

Full Length Research Paper

Detection and characterization of multidrug resistance and extended-spectrum-beta-lactamase-producing (ESBL_s) *Pseudomonas aeruginosa* isolates in teaching hospital

Zahra Tavajjohi¹, Rezvan Moniri^{2*} and Ahmad Khorshidi²

¹Department of Biology, Faculty of Science, Alzahra University, Tehran, Iran.

²Kashan University of Medical Sciences, Kashan, Iran.

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Pseudomonas aeruginosa is the most common pathogen causing nosocomial infections. The objective of this study was to investigate the extended-spectrum-beta-lactamase (ESBLs) producing and multidrug resistance of hospital isolates of *P. aeruginosa* and to determine the presence of several resistance genes. A total of 86 isolates of *P. aeruginosa* were collected from teaching hospital in Kashan, Iran. Susceptibility to eight antimicrobial agents was performed by disk diffusion method. ESBL-phenotypic detection was carried out by double-disk synergy test; and the presence of the genes encoding of *bla*(TEM), *bla*(SHV), *bla*(CTX-M), *bla*(OXA) and *bla*(GES)-like genes was studied by polymerase chain reaction. The prevalence of ESBLs was 8.1%. The presence of genes encoding ESBLs was confirmed in seven isolates, comprising seven *bla*(GES-2), one *bla*(SHV-1), one *bla*(SHV-5) and one *bla*(CTX-M-1) genes. *P. aeruginosa* demonstrated the highest resistance rate to piperacillin (38.4%), 67.5% of isolates were sensitive to imipenem whereas 32.5% were MDR (resistant to three or more classes of antibiotics). A multidrug-resistant (MDR) phenotype occurred frequently in *P. aeruginosa*. *bla*(GES-2), which compromises the efficacy of imipenem detected in all of seven ESBL-producing *P. aeruginosa* strains. Proper infection control practices and barriers are essential to prevent spreading and outbreaks of ESBL-producing and MDR *P. aeruginosa* in our teaching hospital.

Key words: *Pseudomonas aeruginosa*, extended-spectrum-beta-lactamase, multidrug-resistant.

INTRODUCTION

Pseudomonas aeruginosa is widely known as an opportunistic organism, frequently involved in infections of immunosuppressed patients, and also causes outbreaks of hospital-acquired infections (Wirth et al., 2009). *P. aeruginosa* is intrinsically resistant to many antimicrobial agents, including most β -lactams, the older quinolones, chloramphenicol, tetracycline, macrolides, trimethoprim-sulfamethoxazole and rifampin (Dundar and Otkun, 2010; Rossolini and Mantengoli, 2005). Multidrug resistance can be relatively common amongst nosocomial isolates of *P. aeruginosa*, which represent the large majority of

clinical isolates (Rossolini and Mantengoli, 2005). Resistance in *P. aeruginosa* may be mediated via several distinct mechanisms including the production of β -lactamases, efflux pumps, and target-site or outer membrane modifications (Tam et al., 2010). The production of extended-spectrum beta-lactamases (ESBLs) confers resistance at various levels to expanded-spectrum cephalosporins, such as cefotaxime and ceftazidime, and to aztreonam, but not normally to the cephamycins and carbapenems (Mirsalehian et al., 2010). These enzymes are encoded by different genes located on either chromosomes or plasmids (Chuang and Rasmussen, 2006). Two major classification schemes exist for categorizing β -lactamase enzymes: Ambler classes A through D, based on amino acid sequence homology, and Bush-Jacoby-Medeiros groups 1 through

*Corresponding author. E-mail: moniri@kaums.ac.ir. Tel: + 98 361 5550021-25(539). Fax: + 98 361 5551112.

