in their DGGE pattern. Meanwhile, 4 of these samples demonstrated the band corresponding to *K. pneumoniae*, whose number of recovered isolates among these 30 samples was 6.

Sequencing data (Table 2) indicated the presence of the certain bacterial species that were completely missed by culturing techniques or the species that are usually considered as normal flora but could cause infections in the lower airways of CF patients. As expected, the bands whose sequences turned out to be similar to *P. aeruginosa* and *K. pneumoniae* showed complete alignment to the corresponding bands of those bacteria.

DISCUSSION

The main manifest of CF is respiratory infection, which begins in early childhood of these patients. Lung infection and antimicrobial resistance could lead to decline of lung function and pulmonary damage in CF patients. The improvements in molecular methods such as PCR-DGGE allowed us to know microbiota composition that has a significant role in CF lung disease.

According to the culture-based results, *S. aureus* and *P. aeruginosa* are the most common bacteria, respectively. Whereas, *S. aureus* isolates were com-

Table 1. Percentage of prevalence and antibiotic resistance of bacterial isolates from CF patients

Strain	Prevalence (%)	Antibiotic resistance (%)																					
		SXT	GM	CAZ	CP	IPM	NOR	AN	AZM	CC	E	CFM	FOX	CF	OX	CEC	CRO	FF	VA	NA	LEV	P	TOB	AM
P. aeruginosa	26.4	71.4	25.8	0	3.3	7.1	10.5	16.1	-	-	-	87.5	100	-	-	100	46.1	100	-	-	-	-	0	-
S. aureus	38.8	28.5	4	66.6	35.7	0	28.5	-	66.6	42.8	86.9	81.8	0	0	18.1	0	0	5.5	69.5	-	-	85	-	83.3
K. pneumoniae	12.3	57.1	33.3	44.4	21.4	14.2	50	14.2	-	-	-	54.5	33.3	-	-	9.9	27.2	55.5	-	50	25	-	-	-
Enterobacter cloacae	2.45	-	0	-	0	0	-	0	-	-	-	0	-	-	-	-	0	-	-	-	-	-	-	-
Citrobacter sp.	1.65	100	0	-	100	0	-	100	-	-	-	0	0	-	-	-	0	0	-	100	-	-	-	-
S. epidermidis	1.65	0	0	0	0	-	-	-	-	0	-	0	0	-	-	-	-	0	100	-	-	100	-	100
Beta hemolytic *streptococci*	1.65	-	0	0	0	-	100	-	-	-	-	0	-	-	-	-	0	-	0	-	-	0	-	100
Enterococcus faecalis	3.3	-	25	-	0	0	-	50	-	-	-	0	-	-	-	100	-	25	100	-	-	-	-	-
E. coli	3.3	100	50	66.6	75	0	100	25	-	-	-	0	-	-	-	-	0	-	-	-	-	-	-	-
S. viridians	1.65	-	0	0	0	-	100	-	--	--	0	0	-	-	-	0	-	0	100	-	-	0	-	-
S. pneumoniae	0.8	-	0	0	-	-	100	-	-	-	0	0	--	-	-	-	0	-	0	-	-	0	-	0
Edwardsiella	0.8	-	0	0	0	0	-	100	-	-	-	0	0	-	-	-	-	0	-	100	-	-	-	-
Enterobacter gergoviae	0.8	100	100	0	0	0	0	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Klebsiella oxytoca	0.8	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter baumannii	0.8	0	100	100	100	100	100	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Providencia alcalifaciens	0.8	100	100	100	100	100	100	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Burkholderia sp.	0.8	100	100	100	100	100	100	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter johnsonii	0.8	100	100	0	100	100	0	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter asburiae	0.8	100	100	0	0	0	0	100	-	-	-	-	-	-	-	-	--	-	-	-	-	-	-	-
Providencia rettgeri	0.8	0	0	0	100	0	100	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens	0.8	-	100	100	0	0	-	100	-	-	-	-	100	-	-	100	-	-	-	100	-	-	-	-
S. milleri	0.8	-	0	-	-	-	-	-	-	-	100	-	-	-	-	-	0	-	0	-	100	-	-	100
Group D *streptococci*	0.8	-	-	-	-	-	0	-	-	-	100	0	-	-	-	-	-	-	0	-	-	0	-	100
Kluyvera	0.8		100	0	100	100		0				100				100	100							

