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Extended-Spectrum Beta-Lactamases in Cystic Fibrosis Isolates of *Klebsiella pneumoniae*

Roya Rafiee,[1,*] Fereshteh Eftekhar,[2] and Seyed Ahmad Tabatabaii[3]

[1]Radiation Applications Research School, Nuclear Science and Technology Research Institute, Tehran, Iran
[2]Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, G.C., Tehran, Iran
[3]Department of Pediatrics, Shahid Beheshti University of Medical Sciences, M.C., Tehran, Iran

[*]*Corresponding author*: Roya Rafiee, Radiation Applications Research School, Nuclear Science and Technology Research Institute, P.O. Box: 11365-3486, Tehran, Iran. Tel: +98-2182063814, Fax: +98-2188221221, E-mail: roya_rafiee@yahoo.com

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Abstract

Background: The emergence and spread of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* are recognized as a major clinical problem and a public health challenge. *Enterobacteria* are transient colonizers of lungs affected by cystic fibrosis (CF). *Klebsiella pneumoniae* has been shown to cause chronic obstructive pulmonary infections.
Objectives: We aimed to determine the antibiotic resistance profile and ESBL production in *K. pneumoniae* isolates from Iranian patients with CF.
Methods: Sixteen *K. pneumoniae* strains were isolated from the sputum samples of 98 pediatric CF patients at Mofid children's hospital in Tehran, Iran. Antibiotic susceptibility was determined via disc diffusion, and ESBL phenotype was detected by double disc synergy test (DDST). β-lactamase genes, including *TEM*, *SHV*, *CTX*-M, and *OXA* genes, were detected using polymerase chain reaction (PCR) and confirmed by sequencing the PCR products. Finally, genetic fingerprints of the isolates were determined via random amplified polymorphic DNA (RAPD)-PCR method.
Results: Ten isolates had ESBL phenotypes, all of which contained non-ESBL $bla_{\text{TEM-1}}$ genes. Seven isolates harbored $bla_{\text{CTX-M-15}}$ genes (ESBL genes), five of which also showed non-ESBL $bla_{\text{SHV-11}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{OXA-1}}$ genes (1 isolate with $bla_{\text{TEM-1}}$ and 1 isolate with $bla_{\text{TEM-1}}$ plus $bla_{\text{OXA-1}}$ gene). However, *PER-1* and *VEB-1* β-lactamase genes were not detected. Genetic fingerprinting profiles showed heterogeneity among *K. pneumoniae* isolates.
Conclusions: This study highlights not only the presence of multiple β-lactamases, but also the carriage of ESBL $bla_{\text{CTX-M-15}}$ gene among *K. pneumoniae* isolates from CF patients.

Keywords: Cystic Fibrosis, ESBL, RAPD, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{OXA}, *Klebsiella pneumonia*

1. Background

Cystic fibrosis (CF) is an autosomal recessive genetic disorder, caused by one of many different mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which regulates the movement of chloride and sodium ions across epithelial membranes (1). The lungs of patients with CF often become colonized by bacteria from an early age, which can lead to chronic infections.

Bacterial species, most commonly associated with respiratory tract infections in CF patients, include *Staphylococcus aureus*, *Haemophilus influenzae*, and most commonly, *Pseudomonas aeruginosa*, as a frequent cause of acute and chronic lung infections in these patients (2). Other Gram-negative non-fermenting organisms, such as *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex members, and *Alcaligenes xylosoxidans*, have been also reported to cause respiratory infections in CF patients (3).

Members of *Enterobacteriaceae* family are considered as transient colonizers of the airways in CF patients and seem to play a minor role in the development of severe respiratory diseases (4). *Klebsiella pneumoniae* has been shown to cause chronic obstructive pulmonary disease with symptoms such as recurrent cough and acute exacerbation (5). More recently, this organism has been isolated from respiratory infections in CF patients (6-9).

Cephalosporins are often used to treat respiratory tract infections caused by Gram-negative pathogens. However, it is difficult to eradicate these infections mostly due to multidrug-resistant strains, which produce Ambler class A extended-spectrum β-lactamases (ESBLs) (10). ESBLs comprise a rapidly growing group of β-lactamases, encoded by mobile genetic elements, which are capable of hydrolyzing

penicillin, cephalosporins, and aztreonam.

