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Original Article

Microbiology Section

Phenotypic and Genetic Characterization of Carbapenemase and ESBLs Producing Gram-negative Bacteria (GNB) Isolated from Patients with Cystic Fibrosis (CF) in Tehran Hospitals

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ABSTRACT

Background: Cystic Fibrosis (CF) is an autosomal recessive genetic disorder in white populations caused by mutation in a gene that encodes Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. Since frequent respiratory tract infections are the major problem in patients with CF, obligation to identify the causative bacteria and determining their antibiotic resistance pattern is crucial. The purpose of this project was to detect Gram-negative bacteria (GNB) isolated from sputa of CF patients and to determine their antibiotic resistance pattern.

Materials and Methods: The sputum of 52 CF patients, treated as inpatients at hospitals in Tehran, was obtained between November 2011 and June 2012. Samples cultured in selective and non-selective media and GNB recognized by biochemical tests. Antimicrobial susceptibility testing to cephalosporins, aminoglycosides and carbapenems was performed by disk diffusion method and MICs of them were measured. For phenotypic detection of carbapenemase and ESBLs production, the Modified Hodge test, double disk synergy test and the combined disk methods were performed. Subsequently, the genes encoding the extended spectrum beta-lactamases (blaPER, blaCTX-M) and carbapenemases (blaIMP-1, blaGES, blaKPC, blaNDM, blaVIM-1, blaVIM-2, blaSPM, blaSIM) in Gram negative bacteria were targeted among the resistant isolates by using PCR. PFGE was used to determine any genetic relationship among the *Pseudomonas aeruginosa* isolated from these patients.

Results: Fifty five GNB were isolated from 52 sputum samples including *Pseudomonas aeruginosa*, *Klebsiella ozaenae*, *Alcaligenes xylosoxidans*, *Achromobacter denitrificans*, *Klebsiella pneumonia* and *Stenotrophomonas maltophilia*. The rates of resistance to different antibiotic were as follows: cefixime (%80), ceftriaxone (%43), ceftazidime (%45) and meropenem (%7). The prevalence of genes encoding the ESBLs and Carbapenemases among the the phenotypically positive strains were as follows: blaCTX-M (19), blaIMP-1 (2), blaVIM-1 (2) and blaVIM-2 (3) genes respectively. No other genes were detected. PFGE analysis revealed 8 genotypes. Six isolates had mutually 3 similar patterns.

Conclusion: This study showed the existence of important ESBLs and carbapenemases genes among the GNB isolated from patients with CF. Continuous surveillance of ESBLs and Carbapenemases, also identification of their types, in bacteria isolated from these patients have an important clinical impact, since, it can often provide valuable information for effective infection control measures and for the choice of appropriate antimicrobial therapy.

Keywords: Cystic fibrosis, Gram-negative Bacteria (GNB), Carbapenemase, Polymerase Chain Reaction

INTRODUCTION

CF is a severe autosomal recessive genetic disease that was first described in 1936 by the Swiss pathologist, Guido Fanconi, who reported the autopsy and clinical characteristics of three patients with bronchiectasis and pancreatic insufficiency [1]. In 1938, Dorothy Andersen published an autopsy study of 38 infants, described the findings as "cystic fibrosis of the pancreas" and recognized the syndrome as an inherited disease [2].

