Short Communication

Antibiotic Susceptibility and Biofilm Formation of Bacterial Isolates Derived from Pediatric Patients with Cystic Fibrosis from Tehran, Iran

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Abstract

Cystic fibrosis (CF) is a genetic disease with a high rate of morbidity and mortality. Children with CF commonly suffer from recurrent and persistent pulmonary tract infections caused by diverse bacterial pathogens. This study aimed to investigate the prevalence, antimicrobial susceptibility, and biofilm formation of bacterial isolates in pediatric patients with CF. The study population of this cross-sectional study included 8,908 children suspected to have CF by clinical manifestations from March 2015 to August 2017 who were referred to the Tehran Pediatric Central Hospital, Iran. The tests carried out for each participant included screening sweat test, sputum culture, antibiotic susceptibility test using Kirby-Bauer disk diffusion method, and biofilm formation in microtiter plates method. Based on clinical examination and screening sweat test, 183 (2.05 %) out of 8,908 children, were positive for CF. The mean age of children was estimated at 2.93 years, and the majority of them were male (n=103, 56.2%). No gender-specific difference was observed in CF disease in this study (P>0.05). In addition, the results of sputum culture showed that 153 (83.6%) microorganisms (bacteria and fungi) were collected from CF patients. Normal flora was isolated in 30 (16.4%) patients and more than one bacterial species were isolated in 7.2% of patients. The obtained results indicated that _Pseudomonas aeruginosa_ was the most prevalent isolated bacteria followed by _Staphylococcus aureus, and Klebsiella pneumoniae_. Based on the antibiotic susceptibility test results, _P. aeruginosa_ and piperacillin/tazobactam had the highest (11.7%) and the lowest (2.3%) resistance rate against gentamicin, respectively. However, all _K. pneumoniae_ isolates were resistant to Cefotaxime. Among _S. aureus_ isolates, 83.4% and 16.6% were methicillin-susceptible Staphylococcus aureus and Methicillin-resistant Staphylococcus aureus respectively. Concerning biofilm formation, 76%, 67%, and 72.5% of _P. aeruginosa, S. aureus_, and _K. pneumoniae_ isolates were biofilm producers, respectively. Based on the study results, _P. aeruginosa_ was the dominant pathogen in pediatric patients with CF from Tehran, Iran, and most of the pathogens were biofilm producers. No severe antibiotic resistance was observed in the isolates; however, the anti-microbial resistance profile should be carefully checked in CF patients on a regular basis.

Keywords: Antibiotics susceptibility test, Biofilm formation, Cystic fibrosis, Pediatric

Susceptibilité aux Antibiotiques et Formation de Biofilm d'Isolats Bactériens Dérivés de Patients Pédiatriques Atteints de Fibrose Kystique de Téhéran, Iran

Résumé: La fibrose kystique (FK) est une maladie génétique avec un taux élevé de morbidité et de mortalité. Les enfants atteints de mucoviscidose souffrent couramment d'infections des voies pulmonaires récurrentes et persistantes causées par divers agents pathogènes bactériens. Cette étude visait à étudier la prévalence, la