The most frequently encountered ESBLs belong to TEM, SHV, and CTX-M families (11). Another class of enzymes, ie, class D β-lactamases (oxacillinases or OXA-type β-lactamases), was first shown to hydrolyze cloxacillin and oxacillin faster than benzyl penicillin and was later reported to hydrolyze amino- and carboxypenicillin (12, 13). Moreover, production of OXA-type enzymes, such as OXA-23, 40, 51, 58, and 48 groups, has been shown in *K. pneumoniae* (14).

2. Objectives

We aimed to investigate the antibiotic resistance profiles and prevalence of ESBL and OXA type β-lactamase production in *K. pneumoniae* isolates from pediatric CF patients in Tehran, Iran.

3. Methods

3.1. Ethics Statement

Ethical approval was obtained from the ethics committee of Shahid Beheshti University (grant No., D/600/3490).

3.2. Bacterial Isolates

Sixteen *K. pneumoniae* strains were identified among 98 bacterial isolates from the sputum samples of children with CF at Mofid children's hospital in Tehran between July 2012 and February 2013. Among patients with *K. pneumoniae* colonization, 13 (81.25%) were male and 3 (18.75%) were female within the age range of 6 months to 8 years (median, 24 months).

Bacterial isolates were maintained in brain heart infusion broth (Oxoid, UK), containing 10% dimethyl sulfoxide at -20°C until further use. *Klebsiella pneumoniae* 7881 harboring bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ genes, *Pseudomonas aeruginosa* KOAS carrying $bla_{\text{PER-1}}$, *P. aeruginosa* 10.2 harboring $bla_{\text{VEB-1}}$, and *P. aeruginosa* containing bla_{OXA} were obtained from pasteur institute of Iran and used as the positive controls in polymerase chain reaction (PCR) experiments. *Klebsiella pneumoniae* ATCC 10031 was also used as the control in antibiotic susceptibility tests.

3.3. Antimicrobial Susceptibility

Antimicrobial susceptibility was determined by Kirby-Bauer disc diffusion method according to the clinical and laboratory standards institute (CLSI) guidelines (15). The following antibiotic discs (MAST Diagnostics, Merseyside, UK) were used in the present study: ceftazidime (CAZ, 30 μg), ceftriaxone (CRO, 30 μg), cefoxitin (FOX, 30 μg), cefotaxime (CTX, 30 μg), cefepime (CPM, 30 μg), imipenem (IMP, 10 μg), meropenem (MEM, 10 μg), ertapenem (ETP, 10 μg), aztreonam (ATM, 30 μg), piperacillin (PRL, 100 μg), carbenicillin (PY, 100 μg), ticarcillin (TC, 75 μg), amikacin (AK, 30 μg), tobramycin (TN, 10 μg), ciprofloxacin (CIP, 5 μg), co-amoxiclav (augmentin, AUG, 20/10 μg), ampicillin-sulbactam (SAM, 10/10 μg), and piperacillin/tazobactam (PTZ, 100/10 μg). The minimum inhibitory concentration (MIC) of ceftazidime was determined via broth microdilution.

3.4. Screening for ESBL Production

According to the CLSI recommendations, isolates with reduced susceptibility to 1 or more oxyimino-cephalosporins, including ceftazidime ($\leq$ 22 mm), cefotaxime ($\leq$ 22 mm), ceftriaxone ($\leq$ 25 mm), and aztreonam ($\leq$ 27 mm), were selected as probable ESBL producers (15). These organisms were then subjected to the phenotypic confirmatory test, using discs containing cefotaxime (30 μg), cefotaxime plus clavulanic acid (30/10 μg), ceftazidime (30 μg), and ceftazidime plus clavulanic acid (30/10 μg). An increase of $\geq$ 5 mm in the zone diameter for either antibiotic in combination with clavulanic acid or antibiotic alone confirmed the isolate as an ESBL producer.

3.5. PCR Amplification of β-lactamase Genes

DNA extraction was carried out by boiling. Briefly, a loopful of bacterial colonies was suspended in 500 μL of sterile double-distilled water. The suspensions were heated at 100°C for 10 minutes, and cellular debris was removed by centrifugation at 13000 $\times$ g for 10 minutes. The supernatant was used as the DNA template for PCR amplification of ESBL genes, using the primers listed in Table 1.