4, based on substrate and inhibitor profile (Drawz et al., 2010). The activity of Class A enzymes is inhibited in vitro by β -lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam but those belonging to class D are not (Bradford, 2001). Recently, however, several class A, B and D extended spectrum β -lactamases have been reported in *P. aeruginosa* (Park et al., 2005). Clavulanic acid-inhibited ESBLs conferring resistance to expanded spectrum cephalosporins have been reported, first in *Enterobacteriaceae* and then in *Pseudomonas aeruginosa* (Nordmann and Guibert, 1998). The TEM-type ESBLs are derivatives of TEM-1 and TEM-2. TEM-1 is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended- spectrum cephalosporins (Paterson and Bonomo, 2005). The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs (Jacoby, 1997). SHV refers to sulfhydryl variable. SHV-1 is a β -lactamase with activity against penicillins and narrow spectrum cephalosporins such as cephalothin and cephaloridine (Harada et al., 2008).

SHV-5 and SHV-12 are among the most common members of this family (Paterson et al., 2003). The most common group of ESBLs not belonging to the TEM or SHV families was termed CTX-M to highlight their greater activity against cefotaxime than against ceftazidime (Bonnet, 2004) but some of them, such as CTX-M-15 and -19, also hydrolyse ceftazidime efficiently (Poirel et al., 2001) which may complicate their phenotypic recognition (Pitout et al., 2005). CTX-M ESBLs are the most commonly isolated ESBLs in many parts of the world, particularly Europe (Drawz et al., 2010). The OXA-type β -lactamases are so named because of their oxacillin-hydrolyzing abilities. These β -lactamases are characterized by hydrolysis rates for cloxacillin and oxacillin greater than 50% that for benzylpenicillin (Paterson and Bonomo, 2005). GES-1 possesses hydrolytic activity similar to the classic class A ESBLs, is inhibited by β -lactamase inhibitors, and is generally classified into ESBLs (Poirel et al., 2000). However, some of the GES variants, such as GES-2 and 4, also have hydrolytic activity against carbapenems (Poirel and Wiedhagen et al., 2001; Wachino et al., 2004). The occurrence and detection of ESBL in *P. aeruginosa* are undefined in Kashan. This study aimed to investigate the presence and prevalence of *bla*(TEM), *bla*(SHV), *bla*(CTX-M), *bla*(OXA) and *bla*(GES)-like genes, responsible for extended spectrum beta-lactamases (ESBLs) production, antibiotic susceptibilities and multidrug resistance (MDR) of *P. aeruginosa*, isolated from clinical specimens of patients of hospital.

MATERIALS AND METHODS

This descriptive study was carried out in Beheshti hospital from 2010 to 2011 (Kashan city, Isfahan province, Iran). 86 isolates of

P. aeruginosa were isolated from patients enrolled in this study. Conventional biochemical tests were used for identification at the species level in 86 g negative, short rods was based on colonial morphology, oxidase positivity, and the presence of characteristic pigments. The organisms were isolated from urine (n = 23), the trachea (n = 18), stool (n = 17), wound (n = 11), blood (n = 5), bronchoscopy fluid (n = 4), sputum (n = 3), vaginal discharge (n = 2), pleural fluid (n = 1), gastrointestinal fluid (n = 1) and pus (n = 1). All isolates were cultured in Luria-Bertani (LB) broth and grown at 37°C for genomic DNA isolation. The isolates were stored in vials containing LB broth with 50% glycerol at -20°C. The purity of the strains was checked before of analysis. The susceptibilities of the bacterial isolates to eight antimicrobial agents (imipenem 10 μ g, ciprofloxacin 5 μ g, ceftazidime 30 μ g, ceftriaxone 30 μ g, cefotaxime 30 μ g, aztreonam 30 μ g, piperacillin 100 μ g and gentamicin 10 μ g; [Mast Group Ltd., Merseyside, U.K.] were determined by the disk diffusion method according to the criteria published by the Clinical and Laboratory Standards Institute (CLSI). *P. aeruginosa* ATCC 27853 was used as quality control in each susceptibility determination. Multidrug-resistant isolates were defined as those resistant to three or more classes of antipseudomonal agents (carbapenems, penicillins, cephalosporins, monobactams, fluoroquinolones and aminoglycosides). ESBL production in all the isolates was detected by double-disc synergy test as described by Jarlier et al. (1998). Briefly of 30 μ g each ceftazidime, ceftriaxone, cefotaxime and aztreonam disks were placed at a 20 mm (center-to-center) from an amoxicillin-clavulanate 20/10 μ g disk (Mast Group Ltd., Merseyside, U.K.) on Mueller-Hinton agar (Merck, Germany) and incubated at 37°C for 16 h. Enhancement of the zone of inhibition of the oxyimino β -lactam due to synergy of the clavulanate in the amoxicillin-clavulanate disk was considered as evidence of ESBL- production. This criterion also fulfills the CLSI guidelines. This increase occurs because the clavulanic acid present in the amoxyclav disc inactivates the ESBL produced by the test organism.