sensibilité aux antimicrobiens et la formation de biofilm d'isolats bactériens chez les patients pédiatriques atteints de mucoviscidose. La population de cette étude transversale comprenait 8,908 enfants soupçonnés d'être atteints de mucoviscidose par des manifestations cliniques de mars 2015 à août 2017 qui ont été référés à l'hôpital central pédiatrique de Téhéran, en Iran. Les tests effectués pour chaque participant comprenaient un test de dépistage de la sueur, une culture d'expectorations, un test de sensibilité aux antibiotiques utilisant la méthode de diffusion sur disque de Kirby-Bauer et la méthode de formation de biofilm dans des plaques de microtitrage. Sur la base d'un examen clinique et d'un test de dépistage de la sueur, 183 (2.05%) sur 8,908 enfants, étaient positifs pour la mucoviscidose. L'âge moyen des enfants était estimé à 2.93 ans, et la majorité d'entre eux étaient de sexe masculin (n = 103, 56.2%). Aucune différence spécifique au sexe n'a été observée dans la maladie FK dans cette étude (P>0.05). De plus, les résultats de la culture d'expectorations ont montré que 153 (83.6%) microorganismes (bactéries et champignons) ont été collectés chez des patients FK. La flore normale a été isolée chez 30 (16.4%) patients et plus d'une espèce bactérienne a été isolée chez 7.2% des patients. Les résultats obtenus ont indiqué que *Pseudomonas aeruginosa* était la bactérie isolée la plus répandue, suivie de *Staphylococcus aureus* et *Klebsiella pneumonies*. Sur la base des résultats des tests de sensibilité aux antibiotiques, *P. aeruginosa* et la *pipéracilline/tazobactam* avaient respectivement le taux de résistance le plus élevé (11.7%) et le plus faible (2.3%) à la gentamicine. Cependant, tous les isolats de *K. pneumoniae* étaient résistants au Céfotaxime. Parmi les isolats de *S. aureus*, 83,4% et 16,6% étaient respectivement de *Staphylococcus aureus* sensible à la méthicilline et de *Staphylococcus aureus* résistant à la méthicilline. Concernant la formation de biofilm, 76%, 67% et 72.5% des isolats de *P. aeruginosa*, *S. aureus* et *K. pneumoniae* étaient des producteurs de biofilm, respectivement. D'après les résultats de l'étude, *P. aeruginosa* était le pathogène dominant chez les patients pédiatriques atteints de mucoviscidose de Téhéran, en Iran, et la plupart des agents pathogènes étaient des producteurs de biofilm. Aucune résistance aux antibiotiques sévère n'a été observée dans les isolats; cependant, le profil de résistance aux antimicrobiens doit être vérifié régulièrement chez les patients atteints de mucoviscidose.

Mots-clés: Test de sensibilité aux antibiotiques, Formation de biofilm, Fibrose kystique, Pédiatrique

1. Introduction

Cystic fibrosis (CF) patients are susceptible to chronic endobronchial colonization and chronic infections with the microorganism, especially bacterial pathogens (Brennan and Schrijver, 2016; Hisert et al., 2017). *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae,* and gram-negative enteric bacilli are the most important bacteria that infect CF patients. Interestingly, there is a strong correlation between the prevalence of bacterial infection in CF patients and their age or geographical regions (Parkins and Floto, 2015). It should be noted that the colonization and infection of bacteria in the bronchobiliary tree of CF patients leads to the destruction of lung tissue and attenuate their function. Although several antibiotics are prescribed for the treatment and the control of pulmonary infections in CF patients, some bacteria species have recently become resistant to these agents and caused tremendous clinical problems for the management of CF disease (Filkins

and O'Toole, 2015). *P. aeruginosa* strains developed multidrug resistance to carbapenems in recent years, which is a serious concern for the researchers (Chalhoub et al., 2016). Several mechanisms have been described for the antibiotic resistance in *P. aeruginosa*, including reduction of antibiotic penetration, production of metallo beta lactamase (MBLs), formation of biofilm, and the existence of hypermutable strains (Blair et al., 2015). Biofilm formation in the dehydrated mucous layer by *P. aeruginosa* and *S. aureus* may establish a microenvironment for the growth and the protection of the bacteria against the antibiotic effects and immune response (Winstanley et al., 2016). Methicillin resistance *S. aureus* (MRSA) strain is another bacteria strain which displayed a resistant phenotype that is much more difficult to treat and manage in CF patients (Liu et al., 2016). It is worth mentioning that early treatment regimen has not yet been widely accepted in CF patients. Therefore, the main concerns of clinicians include the determination of optimal drug combination,

the method of drug delivery, the efficacy of treatment strategies on pulmonary function, antibiotic resistance, the emergence of other pathogens, and treatment costs (Farrell et al., 2017). Recognition of these bacterial agents, resistance patterns, and the best treatment strategies are important factors affecting the quality of life in CF patients, especially pediatric patients who are more susceptible to infection. This study aimed to investigate the prevalence of bacterial isolates in pediatric patients with CF who were referred to the Tehran Pediatric Central Hospital, Tehran, Iran, from March 2015 to August 2017. The antibiotic susceptibility patterns of the isolates were determined using the Kirby-Bauer disk diffusion method. In addition, the biofilm formation ability of isolates was assessed using the microtiter plate method.

