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Full Length Research Paper

Antimicrobial resistance pattern and prevalence of metallo-β-lactamases in *Pseudomonas aeruginosa* from Saudi Arabia

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To determine the prevalence of metallo-β-lactamases (MBLs) in *Pseudomonas aeruginosa* and to investigate whether MBL genes have spread in MBL-producing isolates. A total of 350 clinical isolates of *P. aeruginosa* were screened for production of MBL. Antibiotic susceptibility testing was determined by E-test strips. Six MBL genes and class 1 integron were tested by polymerase chain reaction (PCR). Positive MBL genes were subjected to sequencing. Matting out assay was carried out. The resistance rates of 350 *P. aeruginosa* isolates to piperacillin, piperacillin/tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, and ciprofloxacin were 54, 50.3, 48.3, 45.14, 40.3, 30.57, 38.6 and 36.85%, respectively. The results of MBL screening revealed that 20.57% (72/350) of *P. aeruginosa* produced MBL. Sixty three (46.46%) of 135 imipenem-resistant isolates were not found to produce MBL. MBL-producing isolates were 100% resistant to β-lactams except aztreonam, which showed resistance rate of 63.88%. Only 20.3 and 5.42% of the MBL-producing isolates were resistant to amikacin and polymyxin B, respectively. PCR and deoxyribonuleic acid (DNA) sequencing investigated revealed that all MBL isolates harbour *bla*$_{VIM\ -2}$ gene. High prevalence of imipenem resistant and MBL-producing *P. aeruginosa* isolates was reported. Imipenem resistance is in increasing and MBL is responsible for 20.57% of the resistance. The *bla*$_{VIM-2}$ is the dominant MBL gene in MBL-producing isolates in Saudi Arabia.

Key words: Carbpenem resistance, Metallo-beta-lactamases, VIM-2, *Pseudomonas aeruginosa*, Saudi Arabia.

INTRODUCTION

Pseudomonas aeruginosa is a versatile nosocomial opportunist, often multiresistant to antibiotics (Zhanel et al., 2010). *P. aeruginosa*, a non-fermenting Gram-nega-tive rod of great clinical and epidemiological relevance in hospital-acquired infections, is more frequently found in intensive care units (ICUs) and is associated with high morbidity and mortality (Agodi et al., 2007; Zavascki et al., 2006). The increasing rates of antibiotic resistance among *P. aeruginosa* are of serious concern. *P. aeruginosa* have significant intrinsic resistance to antibiotics (Bonomo and Szabo, 2006). Antipseudomonal β-lactams such as ticarcillin, piperacillin, ceftazidime, cefepime, aztreonam, and the carbapenems have an important therapeutic value (Bonomo and Szabo, 2006). Resistance to β-lactams may be mediated by upregulated chromosomal (AmpC) β-lactamases, efflux or porin loss or by acquired β-lactamases, included extended-spectrum types (ESBLs) and carbapenemases (Pfeifer et al., 2010). Many different ESBLs have been detected in the species (Mirsalehian et al., 2010), including VEB-,

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Abbreviations: MBLs, metallo-β-lactamases; **PCR,** polymerase chain reaction.

PER-, GES, TEM-, SHV and OXA-types (Weldhagen et al., 2003). The OXA-ESBLs are mutants of OXA-2 and - 10, belonging to class D, whereas the other ESBLs belong to Class A. VEB and PER types were found to be the most common (or least rare) ESBLs in *P. aeruginosa* in several countries, contrasting to the dominance of CTX-M, SHV and TEM ESBLs in Enterobacteriaceae (Woodford et al., 2008, Mirsalehian et al., 2010). The carbapenemases found are mostly metallo- β-lactamases (MBLs), including IMP, VIM, SPM, GIM, AIM, DIM or NDM enzymes, but serine carbapenemases have also been recorded, including KPC and GES variants (Juan and Oliver, 2010; Wang et al., 2010; Yong et al., 2009). The objective of the present study was to determine antimicrobial susceptibly in *P. aeruginosa*, to investigate the prevalence of MBL and to determine the resistance MBL-type genes in MBL-producing isolates collected from Riyadh, Saudi Arabia.