Bacterial DNA was prepared by suspending one or two fresh colonies in 100 ml of sterile distilled water and heating at 95°C for 10 min. After centrifugation, the supernatant was stored at 4°C before analysis. The detection of β -lactamase genes were performed under standard PCR conditions using published set of primers (Table 1). Amplicons were revealed by electrophoresis on a 1.2% agarose gel and a subsequent exposure to UV light in the presence of ethidium bromide.

RESULTS

P. aeruginosa were isolated from 86 patients. *P. aeruginosa* demonstrated the highest resistance rate to piperacillin (38.4%), cefotaxime (30.2%), imipenem (30.2%), ceftriaxone (25.6%), gentamicin (24.4%), ceftazidime (20.9%), aztreonam (19.8%) and ciprofloxacin (17.4%) respectively (Table 2). Nineteen (20.9%) *P. aeruginosa* isolates were resistant to all extended-spectrum cephalosporins tested. Among the total isolates, 7% were susceptible to all studied agents, and 8.1% were resistant to a single agent, predominantly to piperacillin (Table 3). Of the 86 isolates, 28 (32.5%) were multi-drug resistant. From the total MDR strains, 89.3% were resistant to penicillins and 71.4% were resistant to the aminoglycosides. Of the majority of MDR isolates (32.1%) were resistant to seven antimicrobial agents, and this group accounted for 10.5% of all isolates. Using the double-disc synergy test, ESBL-

Table 1. Primers used in this study.

Gene type	Primer	Sequences (5' to 3')	Product size (bp)
TEM-1	TEM-1 A	ATAAAATTCCTTGAAGACGAAA	1080
	TEM-1 B	GACAGTTACCAATGCTTAATCA	
SHV-1	SHV-1 A	TGGTTATGCGTTATATTCGCC	865
	SHV-1 B	GGTTAGCGTTGCCAGTGCT	
SHV-5	SHV-5 A	ACTGAATGAGGCGCTTCC	220
	SHV-5 B	CGCACCCCGCTTGCT	
OXA-1	OXA-1 A	AGCCGTTAAAATTAAGCCC	908
	OXA-1 B	CTTGATTGAAGGGTTGGGCG	
CTX-M-1	CTX-M-1 A	GCGATGTGCAGCACCAGTAA	605
	CTX-M-1 B	GGTTGAGGCTGGGTGAAGTA	
CTX-M-2	CTX-M-2 A	CGGAATTCATGATGACTCAGAGCATTCCG	605
	CTX-M-2 B	GCTCTAGATTATTGCATCAGAAACCGTG	
CTX-M-3	CTX-M-3 A	GGTTAAAAAATCACTGCG	873
	CTX-M-3 B	TTACAAACCGTCGGTGA	
CTX-M-9	CTX-M-9 A	TATTGGGAGTTTGAGATGGT	932
	CTX-M-9 B	TCCTTCAACTCAGCAAAAAGT	
GES-1	GES-1 A	ATGCGCTTCATTCACGCAC	860
	GES-1 B	CTATTTGTCCGTGCTCAGG	
GES-2	GES-2 A	GTTTTGCAATGTGCTCAACG	371
	GES-2 B	TGCCATAGCAATAGGCGTAG	

Table 2. Antimicrobial susceptibility pattern on *P. aeruginosa* isolated from patients of Shahid Beheshti hospital.