The abnormal salt transport in CF became clear during a heat wave in 1952, when children with CF were admitted to hospital with severe dehydration and salt loss [3]. The mechanism for the defect chloride transport in the sweat glands was demonstrated in 1983 [4], and in 1985 the gene was localized to the long arm of chromosome 7. This large gene named CF transmembrane conductance regulator (the CFTR gene), consists of 27 coding exons [5]. The person dies by progressive bronchiectasis and chronic respiratory insufficiency [6,7]. Failure of innate defense mechanisms and the lack of mucocilliary clearance in the airways stimulate primary and recurrent bacterial infections, blockage of airways, inflammation and chronic bacterial infections [8,9]. The bacterial species most commonly associated with respiratory tract infection in CF include common human pathogens such as *Staphylococcus aureus* and *Haemophilus influenzae* as well as several opportunistic pathogens, the most important of which is *Pseudomonas aeruginosa* [10]. With the improved survival, new emergent pathogens in the CF lung as *Burkholderia cepacia complex (BCC)*, *Stenotrophomonas Maltophilia* (SM) and Alcaligenes Xylosoxidans (AX) have been detected in the last years [11]. It is possible to prevent or delay the onset of chronic infections in most patients with CF by eliminating cross-infections and by early aggressive antibiotic treatment of the positive sputum culture [12]. The most antibiotic used for these patients are β-lactams, aminoglycosides, andcorticosteroids. On the other hand microbial resistance against oxyimino-cephalosporins such as ceftriaxone and Ceftazidime (CAZ) and carbapenems is a growing problem in the treatment of infections. This is often caused by the production of Extended-Spectrum Betalactamases (ESBLs) and carbapenemases. ESBLs are frequently identified in *Klebsiella pneumonia* and Escherichia coli [13], but also in other species, such as Citrobacter spp., and Pseudomonas aeroginosa. The most abundant types are represented by SHV, TEM, and CTX-M [14].

The IMP- and VIM-type enzymes are two major groups of carbapenemases [15]. IMP-1 was the first identified acquired MBL [16] and has spread among Enterobacteriaceae, *Pseudomonas aeruginosa*, and other nonfastidious Gram-negative nonfermenters in Japan [17-20].

VIM-1 was identified from a clinical isolate of *P. aeruginosa* in Italy [21], and outbreaks of the VIM-1-producing *P. aeruginosa* isolates have been recognized in Greece [22] as well as Italy [23]. VIM-2 was firstly identified from a clinical isolate of *P. aeruginosa* in France [24]. In this study, we analyzed the pathogens associated with respiratory tract infection in CF patients and investigated the production of ESBLs and carbapenemases. We also used pulse field gel electrophoresis (PFGE) to investigate the possible genetic relationship among the *Pseudomonas aeruginosa* strains colonizing the respiratory tract of these patients.

MATERIAL AND METHODS

Patients and bacterial strains

Sputum of patients with CF treated as in patients at two hospitals in Tehran were collected from November 2011 to June 2012. Ethical clearance was obtained before the collection of samples. Clinical specimens obtained by throat swab or oropharyngeal suction, were cultured in selective and non selective media including (Blood, Chocolate, EMB, MacConkey) agar, *Burkholderiacepacia* Special Agar (BCSA) and Mueller Hinton agar. Isolates were identified based on the biochemical tests and/or the API20E system (France-BioMerieux).

Susceptibility tests

Susceptibility to antibiotics were measured by Kirby Bauer disk diffusion method. The MICs of resistant isolates to cefotaxime, ceftriaxone, imipenem and kanamycin were determined using broth microdilution method. Both tests were performed and interpreted according to the CLSI guidelines (The Clinical and Laboratory Standards Institute). The tested antimicrobial agents (MAST, Co., UK) were Ceftazidime (CAZ), Cefotaxime (CTX), Cefixime (CFM), Cefepime (CFM), Gentamicin (GM), Amikacin (AK), PolymyxinB (PB), Piperacillin/tazobactam (PTZ), Ciprofloxacin (CIP), Ceftriaxone (CRO), Meropenem (MEM), Imipenem (IMP), Ertapenem (ERT). Pseudomonas aeroginosa ATCC27853 was used as positive control.

ESBL activity

ESBL activity in isolates showing resistance to cefotaxime was undertaken by disk synergy testing of CTX in the presence and absence of clavulanic acid which were placed on a plate of Mueller Hinton agar inoculated with suspension (turbidity of 0.5 MacFarland) of isolates. *E. coli* (ATCC25922) was used as positive control reference strain. A positive test result was defined as a ≥ 5 mm difference in the zone diameter between two disks [25].

Screening of Carbapenemase producers

Carbapenem resistant isolates were subjected to a screening test for MBL production using EDTA-disk synergy test and modified Hodge test according to Lee et al., [26] instructions.