MATERIALS AND METHODS

Bacterial strains

Between August and November, 2010, a total of 350 non-duplicate non- consecutive clinical isolates of *P. aeruginosa* were collected from Prince Salman Hospital (PSH), Riyadh, Saudi Arabia. Prince Salman Hospital (PSH) is a 1200-bed tertiary care referral hospital in Riyadh. The isolates were stored at −20°C in try pticase soy broth containing 20% glycerol.

Determination of minimum inhibitory concentrations (MICs)

The MICs were determined for 350 *P. aeruginosa* using E-test (AB BIODISK, Solana, Sweden) as described by the manufacturer. Results were interpreted using CLSI criteria for susceptibility testing (CLSI, 2010). *P. aeruginosa* ATCC 27853 was used as a control strain.

Screening for metallo-β-lactamase (MBL) production

Evaluation of production of MBL was evaluated using E-test MBL strip. Several colonies from a 24 h culture plate were used to prepare the inoculum with a 0.5 McFarland standard density. Mueller-Hinton agar plates were streaked by using cotton swabs. The E-test MBL strips (AB BIODISK, Solana, Sweden) were then applied, and the plates were incubated at 35°C in a ir for 16 to 20 h. A ratio of the MICs of the imipenem to imipenem/EDTA of ≥8 or the presence of a phantom zone was interpreted as being positive for MBL production.

Detection of metallo-β-lactamase (MBL) genes, class 1 integron and gene cassettes by PCR

PCR amplification of bla_{IMP}, bla_{VIM}, bla_{SIM-1}, bla_{GIM-1} and bla_{SPM-1} was performed according to Ellington et al. (2007). Five primer pairs, specific for each family of acquired MBLs, were designed to amplify fragments of 188 bp (IMP), 390 bp (VIM), 271 bp (SPM-1), 477 bp (GIM-1) and 570 bp (SIM-1); these were then evaluated separately and in a multiplex format with all 10 primers. The primer pairs were: IMP family, Imp-F 5′-GGA ATA GAG TGG CTT AAY TCT C-3′/Imp-R 5′-CCA AAC YAC TAS GTT ATC T-3′; VIM family, Vim-F 5′-GAT GGT GTT TGG TCG CAT A-3′/Vim-R 5′-CGA ATG CGC AGC ACC AG-3′; GIM-1, Gim-F 5′-TCG ACA CAC CTT GGT CTG AA-3′/Gim-R 5′-AAC TTC CAA CTT TGC CAT GC-3′; SPM-1, Spm-F 5′-AAA ATC TGG GTA CGC AAA CG-3′/Spm-R 5′-ACA TTA TCC GCT GGA ACA GG-3′; Sim-1, Sim-F 5′-TAC AAG GGA TTC GGC ATC G-3′/Sim-R 5′-TAA TGG CCT GTT CCC ATG TG-3′. DNA template was prepared by emulsifying 5 colonies in 100 µl of PCR grade water and adding 2 µl to the PCR reaction mixture prior to thermal cycling. The cycling conditions were: initial DNA release and denaturation at 94°C for 5 min, followed by 36 cycles of 94°C for 30 s, 52°C for 40 s and 72°C for 50 s, followed by a single, final, elongation step at 72°C for 5 min. NDM was amplified according to Kaase et al. (2011). Class 1 integron was amplified using the previously descriped method (Goldstein et al., 2001). Negative and positive controls were involved in all PCR experiments. A thermal cycler (Techne, UK) was used in this study. The PCR products were confirmed by electrophoresis in agarose containing ethidium bromide using 1x TAE buffer.

DNA sequencing

Purifications of PCR products were carried out using Qiagen PCR purification (Qiagen, Hilden, Germany). PCR purified products of MBL genes were subjected to bidirectional nucleotide sequencing using PCR primers to determine their molecular types. DNA sequences were analyzed using ABI 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's recommendations. The nucleotide and deduced protein sequences were analyzed with BLAST software available over the internet at the National Center for Biotechnology Information (NCBI) Web site (www.ncbi.nlm.nih.gov).

