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 the high rate of morbidity and mortality. Pediatric with CF commonly suffer from recurrent and persistent pulmonary tract infections caused by diverse bacterial pathogens. This study was conducted to investigate the prevalence, antimicrobial susceptibility and biofilm formation of bacterial isolates in pediatric patients with CF. This cross-sectional study was conducted on 8908 children suspected to have CF by clinical manifestations from March 2015 to August 2017 referred to the Tehran Pediatric Central Hospital, Iran. The following investigations were carried out for each participant; screening sweat test, sputum culture, antibiotic susceptibility test using Kirby-Bauer disk diffusion method, and capacity of biofilm formation microtiter plate method. Based on clinical examination and screening sweat test, out of 8,908 children, 183 (2.05 %) were positive for CF with mean age of 2.93 years and gender distribution of 56.2% male (103) and 43.7% female (80). We found that there were no gender-specific differences in CF disease ($P > 0.05$). Results from sputum culture showed that, one hundred fifty-three (83.6%) microorganisms (bacteria and fungi) were isolated. In 30 (16.4%) patients normal flora were isolated and in 7.2% of patient’s more than one bacterial species were isolated. Pseudomonas aeruginosa was the most prevalent isolated bacteria followed by Staphylococcus aureus, and Klebsiella pneumoniae. According to antibiotic susceptibility test results, P. aeruginosa had the highest resistance rate against gentamicin (11.7%), and for piperacillin/tazobactam had the lowest rate (2.3%). However, all K. pneumoniae isolates were resistant to Cefotaxime. Among S. aureus isolates, 83.4% and 16.6% were MSSA and MRSA respectively. Concerning biofilm formation, 76%, 67% and 72.5% of P. aeruginosa,
S. aureus, and K. pneumoniae isolates were biofilm producer, respectively. Our study showed that P. aeruginosa was the dominant pathogen in pediatric patients with CF from Tehran, most of them were biofilm producer. Severe antibiotic resistance in our isolates was not being observed but, the anti-microbial resistance profile in CF patients should be carefully checked on a regular basis.

Keywords: Cystic fibrosis, pediatric, antibiotics susceptibility test, biofilm formation.

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). Among a long list of bacteria infecting CF patients, Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae, and gram-negative enteric bacilli are the most important ones. 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). The colonization and infection of bacteria in the bronchociliary tree of CF patients leads to the destruction of lung tissue, attenuating their function. Although several antibiotics are prescribed for the treatment and the control of pulmonary infections in CF patients, some bacteria species recently become resistant to these agents, causing tremendous clinical problems for the management of CF (Filkins and O’Toole, 2015). In P. aeruginosa strains, resistance to carbapenems has been increased in recent years and multidrug resistance (MDR) of P. aeruginosa is becoming a serious concern (Chalhoub et al., 2016). Several mechanisms have been described for the antibiotic resistance in P. aeruginosa, including decreasing in antibiotic penetration, producing of metallo beta lactamase (MBLs), biofilm formation, 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 from not only the antibiotic effects, but also from the immune response (Winstanley et al., 2016). Another bacteria which displayed a resistant phenotype in CF patients are Methicillin resistance *S. aureus* (MRSA) strains. This resistance is much more difficult to treat and to manage in patients (Liu et al., 2016). It is worth to mention that early treatment regimen has not yet been widely accepted in CF. The main concerns clinicians are faced with are as follows; determination of the optimal drug combination, the method of drug delivery, the efficacy of treatment strategies on pulmonary function, antibiotic resistance, the emergence of other pathogens and the costs of the treatment (Farrell et al., 2017). Recognition of this bacterial agents, resistance pattern and also choosing the best treatment are very important factors affecting quality of life in CF patients, especially pediatric patients because of their susceptibility to infection. In this study, we aimed to investigate prevalence of bacterial isolates from pediatric patients with CF who referred to the Tehran Pediatric Central Hospital from March 2015 to August 2017. The antibiotic susceptibility patterns of the isolates were determined by the Kirby-Bauer disk diffusion method. In addition, we assessed biofilm formation ability of isolates by microtiter plate method.

**Material and Methods**

**Patient’s recruitment**

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

**Sputum culture**
Sputum was weighed prior to dissolving by 0.01% of 1, 4-Dithiothreitol (Merck, Germany) to a final dilution of 1:2. Fifty µl of the diluted sputum were separately cultured on five plates; 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 was incubated at 37 °C for 24 h and the two rest plates were incubated at 37 °C in 5–10% CO$_2$ enriched atmosphere for 48 h. The number of colony forming units (CFU) per ml of sputum were determined by the colony counts on each plate.