The PCR experiments were carried out in reaction mixture volume of 25 μL, containing 10 pM of each primer, 0.2 mM of dNTPs, 0.7 mM of $MgCl_2$, 1 μL of crude DNA template (including the positive controls), and 1 U of Taq polymerase in the reaction buffer, provided by the manufacturer (CinnaGen, Tehran, Iran). Amplifications were carried out in a thermocycler (Techne TC-3000G, UK), using the following program: 5 minutes of denaturation at 94°C, followed by 30 cycles of 95°C for 1 minute, annealing temperature for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes.

The PCR products were separated via electrophoresis on 1.2% Agarose gel, stained with RedSafe (Intron Bio, Korea), and visualized using an image analysis system (UVLtec, St John's innovation centre). The nucleotide sequences were analyzed by searching the GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

Table 1. Primers Used for the Detection of β-lactamase Genes

Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Product Size (bp)	Reference No.
TEM-F	GAGTATTCAACATTTCCGTGTC	45	889	(16)
TEM-R	TAATCAGTGAGGCACCTATCTC			
SHV-F	ATGCGTTATATTCGCCTGTG	58	862	(17)
SHV-R	AGCGTTGCCAGTGCTCGATC			
CTX-M-F	CGCTTTGCGATGTGCAG	63	550	(18)
CTX-M-R	ACCGCGATATCGTTGGT			
PER-1-F	ATGAATGTCATTATAAAAGC	43	920	(18)
PER-1-R	AATTTGGGCTTAGGGCAGAA			
VEB-1-F	CGACTTCCATTTCCCGATGC	55	643	(19)
VEB-1-R	GGACTCTGCAACAAATACGC			
OXA-F	ACACAATACATATCAACTTCGC	60	813	(20)
OXA-R	AGTGTGTTTAGAATGGTGATC			

3.6. RAPD-PCR Fingerprinting

The clonality of *K. pneumoniae* isolates was determined, using random amplified polymorphic DNA (RAPD)-PCR fingerprinting. The PCR reaction mixture contained 1 μM of primer 640 (5'- CGTGGGGCCT-3'; Takapouzist, Tehran, Iran), 1 U of Taq DNA polymerase, 4 mM of $MgCl_2$, 0.4 mM of dNTPs (CinnaGen, Tehran, Iran), and 90 ng of DNA template in a final volume of 25 μL (21).

The following cycling program was applied using a Bioer TC25/HH thermal cycler (Bioer Technology, China): 5 minutes of denaturation at 94°C, followed by 45 cycles of 30 seconds at 94°C, 30 seconds of annealing at 34°C, 30 seconds of extension at 7°C, and a final extension for 10 minutes at 72°C. The PCR products were separated via gel electrophoresis and then stained and visualized as mentioned before. The RAPD-PCR of polymorphic DNA fingerprints was analyzed using NTSYSpc version 2.02 (Setauket, NY, USA).3.7. Statistical Analysis

Chi square test was used for the comparison of antibiotic resistance profiles. Nonparametric two-tailed Mann-Whitney U and Kruskal-Wallis tests were used for comparing the differences in age, gender, and colonization, using SPSS version 20. P value ≤ 0.05 was considered significant.

4. Results

4.1. Antimicrobial Susceptibility

The antibiotic resistance profiles of the isolates are shown in Table 2. Overall, the resistance rates were as follows: ticarcillin (93.8%); ceftazidime, cefotaxime, and carbenicillin (75%); ceftriaxone and piperacillin (68.8%); aztreonam and cotrimoxazole (62.5%); tobramycin (50%); cefepime and co-amoxiclav (37.5%); amikacin and ampicillin-sulbactam (31.3%); cefoxitin and ciprofloxacin (18.8%); and ertapenem (6.3%). All the isolates were susceptible to imipenem, meropenem, and piperacillin/tazobactam. The MIC for ceftazidime ranged from 2 to 128 μg/mL (Table 2).