RESULTS

The antibiotic resistance rates of 350 *P. aeruginosa* isolates are shown in Table 1. Of the total 350 *P. aeruginosa* collected in this study, 135 (38.57) isolates were found resistant to imipenem and 215 (61.43%) isolates were imipenem sensitive.

P. aeruginosa (n=350) isolates were screened for phenotypic production of MBL. The screening of MBL was done using E-test MBL strips. Production of MBL was detected in 72 (20.57%) out of 350 isolates of *P. aeruginosa*.

The antimicrobial resistance rates were higher for MBL-producing isolates than non-MBL-producing *P. aeruginosa* isolates (Table 1). All of MBL-producing *P. aeruginosa* isolates showed 100% resistance to the following antibiotics; piperacillin, piperacillin/tazobactam, ticaricillin, ticaricillin/clavulanic acid, cefoperazone, cefoperazone/sulbactam, cefotaxime, ceftazidime, ceftazidime/clavulanic acid and cefepime. However, the resistance rate for aztreonam was 63.88%. The resistance rates for polymyxin B, ciprofloxacin, amikacin, and gentamicin were 12.66, 63.88, 68.05, and 81.95%, respectively.

The results of MIC distributions for MBL-producing *P. aeruginosa* isolates are shown in Table 2. All MBL-producing *P. aeruginosa* were found to be resistant to imipenem with an MIC value of ≥16 µg/ml.

Table1. Resistance rates for clinical *Pseudomonas aeruginosa.*

Antibiotic		Number (%) of resistant isolates		
		Total isolates (n = 350)	Non-MBL producer (n = 278)	MBL producer (n = 72)
β-lactams	Piperacillin	176 (54%)	117 (42.08%)	72 (100%)
	Piperacillin /Tazobactam	176 (50.3%)	104 (37.4%)	72(100%)
	Ticaricillin	196 (56.3%)	124 (44.6%)	72 (100%)
	Ticaricillin /Clavulanate	173 (49.42%)	101 (36.33%)	72 (100%)
	Cefoperazone	169 (48.28%)	97 (34.89%)	72 (100%)
	Cefoperazone /Sulbactam	157 (44.85%)	85 (30.57%)	72 (100%)
	Cefotaxime	162 (48.28%)	90 (32.37%)	72 (100%)
	Ceftazidime	158 (45.14%)	86 (30.93%)	72 (100%)
	Ceftazidime /Clavulanate	127 (36.28%)	55 (19.78%)	72 (100%)
	Cefepime	141 (40.28%)	69 (42.82%)	72 (100%)
	Aztreonam	107 (30.57)	61 (21.94%)	46 (63.88%)
	Imipenem	135 (38.57%)	63 (22.66%)	72 (100%)
	Meropenem	134 (38.28%)	64 (23.02)	72 (100%)
	Doripenem	130 (37.14%)	59 (21.22)	71 (100%)
Non-β-lactams	Gentamicin	141 (40.28%)	82 (29.50%)	59 (81.95%)
	Amikacin	71 (20.28%)	22 (7.9%)	49 (68.05%)
	Ciprofloxacin	129 (36.85%)	83 (29.85%)	46 (63. 88%)
	Polymyxin B	19 (5.42%)	7 (2.52%)	12 (16.66%)

PCR experiments were used to detect the genotype of MBL genes in MBL-producing isolates. Six different MBL genes (bla_{IMP}, bla_{VIM}, bla_{SIM-1}, bla_{GIM-1}, bla_{SPM-1} and bla_{SNDM-1}) were screened by PCR. The results of PCR indicated that all MBL-producing *P. aeruginosa* harbor VIM type gene. However, other tested MBL genes were negative. DNA sequencing of the purified products of VIM gene revealed that all VIM-like gene belongs to VIM-2. Detection of class 1 integron was done by PCR assay. The results revealed that all MBL-producing *P. aeruginosa* harbor *int1* gene.