Identification of organisms was performed by 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).

**Antimicrobial susceptibility testing**

All culture plates were subjected to Kirby-Bauer disk diffusion method using the following antibiotic disks, amikacin, cefepime, cefotaxime, cefoxitin, ceftazidime, clindamycin, trimethoprim-sulfamethoxazole, erythromycin, gentamicin imipenem, methicillin, penicillin, piperacillin/tazobactam, and vancomycin. Susceptibility to colistin in *P. aeruginosa* isolates and vancomycin and methicillin (cefoxitin strip) in *S. aureus* isolates were determined using E-test strips (MIC Test Strip, Liofilchem, Italy). The results were interpreted according to breakpoints and interpretive recommendation of Liofilchem Company (colistin; susceptible $\leq 2\mu$g/ml, resistant $\geq 4\mu$g/ml, vancomycin; susceptible $<2\mu$g/ml; intermediate 4-8 $\mu$g/ml, resistant $>2\mu$g/ml, cefoxitin; susceptible $\leq 4$, resistant $\geq 8$). 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 as described by the Clinical and Laboratory Standards Institute guidelines (CLSI, 2018).
Biofilm Formation Assay

The capacity of biofilm production in *P. aeruginosa*, *S. aureus*, and *k. pneumoniae* isolates was measured using the microtiter plate method. In brief, 0.5 McFarland turbidity was prepared from overnight cultures of each isolate prior to dilution at 100-fold using tryptic soy broth (TBS) (Liofilchem, Italy). Three hundred 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 washed three times with phosphate-buffered saline (PBS). The plate was fixed by methanol for 15 min and air-dried. Each well was stained with 300 µl of 1% crystal violet solution in water and incubated at room temperature for 30 min. After three times washing with distilled water and drying the plate, the stain was solubilized by 300 µl of 33% of acetic acid. 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 \(\leq \) OD_C); weak producer (OD_C \(\leq\) OD \(\leq\) 2OD_C); moderate-producer (2OD_C \(\leq\) OD \(\leq\) 4OD_C), strong producer (4OD_C \(\leq\) OD).

Statistical analysis

Fisher’s exact and Chi-Square tests and SPSS software version 21 (IBM Corp., Armonk, USA) were used for data statistical analysis. A *P*-value of less than 0.05 was considered statistically significant.

Results

Of 8,908 recruited children either inpatient or outpatient, 4633 (52%) were male and 4275 (48%) female were. One hundred and eighty-three (2.05%) of suspect children were
diagnosed for CF disease with according to the sweat test (Cl ≥ 60 mEq/L). Clinical manifestations with gender distribution of 56.2% male and 43.7% female and mean age (±SD) of 2.93 ± 0.2 years old. Highlighting that there was no gender-specific differences (two-tailed Mann-Whitney U test, P > 0.05).

We collected one hundred fifty-three (83.6%) bacteria and fungi species from CF patients. In 30 (16.4%) patient’s normal flora were diagnosed and 7.2% of the patients were infected by more than one species. In 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 *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.](image)

The results of antibiotic susceptibility showed that in *P. aeruginosa* isolates, the highest resistance rate was found for gentamicin (11.7%), followed by amikacin (7.05%), imipenem (7.05%), and ceftazidime (5.9%) and the lowest resistance rates were found for piperacillin-tazobactam (2.3%) (Figure 2A). All of *P. aeruginosa isolates* in E-test were susceptible to colistin. In *S. aureus* isolates, the highest resistance rate was found for erythromycin,
penicillin, and clindamycin (50%), followed by trimethoprim-sulfamethoxazole (20.8%) and Cefoxitin (16.6%) (Figure 2B). Among *S. aureus*, the rate of MRSA (Cefoxitin strip) was 16.6% and 83.4% of isolates were MSSA and no isolates were found to be vancomycin resistant.