4.2. ESBL Production

The phenotypic test results showed ESBL production in 10 out of 16 *K. pneumoniae* CF isolates (62.5%). The type of produced β-lactamase, as well as the antibiotic resistance profile of the isolates, is shown in Table 2. No significant differences were observed in the antibiotic resistance profiles between ESBL-producing and non-ESBL strains. As shown in Table 2, ten ESBL-positive strains carried $bla_{\text{TEM-1}}$ gene, one of which harbored $bla_{\text{TEM-1}}$ alone and 2 harbored $bla_{\text{TEM-1}}$ plus $bla_{\text{SHV-11}}$ (both non-ESBL β-lactamases). The remaining 7 isolates harbored the ESBL-encoding $bla_{\text{CTX-M-15}}$ gene, five of which also harbored $bla_{\text{SHV-11}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{OXA-1}}$ genes; also, one isolate carried $bla_{\text{TEM-1}}$ and one harbored $bla_{\text{TEM-1}}$ plus $bla_{\text{OXA-1}}$ genes. However, *PER-1* and *VEB-1* β-lactamase genes were not detected. Production of ESBL by *K. pneumoniae* isolates was only observed among patients under 3 years, compared to older patients (Kruskal-Wallis test, $P < 0.05$).

4.3. RAPD-PCR Fingerprinting

The DNA profiles generated by RAPD-PCR showed 19 bands within the range of 200 - 2000 bp (Figure 1). Four major clusters were observed at a similarity level of 70%, and 14 different groups were at a similarity level of 85%,

Table 2. Ceftazidime Susceptibility, β-lactamase Type, and RAPD-PCR Profile of 16 CF *K. pneumoniae* Isolates

Isolate and Gender	Age (Months)	Antibiotic Resistance Profile	CAZ MIC (μg/mL)	PCT	β-lactamase Type	RAPD Cluster
1 M	96	CAZ, FOX, CTX, TC, AUG, TS	16	-	None	1
2 M	84	CAZ, CRO, FOX, CTX, ETP, ATM, PY, TC	16	-	None	2
3 M	48	PY, TC	4	-	None	3
4 F	8	AUG, SAM, TS	4	-	None	2
5 M	12	CAZ, CRO, CTX, CPM, ATM, PRL, PY, TC, AK, TN, CIP, AUG, SAM	16	-	None	4
6 M	60	CAZ, FOX, CPM, TC, SAM, AUG, TS	16	-	None	2
7 M	7	PRL, PY, TC, TS	2	+	TEM-1; SHV-11	4
8 M	24	CAZ, CRO, CTX, CPM, ATM, PRL, PY, TC, AK, TN, TS	128	+	TEM-1; SHV-11; CTX-M-15; OXA-1	4
9 M	11	CAZ, CRO, CTX, ATM, PRL, PY, TC, AK, TN, SAM, TS	128	+	TEM-1; SHV-11; CTX-M-15; OXA-1	4
10 M	8	CRO, CTX, ATM, PRL, PY, TC, TN, TS	32	+	TEM-1; SHV-11; CTX-M-15; OXA-1	2
11 F	18	CAZ, CRO, CTX, ATM, PRL, PY, TC, TS	16	+	TEM-1; SHV-11	2
12 M	24	CAZ, CRO, CTX, ATM, PRL, PY, TC, AK, TN, CIP	32	+	TEM-1; SHV-11; CTX-M-15; OXA-1	4
13 M	24	CAZ, CRO, CTX, PRL, PY, TC, TN, TS	16	+	TEM-1; CTX-M-15	3
14 M	6	CAZ, CRO, CTX, CPM, ATM, PRL, PY, TC, TN, AUG	128	+	TEM-1; SHV-11; CTX-M-15; OXA-1	4
15 M	36	CAZ, CRO, CTX, CPM, ATM, PY, PRL, TC, AK, TN, CIP, AUG, SAM	16	+	TEM-1; CTX-M-15, OXA-1	2
16 F	36	CAZ, CRO, CTX, CPM, ATM, PRL, TC, TS	4	+	TEM-1	2

Abbreviations: AK, amikacin; ATM, aztreonam; AUG, co-amoxiclav; CAZ, ceftazidime; CIP, ciprofloxacin; CPM, cefepime; CRO, ceftriaxone; CTX, cefotaxime; ETP, ertapenem; F, female; FOX, cefoxitin; M, male; PCT, phenotypic confirmatory test; PRL, piperacillin; PY, carbenicillin; SAM, ampicillin-sulbactam; TC, ticarcillin; TN, tobramycin; TS, cotrimoxazole.