Antimicrobial agents (µg)	Resistant isolates (%)	Intermediate isolates (%)	Susceptible isolates (%)	MDR isolates NO. 28 (%)	ESBLs producing isolates NO. 7 (%)
Piperacillin(100)	38.4	0	61.6	89.3	71.4
Cefotaxime (30)	30.2	55.8	14	82.1	57.1
Imipenem (10)	30.2	2.3	67.5	89.3	42.9
Ceftriaxone (30)	25.6	13.9	60.5	71.4	57.1
Gentamicin(10)	24.4	2.3	73.3	71.4	42.9
Ceftazidime 30)	20.9	7	72.1	57.1	28.5
Aztreonam (30)	19.8	11.6	68.6	50	42.9
Ciprofloxacin (5)	17.4	11.6	71	53.6	14.3

producing isolates were detected in only seven of the isolates. Maximum ESBL production was found in trachea and urine samples. Of the seven ESBL-positive isolates, three strains were multi-drug resistant.

PCR was carried out on the genomic DNA of seven

ESBL-positive isolates using primers for the following ESBL-encoding genes: *bla*_{TEM-1}, *bla*_{SHV-1}, *bla*_{SHV-5}, *bla*_{OXA-1}, *bla*_{CTX-M-1}, *bla*_{CTX-M-2}, *bla*_{CTX-M-3}, *bla*_{CTX-M-9}, *bla*_{GES-1} and *bla*_{GES-2}. Four of the ten genes were found alone or in various combinations. Seven strains were positive for

Table 3. Resistance to one or more antimicrobials among *P. aeruginosa* isolated from patients of Shahid Beheshti hospital in Kashan.

No. of agents to which isolates were resistant	Number and total percent of isolates	piperacillin No. (%)	imipenem No. (%)	gentamicin No. (%)	Ciprofloxacin No. (%)	ceftazidime No. (%)	cefotaxime No. (%)	ceftriaxone No. (%)	aztreonam No. (%)
0	46(53.5)								
1	7(8.1)	6(85.7)	1(14.3)						
2	5(5.8)	2(40)				2(40)	3(60)	2(40)	3(50)
3 *	5(5.8)	2(40)	5(100)	3(60)	3(60)		1(20)		1(20)
4 *	2(2.3)	2(100)	2(100)	1(50)	1(50)	1(50)	1(50)		
5 *	5(5.8)	5(100)	3(60)	2(40)	3(60)	2(40)	5(100)	4(80)	1(20)
6 *	4(4.6)	4(100)	3(75)	4(100)	3(75)	1(25)	4(100)	4(100)	1(25)
7 *	9(10.5)	9(100)	9(100)	8(88.9)	2(22.2)	9(100)	9(100)	9(100)	8(88.9)
8 *	3(3.5)	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)

*28 out of 86 (32.5%) isolates were resistant to three or more antimicrobials and defined as MDR.

*bla*_{GES-2} gene. One of seven *P. aeruginosa* isolates had the *bla*_{SHV-1} and *bla*_{SHV-5} genes. Only one isolate was found to harbor the *bla*_{CTX-M-1} gene. However, none of the strains was positive for the *bla*_{TEM-1}, *bla*_{OXA-1}, *bla*_{CTX-M-2}, *bla*_{CTX-M-3}, *bla*_{CTX-M-9} and *bla*_{GES-1} genes.

DISCUSSION

The occurrence of multidrug-resistant *P. aeruginosa* strains is increasing worldwide and limiting our therapeutic options (Bonomo and Szabo, 2006). Our study reported the highest resistance rate to piperacillin, cefotaxime, imipenem, ceftriaxone, gentamicin, ceftazidime, aztreonam, ciprofloxacin respectively. In our study, gentamicin resistance value was higher than values reported in study conducted by Zhanal et al. (2010). In this study, ciprofloxacin exhibited nearly high susceptibility pattern (71%) and Zhanal et al. (2010) reported that 19.0 to 24.1% isolates were resistant to fluoroquinolones.