SXT (co-trimoxazole), GM (gentamicin), CAZ (ceftazidime), CP (ciprofloxacin), IPM (imipenem), NOR (norfloxacin), AN (amikacin), AZM (azithromycin), CC (clindamycin), E (erythromycin), CFM (cefixime), FOX (cefoxitin), CF (cefalotin), OX (oxaciline), CEC (cefaclor), FF (fosfomycin), CRO (ceftriaxone), VA (vancomycin), NA (nalidixic acid), LEV (levofloxacin), P (penicillin), TOB (tobramycin) and AM (ampicillin).
"-" represents no given data.

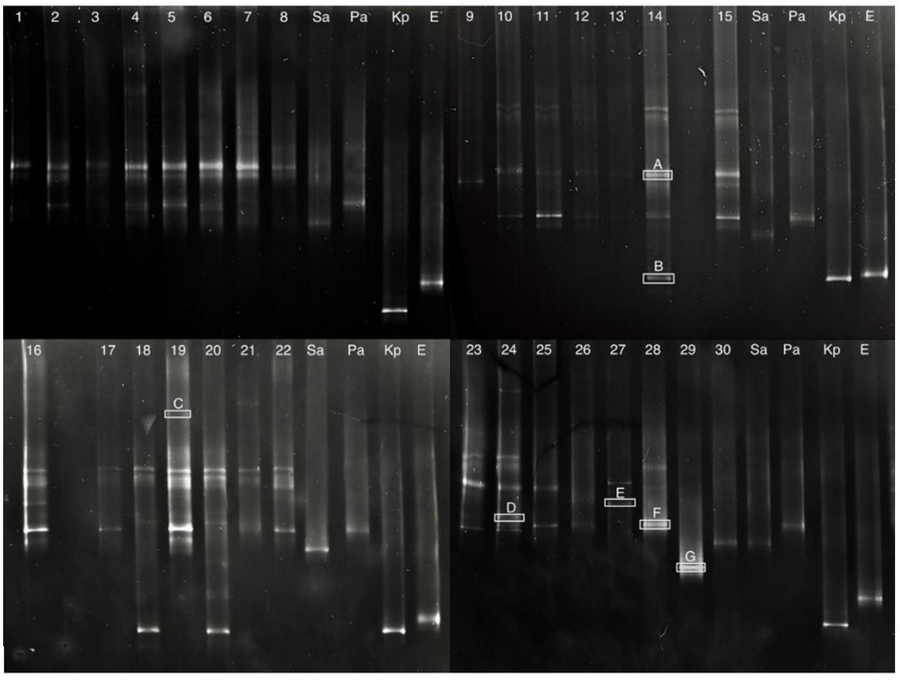

Fig. 1. DGGE profile of the 16S rRNA amplicons from CF respiratory samples (1-30), *S. aureus* (Sa), *P. aeruginosa* (Pa), *K. pneumoniae* (Kp), *E. aerogenes* (E). A-G showcase the position of the sequenced bands.

Table 2. Partial sequencing results of 16s rRNA gene of DGGE gel excised bands

Name	Sequence length (bp)	Sequence ID	Closest BLAST match	
			Species name	Identity (%)
A	160	OK103998	*Streptococcus mitis*	100.00
B	429	OK103999	*Klebsiella pneumoniae*	100.00
C	153	OK104000	*Streptococcus salivarius*	99.35
D	153	OK104001	*Haemophilus parainfluenzae*	98.69
E	425	OK104002	*Lactococcus lactis*	99.53
F	420	OK104003	*Pseudomonas aeruginosa*	100.00
G	432	OK104004	*Stenotrophomonas maltophilia*	100.00

mon in up to 6 years old patients and *P. aeruginosa* was prevalent in older ones. Other studies conducted in Iran reported similar results (23).