Results of antibiotic resistance of other isolates were summarized in Table 1. In brief, all *K. pneumonia* isolates were resistance to cefotaxime and 83.3%, 66.7%, and 66.7% isolates were resistance to cefepime, piperacillin-tazobactam, and trimethoprim-sulfamethoxazole, respectively. From 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
Table 1. The prevalence of antibiotic resistance in K. pneumonia, E. cloacae, E. coli, and S. marcescens isolates in this study.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>K. pneumonia</th>
<th>E. cloacae</th>
<th>E. coli</th>
<th>S. marcescens</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=18 %</td>
<td>N=4 %</td>
<td>N=2 %</td>
<td>N=2 %</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>12 (66.7)</td>
<td>2 (50)</td>
<td>1 (50)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>9 (50)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>9 (50)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Piperacillin-Tazobactam</td>
<td>12 (66.7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>15 (83.3)</td>
<td>0 (0)</td>
<td>1 (50)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>3 (16.7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>18 (100)</td>
<td>0 (0)</td>
<td>1 (50)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

Biofilm Formation Assay

Regarding biofilm formation assay, For P. aeruginosa and S. aureus isolates, OD_{570} was 0.19 ± 0.09 and 0.12 ± 0.02 respectively. OD_{570} of TSB media used as a negative control (cut-off) (0.082±0.01) (figure1). In total, 76.5% of P. aeruginosa isolates were biofilm producer and most of them belong to the weak group and 23.5% of isolates did not produce any biofilm. For S. aureus and K. pneumonia, 67.5% and 72.5% isolates were biofilm-producer. (Table 2).

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

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Non-producing N (%)</th>
<th>Weak N (%)</th>
<th>Moderate N (%)</th>
<th>Strong N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>20 (23.5)</td>
<td>40 (47)</td>
<td>20 (23.5)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>8 (33.3)</td>
<td>9 (37.5)</td>
<td>5 (20.9)</td>
<td>2 (8.3)</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>5 (27.7)</td>
<td>7 (39)</td>
<td>6 (33.3)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
Figure 1. Biofilm formation in microtiter plate method for different isolates of *P. aeruginosa*, *S. aureus*, and *K. pneumonia* with different levels of biofilm production.

**Discussion**

In the present study a partial large number of pediatric patients with CF during was investigated. In CF, respiratory system can be frequently infected by persistent and recurrent bacterial infections leading to a high rate of morbidity and mortality particularly in the presence of risk factors (Filkins and O’Toole, 2015). Among wide variety of microorganisms infected CF patients, *P. aeruginosa*, *Burkholderia cepacia* complex, *S. aureus*, and enteric gram-negative bacilli are the most prevalent ones. It is well-established that *P. aeruginosa* is the most important pathogen in CF airways and its colonization can be occurred after visiting hospitals or clinics (Chiappini et al., 2014). Given to the fact that chronic lung infection can exacerbate the patient’s prognosis, it seems that antibiotic treatment strategies are not effective in the elimination of resistant bacterial pathogens in CF airways. One of the main reasons attributed to this phenomenon is the formation of biofilm by bacterial products. Biofilm formation in CF is assisted by secretion of the 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).