indicating heterogeneity among the isolates (Figure 2). Among 10 ESBL phenotypes, four belonged to cluster 2 (isolates 10, 11, 15, and 16), one belonged to cluster 3 (isolate 13), and five were found in cluster 4 (isolates 7, 8, 9, 11, and 14). Among ESBL-negative isolates, one belonged to cluster 1 (isolate 1), three were in cluster 2 (isolates 2, 4, and 6), and one (isolate 5) was in cluster 4. The results showed no correlation between β-lactamase/ESBL production and specific genetic fingerprints of the isolates.

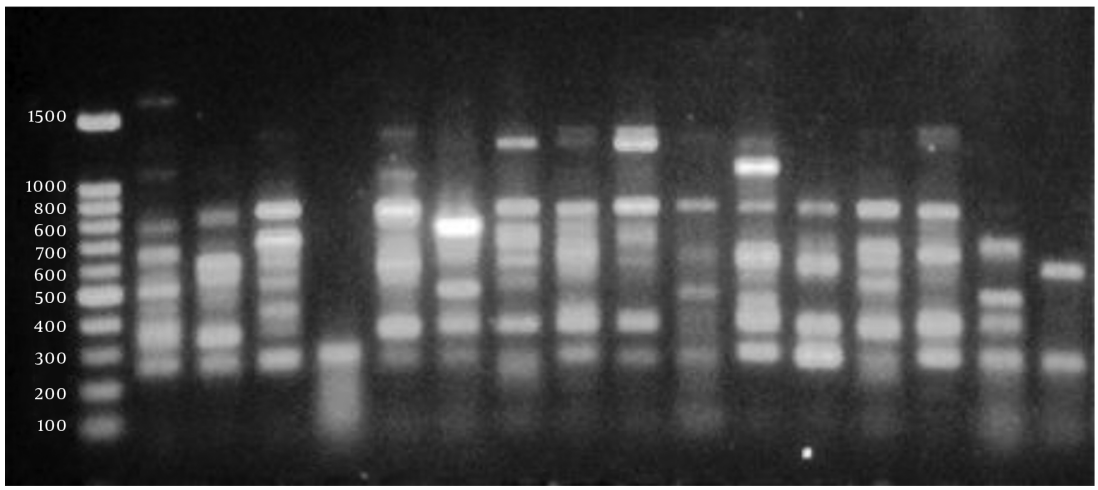
5. Discussion

Members of the *Enterobacteriaceae* family are considered as transient respiratory colonizers, which are not associated with severe diseases in CF patients. Limited reports have shown *K. pneumoniae* isolation from CF patients in Iran and other countries (6-9). In this regard, a study on 129 Iranian pediatric CF patients showed that 11.6% of the isolates were *K. pneumoniae*, although ESBL production by the isolates was not investigated (7).

Another study performed in Iran showed that 4% of Gram-negative bacterial isolates from 52 CF patients were *K. pneumoniae*, none of which produced ESBL (6). In the present study, we found that 16 out of 98 CF isolates were *K. pneumoniae* (16.3%), 10 of which showed the ESBL phenotype. However, 7 strains harbored the ESBL $bla_{\text{CTX-M-15}}$ gene, along with non-ESBL β-lactamase genes. In this regard, Canton et al. reported colonization in a CF patient with a *K. pneumoniae* strain, capable of producing β-lactamase *SHV-5* gene (22). Moreover, Pollini et al. showed that CTX-M group I was predominant in ESBL-producing *Escherichia coli* and *K. pneumoniae* isolates from CF patients (8). Leao et al. also reported 2 cases of *K. pneumoniae* carbapenemase 2-producing isolates from CF patients (4).