Other micro-organisms found in the culture methods follow as *Klebsiella pneumoniae* (12.3%), *Enterococcus faecalis* (3.3%), *Escherichia coli* (3.3%), *Enterobacter cloacae* (2.45%), *Citrobacter* sp. (1.65%), beta-hemolytic streptococci (1.65%), *Staphylococcus epidermidis* (1.65%) and *S. viridians* (1.65%). *S. pneumoniae, Edwardsiella* sp., *Enterobacter gergoviae, Klebsiella oxytoca, Acinetobacter baumannii, Providencia alcalifaciens, Burkholderia* sp., *Acinetobacter johnsonii, Enterobacter asburiae, Providencia rettgeri, Serratia marcescens, S.milleri,* Group D *Streptococcus* and *Kluyvera* found less than 1%.

In our research, *S. aureus* was sensitive to cefoxitin, imipenem, cefalotin, cefaclor and ceftriaxone and *P. aeruginosa* was also sensitive to ceftazidime and tobramycin. Similarly, it was reported that *P. aeruginosa* isolates were sensitive to amikacin and ceftazidime and *S. aureus* isolates were sensitive to linezolid, cefoxitin, teicoplanin, and clindamycin (23).

Emanein et al. reported that although polymyxin B, piperacillin/tazobactam, and meropenem were the most effective antibiotics against *P. aeruginosa* isolates; also, ceftazidime was effective on about 50% of the isolates (24).

The high antibiotic resistance to gentamicin, ciprofloxacin, imipenem, and amikacin among *Burkholderia* sp, *Acinetobacter baumannii, Providencia*

alcalifaciens, and *Acinetobacter johnsonii* isolates (3.3%) was observed as known as MDR. Moreover, the results showed that among *P. aeruginosa* isolates, no MDR was isolated. Other studies reported different frequencies of MDR isolates in Iran. Emaneini et al. described less than half of the *P. aeruginosa* isolates were determined as MDR, whereas, Pournajaf et al. found that 8.4% of *P. aeruginosa* isolates were MDR (24, 25). This difference in antibiotic resistance pattern might be attributed to the different frequent strains in any region as well as antibiotic regimens.

It has recently been demonstrated that there is a large diversity in the CF lung microbiota. The ability of the microbiota to response to changes in lung environmental conditions is enhanced by diversity (26). A critical challenge in CF is to determine microbiota. Although, the limitations of both culture-based and molecular methods in characterizing CF lung infections have become increasingly apparent existing infection control approaches in CF are still limited to classical aerobic culture-based methods that include only screening for the presence or absence of a few bacterial species (5). We could make a point that, although, these regular methods are easy to do, inexpensive and well-defined, molecular approaches have revealed much more diversity in CF lung microbiota (5, 27).

DGGE is considered as a relatively fast, low-cost, and reproducible method to acquire a visual profile of a microbial community (13). Although, DGGE is known to be a technique to evaluate the most prevalent species in the community and to miss less abundant ones, this technique was still more capable of showing diversity than usual culture-dependent methods (13).

Our DGGE results showed that the diversity of the bacterial community was decreased with age. Such a relationship between bacterial diversity and age was believed to be a consequence of antibiotic therapy in CF patients and has been reported in several other studies (28). Loss of bacterial diversity has been frequently reported to be related to dominance of *P. aeruginosa* and the progression of inflammatory diseases (28, 29).