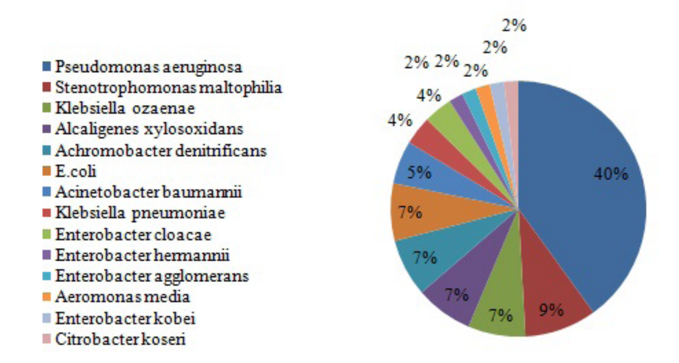
DNA extraction: DNA templates for polymerase chain reaction PCR was extracted by suspending 4-5 colonies of an overnight growth of isolate on Mueller Hinton agar, in 500µl of double distilled water. The suspension was boiled at 100°C for 10 minutes and frozen for 5 minutes. Then, it was centrifuged at 19000 rpm for 5 minutes. An aliquot in 1µl of the supernatant was used as DNA template for PCR [27].

PCR procedure:

Isolates included in this study were screened by PCR for the following ESBLs and carbapenemases encoding genes: ESBLs (*bla*PER, *bla*CTX-M) and Carbapenemases (*bla*IMP-1, *bla*GES, *blaKPC, blaNDM, blaVIM-1, blaVIM-2, blaSPM, blaSIM).* K. pneumonia 7881 containing bla_{CTX-M}, *P. aeruginosa* KOAS containing bla_{PER} gene, A. baumannii AC54/97 producing blaIMP gene, *P. aeruginosa*PO510 producing bla_{VIM-1}, *P. aeruginosa* COL-1

Primer	Gene	Sequence (5'-3')	Size (bp)	References
CTX_M-F	bla_{CTX-M}	CGCTTTGCGATGTGCAG	550	[28]
CTX_M-R		ACCGCGATATCGTTGGT		
PER-F	bla_{PER}	AATTTGGGCTTAGGGCAGAA	925	[29]
PER-R		ATGAATGTCATTATAAAAGC		
KPC-F	bla_{KPC}	CTTGCTGCCGCTGTGCTG	489	[30]
KPC-R		GCAGGTTCCGGTTTTGTCTC		
GES-F	bla_{GES}	ATGCGCTTCATTCACGCAC	840	[31]
GES-R		CTATTTGTCCGTGCTCAGG		
NDM-F	bla_{NDM}	ACCGCCTGGACCGATGACCA	263	[32]
NDM-R		GCCAAAGTTGGGCGCGGTTG		
VIM1-F	bla_{VIM-1}	AGTGGTGAGTATCCGACA	261	[33]
VIM1-R		ATGAAAGTGCGTGGAGAC		
VIM2-F	bla_{VIM-2}	ATGTTCAAACTTTTGAGTAAG	801	[33]
VIM2-R		CTACTCAACGACTGAGCG		
IMP1-F	bla_{IMP-1}	ACCGCAGCAGAGTCTTTGCC	587	[33]
IMP1-R		ACAACCAGTTTTGCCTTACC		
SPM-F	bla_{SPM}	GCGTTTTGTTTGTTGCTC	786	[33]
SPM-R		TTGGGGATGTGAGACTAC		
SIM-F	bla_{SIM}	TACAAGGGATTCGGCATCG	571	[34]
SIM-R		TAATGGCCTGTTCCCATGTG		
OXA_{48}	bla_{OXA-48}	TTGGTGGCATCGATTATCGG	743	[35]
OXA_{48}		GAGCACTTCTTTTGTGATGGC		

[Table/Fig-1]: Primers used for PCR amplification of different genes encoding ESBLs and carabapenemases



[Table/Fig-2]: Percentage of isolates

Antibiotic	Number of resistant isolates (%)
Cefotaxime	26(47.3%)
Ceftazidime	25(45.5%)
Ceftriaxone	24(43.6%)
Cefexime	44(80%)
Imipenem	7(12.7%)
Meropenem	11(20%)
Gentamicin	14(24.5%)
Ciprofloxacin	10(18%)
Piperacillin/Tazobactam	11(20%)

[Table/Fig-3]: The number of resistant bacteria to different antibiotics producing bla$_{VIM-2}$ and *P. aeruginosa*16 producing bla$_{SPM-1}$ (Kindly provided by Patrice Nordmann)strains were used as positive controls. The sequences of primers are shown in [Table/Fig-1].