Cephalosporins, are known anti-pseudomonal drugs, especially the third-generation ceftazidime, that has demonstrated high susceptibility pattern (72.1%) with *P. aeruginosa* isolates, however cefotaxime and ceftriaxone were the two of the cephalosporin drug tested in this study, with susceptibility value of 14 and 60.5% respectively. These low resistance value observed were comparable with the report from Malaysia of 40 and 31% (Jombo et al., 2008). In our study, prevalence of MDR *P. aeruginosa* isolates, was 32.5% which lower than values reported in other studies in Iran (Mirsalehian et al., 2010), Malaysia (Lim et al., 2009) and Pakistan (Ullah et al., 2009) and this rate is lower than study was performed in this hospital five years ago (Moniri et al., 2005). In contrast, Tam et al. (2010) reported that 14.0% of *P. aeruginosa* isolates were MDR. The multidrug-resistant phenotype in *P. aeruginosa* could be mediated by several mechanisms including multi-drug efflux systems, enzyme production, outer membrane protein (porin) loss and target mutations. Inappropriate empirical therapy has

been associated with increased mortality in *P. aeruginosa* infections; delays in starting appropriate therapy may contribute to increased length of hospital stay and persistence of infection. In addition, worse clinical outcomes may be associated with MDR infections owing to limited effective antimicrobial options. Our study reported 8.1% ESBL production among *P. aeruginosa* isolates. The studies conducted by others depicted low rates, 3.7% (Woodford et al., 2008), 4.2% (Lim et al., 2009), 7.7% (Jacobson et al., 1995) respectively, of ESBL production in *P. aeruginosa*. The results of various studies, showed high rate 20.3% (Aggarwal et al., 2008), 39.41% (Mirsalehian et al., 2010) and 35.85% (Ullah et al., 2009) of ESBLs in the samples of *P. aeruginosa* isolates examined. The other genes *bla*_{TEM-1}, *bla*_{OXA-1}, *bla*_{CTX-M-2}, *bla*_{CTX-M-3}, *bla*_{CTX-M-9} and *bla*_{GES-1} genes were recently reported in *P. aeruginosa* were not detected. The first report of the SHV-1 penicillinase in epidemic *P. aeruginosa* strain showed by Kalai Blagui et al., 2009. Seven *P. aeruginosa* isolates producing the clavulanic

acid-inhibited extended-spectrum beta-lactamase SHV-5 were isolated in the hospital in Athens, Greece (Poirel et al., 2004).

In our study, the *bla*_{GES-2} gene was detected in all ESBL producing isolate. This was not accordance with other results in Middle East and other study in Iran. Recent reports from Brazil showed that *bla*_{GES-1} are present in *P. aeruginosa* strains (Castanheira et al., 2004). In this study, we describe the presence of CTX-M-1 β -lactamase in 1 clinical bacterial strains of *P. aeruginosa*. The first description of the presence of CTX-M ESBLs in *P. aeruginosa* and *S. maltophilia* reported by Al Naiemi et al. (2006). The CTX-M ESBLs provide *P. aeruginosa* with an additional powerful resistance mechanism with potential serious clinical implications, including limitation of the therapeutic options. ESBLs producing organisms pose unique challenges to clinical microbiologists, clinicians, infection control professionals and scientists engaged in finding new antibacterial agents. The incidence of ESBLs varies with geographic location and time. ESBL producing strains are usually found in those areas of hospitals where antibiotic use is frequent and the patient's condition is critical. These resistant organisms are clinically important because they result in increased morbidity and mortality. In addition, ESBL producing bacteria are frequently resistant to many classes of antibiotics, resulting in difficult-to-treat infections. Currently, carbapenems are regarded as the drugs of choice for treatment of infections caused by ESBL-producing organisms. Unfortunately, use of carbapenems has been associated with the emergence of carbapenem-resistant *Pseudomonas* spp. The development and spread of ESBLs is most likely caused by the overuse of expanded-spectrum cephalosporins in the hospital setting. Proper infection control practices and barriers are essential to prevent spreading and outbreaks of ESBL-producing bacteria.

In conclusion, gentamicin still shows the highest potency against *P. aeruginosa* in our hospital. However, emerging of resistance to carbapenems, in particular resistance to imipenem, may limit their prescription in the future. The prevalence of ESBL-producing *P. aeruginosa* occurred in 8.1% of isolates and MDR phenotype was common in *P. aeruginosa*. The most prevalent genes among ESBL producers were *bla*_{GES-2}, which compromises the efficacy of imipenem, tend to be geographically restricted.

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