2. Material and Methods

2.1. Patient Recruitment

This descriptive study included 8,908 pediatric patients who were suspected to have CF by clinical manifestations and were referred to the Tehran Pediatric Central Hospital, Tehran, Iran, from March 2015 to August 2017. Each participant had two sweat tests (Gibson and Cooke technique) and a sputum sample was also collected for microbial culture in case the two sweat tests were positive for CF (O'Sullivan, 2016).

2.2. Sputum Culture

The patient's Sputum was weighed prior to dissolving by 0.01% of 1, 4-Dithiothreitol (Merck, Germany) to reach a final dilution of 1:2. Afterward, 50 μl of the diluted sputum was separately cultured on five plates, including two plates of blood-agar (Liofilchem, Italy), two plates of chocolate agar (Liofilchem, Italy) with bacitracin (Becton Dickinson, Germany), and one plate of MacConkey agar (Liofilchem, Italy). One plate of each culture was incubated at 37 °C for 24 h and the two remaining plates were incubated at 37 °C in a 5–10% CO_2 enriched atmosphere for 48 h. The number of colony-forming units (CFU) per ml of sputum was determined by the colony counts on each plate. Identification of organisms was performed using routine

techniques, including morphological and biochemical tests. Bacterial isolates were maintained as stock cultures at −20°C in tryptic soy broth (TSB) supplemented with 15% glycerol until further examination (Tille, 2017).

2.3. Antimicrobial Susceptibility Testing

All culture plates were subjected to the Kirby-Bauer disk diffusion method using amikacin, cefepime, cefotaxime cefoxitin, ceftazidime, clindamycin, trimethoprim-sulfamethoxazole, erythromycin, gentamicin imipenem, methicillin, penicillin, piperacillin/tazobactam, and vancomycin antibiotic disks. Susceptibility of *P. aeruginosa* and *S. aureus* isolates to vancomycin, as well as colistin and methicillin (cefoxitin strip), respectively, were determined using E-test strips (MIC Test Strip, Liofilchem, Italy). The results were interpreted according to the breakpoints and recommendations of Liofilchem Company (colistin; susceptible ≤2μg/ml, resistant ≥4μg/ml, vancomycin; susceptible <2μg/ml; intermediate 4-8 μg/ml, resistant ≥2μg/ml, cefoxitin; susceptible ≤4, resistant ≥8). The isolates showing intermediate levels of susceptibility were classified as resistant. *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 were used as the quality control strains in susceptibility testing. The results were evaluated by the guidelines of the Clinical and Laboratory Standards Institute.

2.4. Biofilm Formation Assay

The capacity of biofilm formation in *P. aruginosa*, *S. aureus*, and *k. pneumoniae* isolates were measured using the microtiter plate method. In brief, 0.5 McFarland turbidity was prepared from overnight cultures of each isolate prior to 100-fold dilution using TBS (Liofilchem, Italy). Subsequently, 300 μl of this suspension was inoculated in triplicate on 96-well flat-bottomed polystyrene plate (jet biofil, China) and incubated overnight at 37°C. The content of each well was then discarded and wells were washed three times with phosphate-buffered saline. Afterward, methanol fixation was conducted for 15 min and the plate was then air-dried. Each well was stained with 300 μl of 1%

crystal violet solution in water and incubated at room temperature for 30 min. Subsequently, the stain was solubilized by 300 μl of acetic acid 33% after the plate was washed three times with distilled water and then dried. The optical density (OD) of each well was read at 570 nm using an ELISA reader (Biotech, USA). The cut-off OD control for the microtiter plate was defined as three standard deviations (SD) plus the mean OD of the negative control. Based on the OD average values, the results of biofilm formation assay were analyzed as follows; non-biofilm producer (OD≤OD$_C$); weak producer (OD$_C$≤ OD≤2OD$_C$); moderate-producer (2OD$_C$≤OD≤4OD$_C$), and strong producer (4OD$_C$≤OD).