To identify the size of genes, PCR products were run on %1 agarose gel and visualized by gel documentation.

Genotyping

DNA typing was performed by PFGE according to the protocol described by Nikbin et al., [36]. PFGE has been widely used to type various microorganisms in both outbreak and population based studies. The percentage of relatedness were calculated by usage of the Dice coefficient. DNA patterns were aslo analyzed virtually as instructed by Tenover et al [37]. Accordingly, strains with up to three band differences were considered closely related, strains with four to six band differences were considered possibly related, and strains with greater than six band differences were considered unrelated.

RESULTS

Study population

The study population consisted of 52 CF patients from two Paediatric hospitals in Tehran. Patients ranged in ages from 1.5 months to 16 years. The male: female ratio was 2:1.

Sample processing

All throat swabs, oropharyngeal suctions and sputa were cultured. A total of 55 GNB were isolated from 52 samples. Six samples contained just Gram-positive pathogens. Also 1 fungus was isolated. No organisms were isolated from 5 samples.

Fifty five isolates belonging to different species of GNB were isolated [Table/Fig-2].

Antimicrobial susceptibility testing

The results of susceptibility on 55 isolates of GNB are shown in [Table/Fig-3].

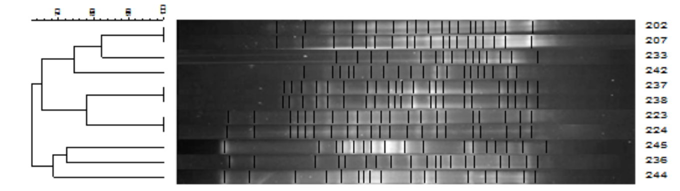
Anti-biogram showed that 89.09% of isolates were resistant to at least one of the third generation cephalosporins.

MIC tests showed that 40% [22] of isolates were resistant to cefotaxime. Of these, 16 (72.72%) were positive in combined disk tests for ESBLs detection. Eight isolates (14.51%) were resistant to meropenem. The modified Hodge test was positive in 3 isolates. The EDTA disk synergy was also positive in 4 of 11 imipenem resistant isolates.

PCR: PCR demonstrated that 19 of isolates contained *bla*$_{CTX-M}$.Of 17 carbapenem resistant isolates, *bla*$_{IMP-1}$were detected in 2, *bla*$_{VIM-1}$ in 2 and *bla*$_{VIM-2}$ in 3 respectively. No other genes were detected.The

Isolates	bla$_{CTX-M}$	bla$_{IMP-1}$	bla$_{VIM-1}$	bla$_{VIM-2}$
P. aeruginosa	5	-	-	-
K.ozaenae	2	-	-	-
A. xylosoxidans	2	2	2	3
A. denitrificans	1	-	-	-
E.coli	4	-	-	-
S. maltophilia	-	-	-	-
A. baumannii	-	-	-	-
K. pneumonia	-	-	-	-
E. cloacae	1	-	-	-
E. hermannii	1	-	-	-
E. agglomerans	1	-	-	-
Aeromonas media	-	-	-	-
E. kobei	1	-	-	-
C. koseri	1	-	-	-

[Table/Fig-4]: Prevalence of some of ESBL and Carbapenemase genes in isolates.



[Table/Fig-5]: Dendrogram of PFGE results for *P. aeruginosa* isolates. It revealed 8 different clusters

number of isolates within every species containing *bla*$_{CTX-M}$, *bla*$_{IMP-1}$, *bla*$_{VIM-1}$, *bla*$_{VIM-2}$ and *bla*$_{PE}$ Rare shown in [Table/Fig-4].

PFGE: The PFGE patterns obtained from 11 strains of *P. aeruginosa* are shown in [Table/Fig-5]. It was used to assess the clonality of them. The PFGE analysis revealed 8 different clusters. Of these isolates 6 were mutually similar. They were collected from one hospital. 3 clusters from 3 isolates obtained from another hospital were completely different. No similarity were seen between isolates of two hospitals.