Conjugation experiments by broth matting technique, using an azide-resistant mutant of *E. coli* JM 105 as the recipient, were used to transfer the imipenem resistant determinants. Transconjugates were obtained for 57 out of 72 MBL producers by conjugation experiments and the remaining failed.

DISCUSSION

Carbapenems are among the best choices for the treatment of infection caused by multidrug resistant gram negative rods. The frequency of imipenem resistance among *P. aeruginosa* isolates in Saudi Arabia was 11% in 1998 (Bukharie and Mowafi, 2010), 9.3% in 1999 (Al-Jasser and Elkhizzi, 2004), 20% in 2004 (Bukharie and Mowafi, 2010) and 16.3% in 2007 (Al-Agamy et al., 2009); however, in the current study, the resistance rate to imipenem was increased to 38.57%. It was noticed that the resistance rates of most tested antibiotics in the studies performed in 1998, 1999, 2004, and 2007 (Al-Jasser and Elkhizzi, 2004; Al-Agamy et al., 2009; Bukharie and Mowafi, 2010) were lower than in the present study. In Iran, the resistance rate of *P. aeruginosa* to imipenem was 12.45% (76/610) lower than reported in the present study (Shahcheraghi et al., 2010).

The antimicrobial resistance rates are increasing for most tested agents in Saudi Arabia. This can be explained in part by increasing in consumption of antimicrobial agents in last decade and this point to increase in a selective pressure of antibiotics on *P. aeruginosa* and consequently the bacteria modify or alter the resistant mechanisms. About of 94.5% of *P. aeruginosa* isolates were sensitive to polymyxin B. The resistance to polymyxin B was increased from 3.7% (Al-Agamy et al., 2009) to 5.5% in this study. The resistance rates of the tested antimicrobial agents were higher for MBL-producing than non-MBL-producing *P. aeruginosa* isolates. 2.5% of non-MBL-producing isolates were susceptible to polymyxin B while 16.66 % of MBL-producing isolates were resistant.

The resistance to carbapenems is mainly due to production of carbapenemases. The carbapenemases found are mostly MBLs, including IMP, VIM, SPM, GIM, AIM, DIM or NDM enzymes, but serine carbapenemases have also been recorded, including KPC and GES variants (Juan and Oliver, 2010; Wang et al., 2010; Yong et al., 2009). Based on the results of E-test MBL strips, 53.33% of imipenem-resistant isolates were positive for production of MBL and 46.66% of them were MBL negative. This indicated that there are other resistance mechanisms to carbapenem such as class A carbapenemases, including KPC and GES variants and

Table 2. MIC distribution of MBL-producing-*Pseudomonas aeruginosa* isolates.

Antibiotics	Distribution of MIC(µg/ml) for 72 MBL -producing *P. aeruginosa* isolates																				
	≥256	192	128	96	64	48	32	24	16	12	8	6	4	3	2	1.5	1	0.75	0.5	0.38	≤0.25
Piperacillin	72	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Piperacillin/tazobactam	70	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ticaricillin	72	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ticaricillin/clavulante	71	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cefoperazone	65	3	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cefoperazone/sulbactam	59	2	6	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cefotaxime	72	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ceftazidime	72	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ceftazidime/clavulante	70	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aztreonam	30	4	6	2	3	1	0	0	7	8	10	1	0	0	0	0	0	0	0	0	0
Cefepime	61	5	3	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Imipenem	0	0	0	0	0	0	72	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Meropenem	0	0	0	0	0	0	72	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Doripenem	0	0	0	0	0	0	71	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Gentamicin	49	0	4	2	1	3	0	2	6	3	5	0	0	0	0	0	0	0	0	0	0
Amikacin	65	10	10	35	30	15	10	0	0	5	5	10	15	5	5	0	0	0	0	0	0
Ciprofloxacin	0	0	0	0	0	0	46	0	0	0	0	0	0	0	0	15	10	1	0	0	0
Polymyxin B	6	1	0	0	5	0	0	0	0	0	22	17	14	0	0	0	0	0	0	0	0