2.5. Statistical Analysis

Data were analyzed in SPSS software (version 21; IBM Corp., Armonk, USA) through Fisher's exact and Chi-Square tests. A p-value less than 0.05 was considered statistically significant.

3. Results

Out of 8,908 recruited children (either inpatient or outpatient), 4,633 (52%) and 4,275 (48%) patients were male and female, respectively. Based on the results of the sweat test, 183 (2.05%) suspected children were diagnosed with CF disease (Cl≥60 mEq/L). Clinical manifestations were 56.2% and 43.7% in male and female patients, respectively, with the mean age ±SD of 2.93±0.2 years, indicating that there were no gender-specific differences (two-tailed Mann-Whitney U test, P>0.05).

In total, 153 (83.6%) bacteria and fungi species were collected from CF patients. It should be noted that 30 (16.4%) patients were diagnosed with normal flora and 7.2% of the patients were infected by more than one species. Out of all culture-positive patients 85 (55.5%), 24 (15.6%), 16 (10.4%), 18 (11.7%), 4 (2.6%), 2 (1.3%), 2 (1.3%), 2 (1.3%) were infected by *P. aeruginosa*, *S. aureus*, *Candida albicans*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli*, *Serratia marcescens*, and *Streptococcus pyogenes*, respectively (Figure 1).
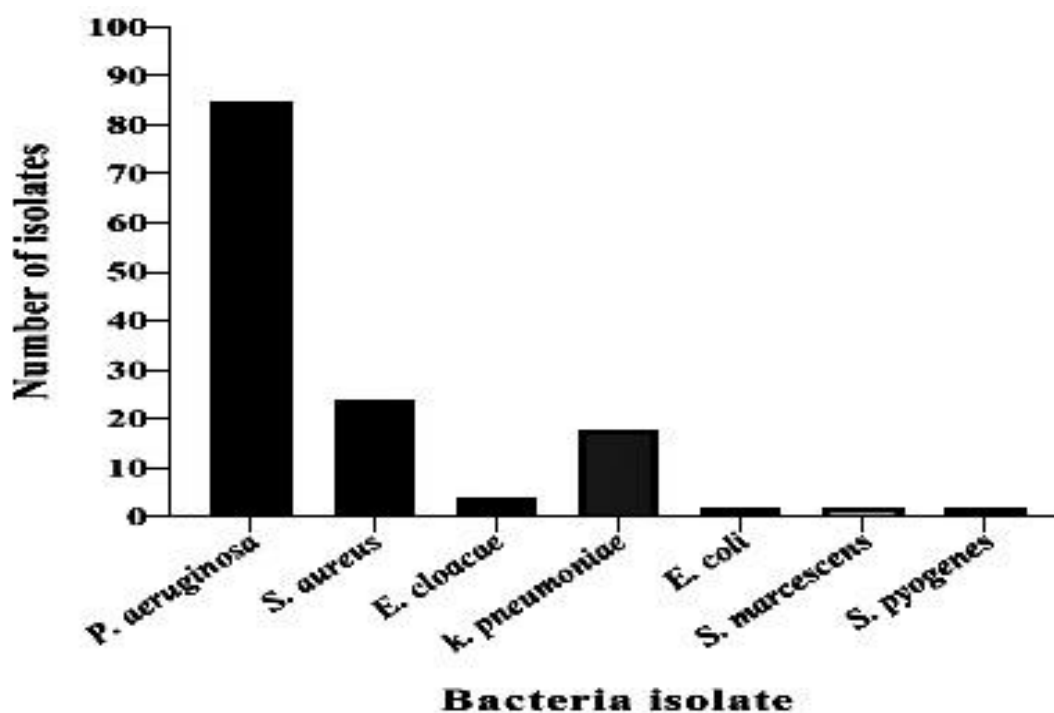

Figure 1. The Frequency of bacterial isolates collected from CF patients.