Our results indicated that 2.05% of all suspected patients who were admitted to the CF clinic had a positive sweat test. The mean age of all patients was 2.93 years old and we failed to find any gender specific differences between our samples ($P > 0.05$). In total, 83% of CF patients were positive in microbiological analysis with the bacterial frequency of 74.9% and only 10.4% of patients were infected with $C. \textit{albicans}$ isolates. In 16.4% of patients, normal flora were diagnosed and 7.2% were concomitantly infected with two bacterial genera. Among all isolated bacteria, $P. \textit{aeruginosa}$ was the most prevalent pathogen, suggesting that the primary control of $P. \textit{aeruginosa}$ colonization may culminate in a good outcome in CF patients. Moreover, we found that after $P. \textit{aeruginosa}$, the two most common isolates were $S. \textit{aureus}$ and $k. \textit{pneumonia}$ respectively. These findings were in agreement with other studies suggested that chronic pulmonary infections in CF patients are caused mainly by $P. \textit{aeruginosa}$ and $S. \textit{aureus}$ (Ahlgren et al., 2015). In contrast, the CF foundation report showed that 27.5% of CF patients were positive for $P. \textit{aeruginosa}$ in sputum culture. This discrepancy may be related to some factors including the transition colonization with $P. \textit{aeruginosa}$, low age of patients and inappropriate management and treatment of CF patients. On the other hand, the prevalence of $P. \textit{aeruginosa}$ continues to decline over time while multidrug-resistant $P. \textit{aeruginosa}$ (MDR-PA) has yet remained (Mogayzel Jr et al., 2014). Some studies observed that $S. \textit{aureus}$ and $H. \textit{influenzae}$ are the most isolated bacteria from CF patients throughout the first decade of life, while infection with $P. \textit{aeruginosa}$ mostly increases in the following decades of life (Valenza et al., 2008). Although, $P. \textit{aeruginosa}$ is a classical CF pathogens colonizer, non-classical pathogens have tremendous diversity based on different areas of the lung and datasets (Parkins and Floto, 2015). In our study, most non-classical species were $S. \textit{aureus}$ and $k. \textit{pneumoniae}$. In infants and children suffering from
CF, *S. aureus* is one of the earliest pathogens. 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 and it was in accordance with the results of other counties. In contrast to our results, it has 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 long duration of hospitalization, worsening 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 CF patients with positive MRSA was 1.27 times greater than who were MRSA-negative (Jennings et al., 2017). Contamination of the sputa sample with oral flora is a problematic issue in CF patients. So, sputum sample in CF patients should be collected in an aseptic method and in some cases serially produced sputum samples can be helpful. Azithromycin, ceftazidime, and Ciprofloxacin have been suggested as therapies for *P. aeruginosa* eradication in CF patients (Talwalkar and Murray, 2016). In our study, antibiotic susceptibility profiles of *P. aeruginosa* isolates showed that piperacillin-tazobactam was the most effective antibiotics. During intensification of infections, imipenem or ceftazidime in combination with an aminoglycoside, such as amikacin could be a promising agents for the antibacterial therapy (Talwalkar and Murray, 2016). In contrast to these agents, we found that most of bacterial isolates displayed a resistant phenotype to gentamicin and ceftazidime. In addition, imipenem and amikacin resistance were also observed in *P. aeruginosa* isolates. In agreement with our results, another study conducted in Iran also showed that the most common bacteria isolated from pediatric CF patients were *P. aeruginosa, S. aureus, and K. pneumoniae*. Their study results demonstrated that the most effective antibiotics against these microorganisms were vancomycin, rifampin, and imipenem. In addition, resistance to aminoglycoside was observed (Aghamohammadi et al., 2019). Having established that *P.
*P. aeruginosa* is the most common pathogen in CF patients, it’s not surprising that a wide range of antibiotics regimens have been prescribed for this infection. Moreover, recent studies reported that the antibiotic resistance and virulence of bacterial pathogens in the CF lung might have a tight association with the formation of biofilm (Olsen, 2015). It has been suggested that different morphological colonies can be accumulated in the airways of CF patients based on their stage of colonization. In the late colonization stage, the bacterial density overwhelmingly increases and morphological phenotype shifts to produce biofilm in the CF airway in order to prevent the penetration of antibiotics. This phenomenon can subsequently worsen CF patients outcome and reduce the efficacy of antimicrobial treatment strategies (Bowler, 2018). Interestingly, our results showed that 76%, 67% and 72.5% of *P. aeruginosa*, *S. aureus*, and *K. pneumoniae* isolates were biofilm producer, respectively. This finding was in consistent with another study outlined that *P. aeruginosa* isolates could colonize in CF respiratory tract by producing biofilm (Hill et al., 2005). Likewise, in accordance with the previous reports, the results of this study also showed that most of the isolates from CF patients were capable to form a biofilm, highlighting that a higher concentration of antibiotics prescribed for patients may prevent from biofilm formation during the stages of colonization. The prevalence of different bacterial pathogens in the sputum of pediatric CF patients should be carefully checked on a regular basis. Our study showed that *P. aeruginosa* was the most dominant bacterial pathogens in pediatrics with CF, most of them were biofilm producer. Although, severe antibiotic resistance in our isolates was not being observed, the anti-microbial resistance profile in CF patients should be investigated. The role of other less common pathogens in the pathogenesis of CF patients remains to be clarified and further studies are required to validate the association between biofilm formation and clinical condition of CF patients.

**Ethics approval and consent to participate**
The ethics committee of the Shahid Beheshti University of Medical Sciences approved the study (IR.SBMU.MSP.REC.1397.52). From the parents or guardians of all children, verbal consent was obtained to participate in this study. First, the main investigator introduced herself and the study’s objectives then, the sweat test was performed for all participants after that, sputa samples were collected. Finally, they were ensured that their name and information would be kept. This is considered to be an acceptable consent procedure by the ethics committees at the institutional level (Shahid Beheshti University of Medical Sciences).

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

MK performed the microbiological analyses and drafted the manuscript, BN coordinated patient samples collection, HH revised the manuscript, ZG supervised the study, revised the manuscript and gave final approval of the version to be published. All authors read and approved the final manuscript.

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Reference