MBLs were not the sole mechanism of carbapenem resistance in the present study. In spite of class A, carbapenemases was not tested in our study, however, the results had proof these enzymes may be present in Saudi *P. aeruginosa* isolates. In a previous study in Saudi Arabia, the prevalence of MBL in *P. aeruginosa* was 16.3% (Al-Agamy et al., 2009). In the present study, the prevalence of MBL-producing *P. aeruginosa* was 20.57% and this prevalence was comparatively high. In Spain, the prevalence of MBL-producing *P. aeruginosa* was 6.9% (12/175) and all MBL-producers showed high-level resistance (MIC 32 mg/L) to all three carbapenems (imipenem, meropenem and doripenem) (Riera et al., 2011). In some countries, such as Korea and Brazil, MBL-producing *P. aeruginosa* constitutes nearly 10% (Gales et al., 2003; Lee et al., 2003), whereas in USA, the prevalence of MBL (1%) is still comparatively small (Karlowsky et al., 2003; Aboufaycal et al., 2007). On the other hand, the prevalence of MBL was high in many countries. In India, 28.57% of *P. aeruginosa* produce MBL (De et al., 2010). All imipenem resistant isolates in our study was due to production of MBL while in other countries such as Spain, the imipenem resistance is high (20%) while only 1% able to produce MBL. In addition, imipenem-susceptible isolates were not produce MBL. In Spain, the imipenem resistance is high (20%) and MBL is responsible for 1% of the resistance (Gutièrrez et al., 2007). However, in the present study, the imipenem resistance is high (38.6%) and MBL is responsible for 20.57% of the resistance.

MBL-producing *P. aeruginosa* were investigated for the presence of the reported MBL genes including bla_{IMP}, bla_{VIM}, bla_{GIM} bla_{SIM}, bla_{SPM} and bla_{NDM} alleles and class 1 integron. All MBL-producing *P. aeruginosa* were found to harbor bla_{VIM} like determinants while none of them carried a bla_{IMP}, bla_{GIM} bla_{SIM} bla_{SPM} and bla_{NDM} determinants. All isolates carried class 1 integron, which plays important role in horizontal transfer of resistant genes including MBL gene. VIM was the predominant metallo-enzyme found, as is typical worldwide for *P. aeruginosa* (Walsh et al., 2005), the findings of this result is in agreement with our results. In the present study, DNA sequence of revealed that all VIM MBL genes are bla_{VIM-2}. VIM-2 was first detected in France from *P. aeruginosa* and *K. pneumoniae* and VIM-2 is the prodominant MBL in France (Lauretti et al., 1999; Guerin et al., 2005). *P. aeruginosa* strain producing VIM-2 was described from patient coming from Saudi Arabia (Guerin et al., 2005). In the present study, all MBL and from these results we can conclude that bla_{VIM-2} is the prevalent MBL among Saudi Arabia

P. aeruginosa isolates. All the *bla*$_{VIM-2}$ positive isolates were multi resistant to ceftazidime, cefoperazone, cefoperazone/sulbactam, cefotaxime, ceftazidime, piperacilin, piperacilin/ tazobactam, ticaricillin, ticaricillin/ clavulanic acid, imipenem and meropenem. Only one isolate showed susceptibility to doripenem. MBLs can hydrolyze all β-lactams except aztreonam but our study showed that 64% of MBL-producing isolates were resistant to aztreonam. This could be due to the existence of other mechanisms such as ESBL production, efflux pumps and cephalosporinase hyperproduction (Walsh et al., 2005).

Rapid detection of MBL-producing *P. aeruginosa* isolates could be helpful for epidemiological purposes and for monitoring the emergence of MBL-producing *P. aeruginosa* isolates in clinical setting. The detection of such isolates could help rapidly establish standards for hospital infection control measures to minimize the spreading of these resistant determinants.

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