The results of antibiotic susceptibility testing showed that in *p. aeruginosa* isolates, the highest resistance rate was observed in gentamicin (11.7%), followed by amikacin (7.05%), imipenem (7.05%), and ceftazidime (5.9%) and the lowest resistance rates were observed in piperacillin-tazobactam (2.3%; Figure 2A). All *P. aeruginosa isolates* in E-test were susceptible to colistin. In *S. aureus* isolates, the highest resistance rate was observed in erythromycin, penicillin, and clindamycin (50%), followed by trimethoprim-sulfamethoxazole (20.8%) and Cefoxitin (16.6%; Figure 2B). Among *S. aureus* isolates, the rate of

MRSA and MSSA were determined at 16.6% and 83.4%, respectively, and no isolates were found to be resistant to vancomycin.

Results of antibiotic resistance of other isolates were summarized in Table 1. In brief, all *K. pneumonia* isolates were resistant to cefotaxime, and 83.3%, 66.7%, and 66.7% of isolates were resistant to cefepime, piperacillin-tazobactam, and trimethoprim-sulfamethoxazole, respectively. Out of four isolates of *E. cloacae,* 50% were resistant only to trimethoprim-sulfamethoxazole, and the other antibiotics were effective against this bacteria.
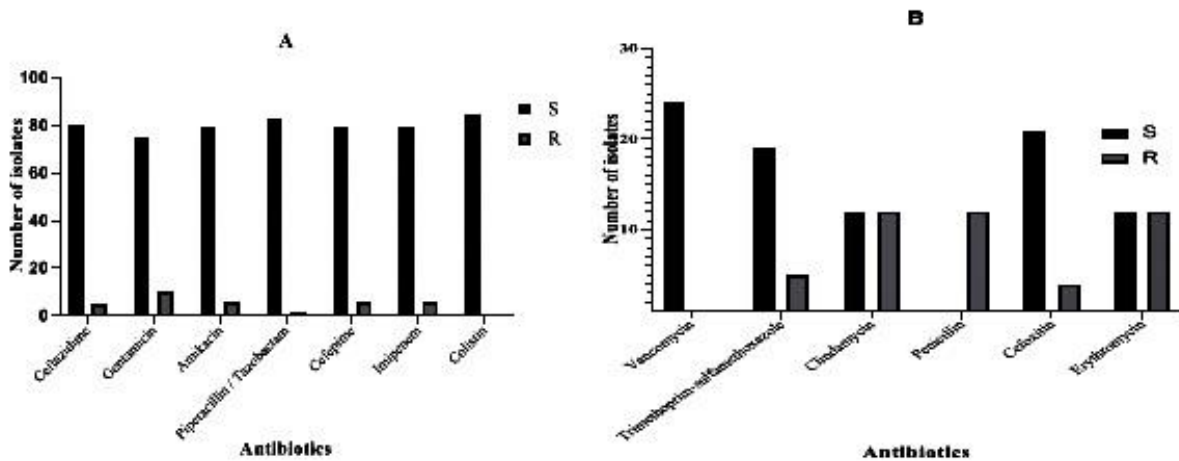

Figure 2. Antibiotic susceptibility in (**A**) *P. aeruginosa* isolates and (**B**) *S. aureus* isolate

Table1. The prevalence of antibiotic resistance in *K. pneumonia*, *E. cloacae*, *E. coli*, and *S. marcescens* isolates.