We found that the DGGE technique could be a more efficient way to detect the presence of certain bacterial species such as *P. aeruginosa* than the culture methods. Moreover, *P. aeruginosa* is one of the classic CF pathogens which are proven to have an influence on disease state and severity (30). Such clinically significant species could be missed through the culture methods due to the emergence of auxotrophic strains, but they might be recovered using molecular methods (31). It is worth noting that molecular approaches could be deceiving since DNA from dead bacterial cells might get stuck in CF patients' thick mucosal secretions. Nevertheless, culture method seemed to be more proper way to detect the presence of *S. aureus* than PCR-DGGE. This result is probably due to the improper DNA extraction method in the lysis of bacteria having a thick cell wall such as staphylococci, which leads to the underestimation of their population in molecular techniques (32).

Our sequencing results indicated the presence of oral streptococci in the airway microbiota of CF patients. Streptococcal species could be missed in regular cultures because of their special cultivation requirements (33, 34). Streptococcal species have been increasingly reported in respiratory samples of CF patients (33). Although their mechanism of action is yet to be understood, they are shown to be related to less severe diseases and reduced inflammation; therefore, their presence might be clinically significant (29).

Haemophilus species are known to be one of the most common residents in the respiratory tract of younger CF children, and the presence of *H. parainfluenzae* among lower respiratory microbiota has been reported in numerous articles (34, 35). It is believed that its presence is associated with poor lung function in CF infants (36).

The culture methods used in the current study failed to detect the presence of *Haemophilus* species in patients but molecular methods showed the existence of *H. parainfluenzae* in at least one sample.

Though *Stenotrophomonas maltophilia* infections are receiving more and more attention due to their correlation with more severe disease and decline of lung function and even have been recommended as a marker of disease severity, the regular culture methods used in this study have failed to recognize it in CF samples. The presence of this bacteria in at least one patient was approved by sequencing (37, 38).

CONCLUSION

In conclusion, *S. aureus* and *P. aeruginosa* were the most frequent airway pathogens in our CF patients; also, the appearance of MDR isolates is a ma-

jor concern. Failure to eradicate the pathogens might give rise to lung exacerbation; therefore, it is suggested to investigate the antibiotic resistance pattern in order to choose the appropriate antibiotic therapy. Comprehension of lung microbiota, its diversity, and changes might lead to improved treatments or a decrease in eradication failure of pulmonary infection. It seems molecular methods provide a more accurate profile of microbiota than the culture methods.

ACKNOWLEDGEMENTS

The authors wish to thank Alzahra University's vice-chancellor of research and Immunoregulation Research Center, Shahed University for the financial support and the Pediatric Pulmonary and Sleep Medicine Department, Children's Medical Center, for providing the bacterial isolates.