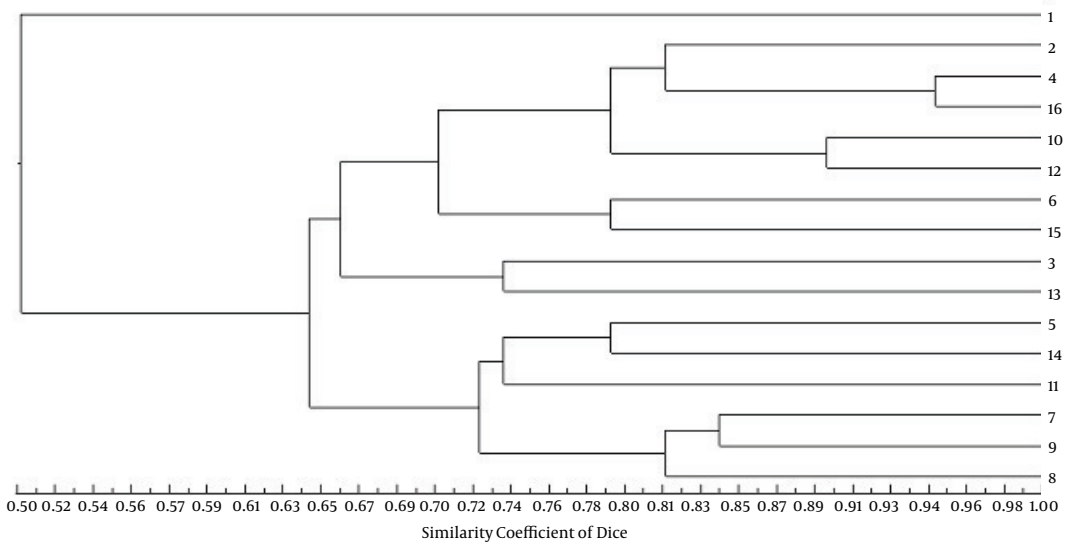
Emergence of OXA β-lactamases in the *Enterobacteriaceae* family, particularly *Klebsiella* species, is of major significance, as these bacteria are true pathogens capable of infecting immunocompromised individuals. The first sequenced $bla_{\text{OXA-1}}$ gene was part of a transposon, carried by plasmid RGN238 (14). Coproduction of CTX-M and OXA enzymes by *K. pneumoniae* enhances resistance to β-lactamase inhibitors, presumably explaining their nonsusceptibility to amoxicillin/clavulanate (23). All our ESBL-positive isolates carried the non-ESBL *TEM-1* gene, and

Figure 1. RAPD Profiles of 16 *K. pneumoniae* CF Isolates



DNA molecular weight marker (100 bp DNA ladder; CinnaGen) and isolates 1 - 16 are shown from left to right.

Figure 2. Cluster Analysis of *K. pneumoniae* Isolates Based on RAPD Fingerprints Using Dice Similarity Coefficient



The isolate numbers are shown on the right.

the majority harbored the *SHV-11* gene along with ESBL $bla_{\text{CTX-M-15}}$ gene.

TEM-1 hydrolyzes ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin and shows negligible activity against extended-spectrum cephalosporins (24). Also, *K. pneumoniae* strains naturally possess a single chromosomal copy of $bla_{\text{SHV-1}}$ or its derivative $bla_{\text{SHV-11}}$ (25). We showed that 6 isolates carried $bla_{\text{SHV-11}}$, 5 of which also harbored $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{OXA-1}}$ genes. Finally, using RAPD-PCR, we found distinct heterogeneity among *K. pneumoniae* isolates (both non-ESBL and ESBL producers), and no association was observed between ESBL production and genetic fingerprints.

Colonization of CF patients by multidrug-resistant ESBL-producing *K. pneumoniae*, whether transient or not, can be important in the development of chronic airway in-

flammation and pulmonary exacerbation. Since β-lactam antibiotics (eg, ceftazidime and cefepime), used for the treatment of CF airway infections, are ineffective against ESBL-producing *Enterobacteriaceae*, persistence of these pathogens can complicate the diagnostic procedures and majorly influence the selection of therapeutic regimens.

6. Conclusion

The results of the present research are alarming, as multidrug-resistant pathogens can provide a reservoir for dissemination of drug-resistant genes among other bacterial species.

Footnotes

Authors' Contribution: Fereshteh Eftekhar was responsible for designing the research project, interpretation of the results, and final preparation of the manuscript; Roya Rafiee carried out the research, collected the data, and wrote the first draft of the manuscript; Seyed Ahmad Tabatabaii provided the bacterial isolates and contributed to the selection of antibiotics for the study.

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