Antibiotic	K. pneumonia		E. cloacae		E. coli		S. marcescens	
	N=18	%	N=4	%	N=2	%	N=2	%
Trimethoprim-sulfamethoxazole	12	66.7	2	50	1	50	0	0
Gentamicin	9	50	0	0	0	0	2	100
Amikacin	9	50	0	0	0	0	2	100
Piperacillin-Tazobactam	12	66.7	0	0	0	0	1	50
Cefepime	15	83.3	0	0	1	50	2	100
Imipenem	3	16.7	0	0	0	0	0	0
Cefotaxime	18	100	0	0	1	50	2	100

3.1. Biofilm Formation Assay

Regarding biofilm formation assay, OD_{570} for *P. aeruginosa and S. aureus* isolates were 0.19±0.09 and 0.12±0.02, respectively. Moreover, OD_{570} for TSB media used as a negative control (cut-off) was calculated at 0.082±0.01 (Figure 3). In total, 76.5% of *P.* *aeruginosa* isolates were biofilm producers and most of them belonged to the weak group. Moreover, 23.5% of isolates did not produce any biofilm. In addition, 67% and 72.5% of isolates of *S. aureus* and *K. pneumonia,* were biofilm-producer (Table 2).
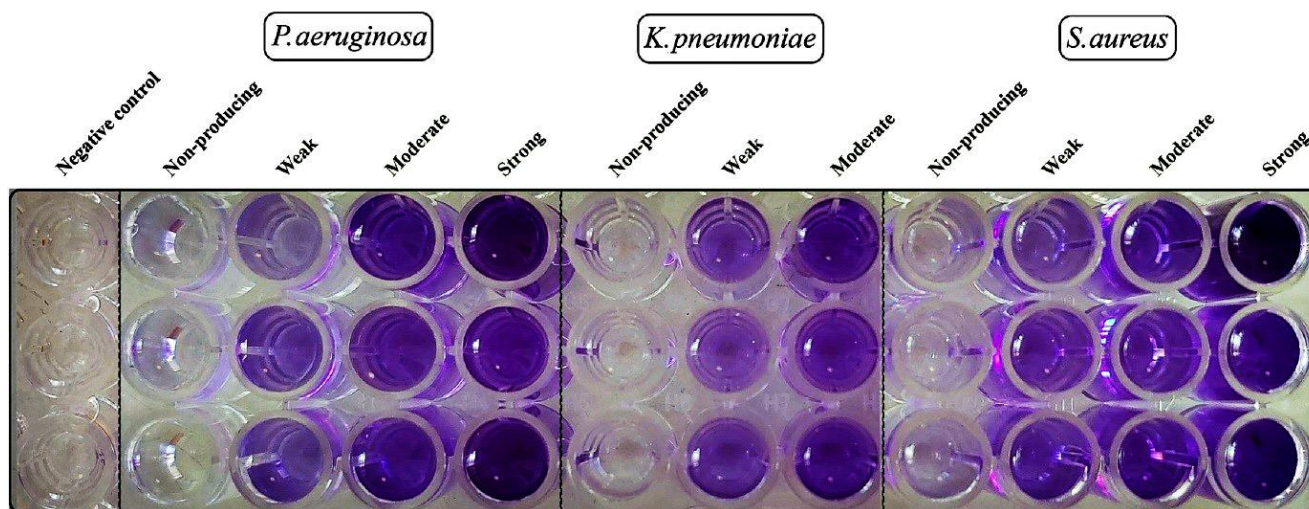

Figure 3. Biofilm formation in microtiter plate method for different isolates of *P. aeruginosa*, *S. aureus*, and *K. pneumonia* with different levels of biofilm formation.

Table 2. Status of biofilm formation among *P. aeruginosa*, *S. aureus*, and *K. pneumonia* isolates collected from CF patients.