REFERENCES

1. Bhagirath AY, Li Y, Somayajula D, Dadashi M, Badr S, Duan K. Cystic fibrosis lung environment and *Pseudomonas aeruginosa* infection. *BMC Pulm Med* 2016; 16: 174.
2. Charman S, McClenaghan E, Cosgriff R, Lee A, Carr S. UK Cystic Fibrosis Registry annual data report, 2018. Cyst Fibros Trust London, United Kingdom. 2019. https://www.cysticfibrosis.org.uk/sites/default/files/2020-12/2018%20Registry%20Annual%20Data%20Report.pdf
3. Vandeplassche E, Tavernier S, Coenye T, Crabbé A. Influence of the lung microbiome on antibiotic susceptibility of cystic fibrosis pathogens. *Eur Respir Rev* 2019; 28: 190041.
4. Huang YJ, LiPuma JJ. The microbiome in cystic fibrosis. *Clin Chest Med* 2016; 37: 59-67.
5. Cuthbertson L, Walker AW, Oliver AE, Rogers GB, Rivett DW, Hampton TH, et al. Lung function and microbiota diversity in cystic fibrosis. *Microbiome* 2020; 8: 45.
6. Vandeplassche E, Sass A, Ostyn L, Burmølle M, Kragh KN, Bjarnsholt T, et al. Antibiotic susceptibility of cystic fibrosis lung microbiome members in a multispecies biofilm. *Biofilm* 2020; 2: 100031.
7. Garcia-Nuñez M, Garcia-Gonzalez M, Pomares X, Montón C, Millares L, Quero S, et al. The respiratory microbiome in cystic fibrosis: compartment patterns and clinical relationships in early stage disease. *Front Microbiol* 2020; 11: 1463.
8. O'Brien TJ, Welch M. A continuous-flow model for *in vitro* cultivation of mixed microbial populations associated with cystic fibrosis airway infections. *Front Microbiol* 2019; 10: 2713.
9. Liu T, Jia T, Chen J, Liu X, Zhao M, Liu P. Analysis of microbial diversity in Shenqu with different fermentation times by PCR-DGGE. *Braz J Microbiol* 2017; 48: 246-250.
10. Siqueira JF, Sakamoto M, Rosado AS. Microbial community profiling using terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE). *Methods Mol Biol* 2017; 1537: 139-152.
11. Bluth MJ, Bluth MH. Molecular pathology techniques: advances in 2018. *Clin Lab Med* 2018; 38: 215-236.
12. Phadke S, Salvador AF, Alves JI, Bretschger O, Alves MM, Pereira MA. Harnessing the power of PCR molecular fingerprinting methods and next generation sequencing for understanding structure and function in microbial communities. *Methods Mol Biol* 2017; 1620: 225-248.
13. Singh, Akhlash. (2021). Genomic Techniques Used to Investigate the Human Gut Microbiota. 10.5772/intechopen.91808.
14. Moazami-Goudarzi S, Eftekhar F. Multidrug resistance and integron carriage in clinical isolates of P*seudomonas aeruginosa* in Tehran, Iran. *Turk J Med Sci* 2015;45:789-793.
15. Clinical and Laboratory Standards Institute (2016) CLSI Document M100- S21. Performances Standards for Antimicrobial Susceptibility Testing. 26th Edition Informational Supplement, Wayne.
16. Willner D, Daly J, Whiley D, Grimwood K, Wainwright CE, Hugenholtz P. Comparison of DNA extraction methods for microbial community profiling with an application to pediatric bronchoalveolar lavage samples. *PLoS One* 2012; 7(4): e34605.
17. Sundström K, Mishra PP, Pyysalo MJ, Lehtimäki T, Karhunen PJ, Pessi T. Similarity of salivary microbiome in parents and adult children. *PeerJ* 2020; 8: e8799.
18. Fasoli S, Marzotto M, Rizzotti L, Rossi F, Dellaglio F, Torriani S. Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *Int J Food Microbiol* 2003; 82: 59-70.
19. Hong SW, Lee JS, Chung KS. Polymerase chain reaction-denaturing gradient gel electrophoresis analysis of bacterial community structure in the food, intestines, and feces of earthworms. *J Microbiol* 2011; 49: 544-550.
20. Nelson A, De Soyza A, Bourke SJ, Perry JD, Cummings SP. Assessment of sample handling practices on microbial activity in sputum samples from patients with cystic fibrosis. *Lett Appl Microbiol* 2010; 51: 272-