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DISCUSSION

CF is the most common life-shortening autosomal recessive disease in the white population and afflicts about 60,000 patients worldwide, approximately 30,000 of whom are cared for in the United States [11]. The abnormal characteristic of this disease is the movement of water and ions through the epithelial cells that leads to formation of a dense mucosa and decrease in mucosal clearance in the lungs [38].

The microbiology of CF pulmonary infection has changed over the past 5 decades, as modern therapies enable patients with CF to live longer. Unusual pathogens and highly antibiotic-resistant organisms are increasingly recovered from patients with more advanced disease.

One of the major aspects of this study is reporting the broad range of bacteria identified in sputum and throat swab cultures of 52 CF patients. We isolated *P. aeruginosa, K. zaenae, A. xylosoxidans, A. denitrificans, K. pneumoniae, S. maltophilia, E. hermannii, E. agglomerans, E. cloacae, E. kobei* and *C. koseri*. As reported by Paixão et al., in 2010, *P. aeruginosa* as is the most frequent pathogen in CF patients [39]. We obtained the same result. Studies from Iran concerning the infective microorganisms among patients with CF is limited. In 2006 and 2010 Eftekhar et al., [40] and Khanbabaei et al., [41], detected *P. aeruginosa* as the most common agent and they reported that 85.7% of microorganisms were susceptible to ceftazidime. In another research in Iran which was done by Forozeshfard et al., [42], 72% of *P. aeruginosa isolates* from sputa of CF patients were susceptible to ceftazidime, and none of them showed resistance to imipenem. While in this study 44.45% of pathogens were resistant to ceftazidime. The reason might be extra administration and usage of ceftazidime and/or obtaining resistant genes which was widelyspread in the hospital by pathogens causing infection in CF patients.Fortunately in all studies including ours, imipenem was the most effective antibiotic. This may be due to limited administration of this drug tothese patients.Among a variety of drug-resistance traits, ESBL-producing GNB with resistance to newer cephalosporins have been posing a significant challenge in clinical practice. CTX M-1 had been observed in *P. aeruginosa* and *S. Maltophilia isolated* from patients with CF in Greece [43]. Multidrug-resistant *P. aeruginosa* isolate co-expressing extended-spectrum β-lactamase PER-1 and metallo-β-lactamase VIM-2 had been recovered from a 2-year-old child suffering from pneumonia in an underlying context of CF in Turkey [44]. A VIM-2 enzyme was detected in *P. aeruginosa* isolates in CF patents in Portugal [45].

The genes encoding carbapene mases and ESBLs were detected among the isolates in this study too. Carbapenemases represent the most versatile family of β-lactamases, with a breadth of spectrum unrivaled by other β-lactam-hydrolyzing enzymes. Until the early 1990s, all carbapenemases were described as species-specific, chromosomally encoded β-lactamases, each with a well-defined set of characteristics.

In a study in Iran, strains of *P. aeruginosa* isolated from CF patients were checked for production of MBLs using PCR targeting blaVIMand none of clinical isolates was positive for it [42]. Among MBL genes, VIM-type had been detected in *P. aeruginosa* in Iran [46], NDM-1 in *K. pneumonia* [47] and SPM-1, GES-1, OXA-23,OXA51 in A. *baumanii* [48]. In the current study, we identified some of the genes encoding Carbapenemases and ESBLs among the isolates and these were bla_{PER}, bla_{IMP-1}, $bla_{VIM-1}$$bla_{VIM-2}$and bla_{CTX-M}. With the incidence of 34.54% (n=19), $bla_{CTX-M}$was the most prevalent gene. Despite the prevalence of genes encoding carbapenemases and ESBLs in Iran, this is the first description of them which were isolated from CF patients. The clonal relationship between *P. aeruginosa* isolates was studied by PFGE and while 6 isolates were mutually related, a close relationships among 7 isolates was observed. Three CTX-M producing isolates showed closely related patterns.They belonged to the same hospital. Other isolates (n=4) were genetically distinct.

In conclusion, as the transmission of isolates in CF patients is not well specified, therefore it is important to separate patients, allocate a special center for them and design infection control policies. Also we suggest that careful supervision of the prevalence of antibiotic resistance in these patients should be established.

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