Bacteria	Biofilm formation			
	Non-producing N (%)	Weak N (%)	Moderate N (%)	Strong N (%)
P. aeruginosa	20 (23.5)	40 (47)	20 (23.5)	5 (6)
S. aureus	8 (33.3)	9 (37.5)	5 (20.9))	2 (8.3)
K. pneumoniae	5 (27.7)	7 (39)	6 (33.3)	0

4. Discussion

In the present study, a rather large number of pediatric patients with CF was investigated. The respiratory system of CF patients can be frequently infected by persistent and recurrent bacterial infections which in turn leads to a high rate of morbidity and mortality, particularly in the presence of risk factors (Filkins and O'Toole, 2015). It should be noted that *P. aeruginosa*, *Burkholderia cepacia* complex, *S.*

aureus, and enteric gram-negative bacilli are the most prevalent microorganisms that infect CF patients. It is well-established that *P.* aeruginosa is the most important pathogen in the airways of CF patients and its colonization can occur after visiting hospitals or clinics (Chiappini et al., 2014). Given the fact that chronic lung infection can exacerbate the prognosis of CF patients, it seems that antibiotic treatment strategies are not effective in the elimination of resistant bacterial pathogens in CF airways. This phenomenon can be mainly attributed to the formation of biofilm by bacterial products. Biofilm formation in CF is assisted by the secretion of a thick mucus layer in the airway, which provides an anaerobic, supportive, and nutritive microenvironment for the growth of the microbial population (Høiby et al., 2017; Montgomery et al., 2017).

Based on the results, 2.05% of all suspected patients admitted to the CF clinic had a positive sweat test. The mean age of all patients was estimated at 2.93 years, and no gender-specific differences were observed between the study samples (P>0.05). In total, microbiological analysis revealed that only 83% of CF patients were positive, with a bacterial frequency of 74.9%, and that only 10.4% of patients were infected with *C. albicans* isolates. Normal flora was diagnosed in 16.4% of patients, and 7.2% were concomitantly infected with two bacterial genera. Among all isolated bacteria, *P. aeruginosa* was the most prevalent pathogen suggesting that the primary control of *P. aeruginosa* colonization may result in a good outcome in CF patients. Moreover, it was found that the two most common isolates after *P. aeruginosa* were *S. aureus* and *k. pneumonia,* respectively. These findings were in line with those of other studies that suggested chronic pulmonary infections in CF patients were caused mainly by *P. aeruginosa* and *S. aureus* (Ahlgren et al., 2015). In contrast, the CF foundation report showed that based on the results

of sputum culture, 27.5% of CF patients were positive for *P. aeruginosa*. This discrepancy may be related to factors such as the colonization with *P. aeruginosa*, low age of patients, and inappropriate management and treatment of CF patients. On the other hand, the prevalence of *P. aeruginosa* continued to decline over time while multidrug-resistant *P. aeruginosa* (MDR-PA) remained at the same rate (Mogayzel Jr et al., 2014). Based on the results of some studies *S. aureus* and *H. influenzae* were the most isolated bacteria from CF patients throughout the first decade of life, while infection with *P. aeruginosa* mostly increased in the following decades of life (Valenza et al., 2008). Although*, P. aeruginosa* is a classical CF pathogen, non-classical pathogens have tremendous diversity based on different areas of the lung and datasets (Parkins and Floto, 2015). In this study, the most non-classical species included *S. aureus* and *k. pneumoniae*.

It should be noted that *S. aureus* is one of the earliest pathogens in infants and children suffering from CF. The high incidence of MRSA strains has attracted tremendous attention to this pathogen in the last decades (Goss and Muhlebach, 2011). In this study, the rate of MRSA was considerably lower, which was in line with the results of other studies. However, in contrast to the results of this study, it was reported that 25% of CF patients in the United States were infected with MRSA isolates in respiratory culture. It was also mentioned that persistent MRSA infection can lead to a long duration of hospitalization, worsening of lung function, and reduction of life expectancy in CF patients (Goss and Muhlebach, 2011; Foundation, 2013; Jennings et al., 2017). Meanwhile, the risk of death in MRSA-positive CF patients was 1.27 times higher than those who were MRSA-negative (Jennings et al., 2017). Contamination of the sputum sample with oral flora is a problematic issue in CF patients. Therefore, sputum samples in CF