277.
21. Ding C, Adrian L, Peng Y, He J. 16S rRNA gene-based primer pair showed high specificity and quantification accuracy in detecting freshwater *Brocadiales anammox* bacteria. *FEMS Microbiol Ecol* 2020; 96: fiaa013.
22. Hahn A, Burrell A, Fanous H, Chaney H, Sami I, Perez GF, et al. Antibiotic multidrug resistance in the cystic fibrosis airway microbiome is associated with decreased diversity. *Heliyon* 2018; 4(9): e00795.
23. Kodori M, Nikmanesh B, Hakimi H, Ghalavand Z. Antibiotic susceptibility and biofilm formation of bacterial isolates derived from pediatric patients with cystic fibrosis from Tehran, Iran. *Arch Razi Inst* 2021;76:397-406.
24. Emaneini M, Kalantar-Neyestanaki D, Jabalameli L, Hashemi M, Beigverdi R, Jabalameli F. Molecular analysis and antimicrobial resistance pattern of distinct strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients in Iran. *Iran J Microbiol* 2019; 11: 98-107.
25. Pournajaf A, Razavi S, Irajian G, Ardebili A, Erfani Y, Solgi S, et al. Integron types, antimicrobial resistance genes, virulence gene profile, alginate production and biofilm formation in Iranian cystic fibrosis Pseudomonas aeruginosa isolates. *Infez Med* 2018; 26: 226-236.
26. Kordes A, Preusse M, Willger SD, Braubach P, Jonigk D, Haverich A, et al. Genetically diverse *Pseudomonas aeruginosa* populations display similar transcriptomic profiles in a cystic fibrosis explanted lung. *Nat Commun* 2019; 10: 3397.
27. Mahboubi MA, Carmody LA, Foster BK, Kalikin LM, VanDevanter DR, LiPuma JJ. Culture-based and culture-independent bacteriologic analysis of cystic fibrosis respiratory specimens. *J Clin Microbiol* 2016; 54: 613-619.
28. Muhlebach MS, Zorn BT, Esther CR, Hatch JE, Murray CP, Turkovic L, et al. Initial acquisition and succession of the cystic fibrosis lung microbiome is associated with disease progression in infants and preschool children. *PLoS Pathog* 2018; 14(1): e1006798.
29. Acosta N, Heirali A, Somayaji R, Surette MG, Workentine ML, Sibley CD, et al. Sputum microbiota is predictive of long-term clinical outcomes in young adults with cystic fibrosis. *Thorax* 2018; 73: 1016-1025.
30. Parkins MD, Somayaji R, Waters VJ. Epidemiology, biology, and impact of clonal *Pseudomonas aeruginosa* infections in cystic fibrosis. *Clin Microbiol Rev* 2018; 31(4): e00019-18.
31. Planet PJ. Adaptation and evolution of pathogens in the Cystic Fibrosis Lung. *J Pediatric Infect Dis Soc* 2022; 11(Supplement_2): S23-S31.
32. Brooks JP, Edwards DJ, Harwich MD, Rivera MC, Fettweis JM, Serrano MG, et al. The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiol* 2015; 15: 66.
33. Scott JE, O'Toole GA. The yin and yang of Streptococcus lung infections in cystic fibrosis: a model for studying polymicrobial interactions. *J Bacteriol* 2019; 201(11): e00115-19.
34. Zachariah P, Ryan C, Nadimpalli S, Coscia G, Kolb M, Smith H, et al. Culture-independent analysis of pediatric bronchoalveolar lavage specimens. *Ann Am Thorac Soc* 2018; 15: 1047-1056.
35. Ahmed B, Cox MJ, Cuthbertson L, James P, Cookson WOC, Davies JC, et al. Longitudinal development of the airway microbiota in infants with cystic fibrosis. *Sci Rep* 2019; 9: 5143.
36. Law DKS, Shuel M, Bekal S, Bryce E, Tsang RSW. Genetic detection of quinolone resistance in *Haemophilus parainfluenzae*: mutations in the quinolone resistance-determining regions of gyrA and parC. *Can J Infect Dis Med Microbiol* 2010; 21(1): e20-22.
37. Barsky EE, Williams KA, Priebe GP, Sawicki GS. Incident *Stenotrophomonas maltophilia* infection and lung function decline in cystic fibrosis. *Pediatr Pulmonol* 2017; 52: 1276-1282.
38. Berdah L, Taytard J, Leyronnas S, Clement A, Boelle P-Y, Corvol H. *Stenotrophomonas maltophilia*: a marker of lung disease severity. *Pediatr Pulmonol* 2018; 53: 426-430.