patients should be collected using an aseptic method, and serially produced sputum samples can be helpful in some cases. Azithromycin, ceftazidime, and Ciprofloxacin have been suggested as therapies for *P. aeruginosa* eradication in CF patients (Talwalkar and Murray, 2016). In this study, antibiotic susceptibility profiles of *P. aeruginosa* isolates showed that piperacillin-tazobactam was the most effective antibiotic. During intensification of infections, imipenem or ceftazidime in combination with an aminoglycoside, such as amikacin, could be promising agents for the antibacterial therapy (Talwalkar and Murray, 2016). In contrast to these agents, it was found that most bacterial isolates displayed a resistant phenotype to gentamicin and ceftazidime. In addition, imipenem and amikacin resistance was observed in *P. aeruginosa* isolates as well.

In agreement with the results of the present study, another study conducted in Iran showed that *P. aeruginosa*, *S. aureus, and K. pneumoniae* were the most common bacteria isolated from pediatric CF patients. The results of their study demonstrated that vancomycin, rifampin, and imipenem were the most effective antibiotics against these microorganisms. In addition, resistance to aminoglycoside was observed (Aghamohammadi et al., 2019). Regarding the fact that *P. aeruginosa* is the most common pathogen in CF patients, it's not surprising that a wide range of antibiotic regimens has been prescribed for this infection. Moreover, based on the results of the recent studies, the antibiotic resistance and virulence of bacterial pathogens in the CF lung might have a tight association with the formation of biofilm (Olsen, 2015). Furthermore, it has been suggested that different morphological colonies can be accumulated in the airways of CF patients based on the stage of colonization. The bacterial density overwhelmingly increases in the late colonization stage and morphological phenotype shifts to produce biofilm in the CF airway to prevent the penetration of antibiotics. This phenomenon can subsequently worsen CF patients' symptoms and reduce the efficacy of antimicrobial treatment strategies (Bowler, 2018). Interestingly, the results of the present study showed that 76%, 67%, and 72.5% of *P. aeruginosa*, *S. aureus,* and *K. pneumoniae* isolates were biofilm producers, respectively. This finding was consistent with that of another study that indicated *P. aeruginosa* isolates could be colonized in the CF respiratory tract by the production of biofilm (Hill et al., 2005). Moreover, in line with the previous reports, the results of this study also indicated that most of the isolates from CF patients were able to form a biofilm, suggesting that a higher concentration of antibiotics prescribed for patients may prevent the formation of biofilm during the stages of colonization. It should be noted that the prevalence of different bacterial pathogens in the sputum of pediatric CF patients should be carefully checked on a regular basis. Based on the results of this study, *P. aeruginosa* was the most dominant bacterial pathogen in pediatric patients with CF, and most of these pathogens were biofilm producers. Although severe antibiotic resistance was not observed in the isolates in this study, the anti-microbial resistance profile in CF patients should be investigated on the regular basis. The role of other less common pathogens in the pathogenesis of CF disease remained to be clarified, and further studies are required to validate the association between biofilm formation and clinical condition of CF patients.

Authors' Contribution

M. K. performed the microbiological analyses and drafted the manuscript,

B. N. coordinated collection of patients' samples,

H. H. revised the manuscript,

Z. G. supervised the study, revised the manuscript and approved of the final version.

All authors read and approved the final manuscript.

Ethics

This study was approved by the Ethics Committee of the Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1397.52). In addition, verbal consent was obtained from the parents or guardians of all children before participation in this study. Initially, the main investigator introduced herself and explained the study objectives to the participants. Afterward, the sweat test was performed on all participants, and sputum samples were collected. Eventually, they were ensured that their name and information would be kept confidential. This is considered to be an acceptable consent procedure by the Ethics Committees at the institutional level (Shahid Beheshti University of Medical Sciences, Tehran, Iran).

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of the present study.

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