



Prevalence of metallo- β -lactamases in clinical isolates of *Pseudomonas aeruginosa* from King Abdulaziz University Hospital in Jeddah

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen associated with a wide range of nosocomial infections. A total of 75 *P. aeruginosa* clinical isolates were obtained from microbiology lab at King Abdulaziz University Hospital, Jeddah, Kingdom of Saudi Arabia. The antimicrobial susceptibility testing of these isolates was done using Kirby Bauer disc diffusion method as a preliminary screening method for detection of the β -lactamase resistant isolates. The results showed that 80% (60/75) of the tested isolates were resistant to ceftizoxime. Ethylene diamine tetraacetic acid disc synergy test was applied on the resistant isolates to detect the metallo- β -lactamase producers. According to the obtained results, 45% (27 / 60 isolates) of the resistant isolates were found to be metallo- β -lactamase producers for which a confirmatory test for the presence of *bla*_{IMP-1} and/or *bla*_{VIM-1} genes was carried out by polymerase chain reaction amplification technique. The results revealed that 67.7% (18 isolates) and 33.3% (9 isolates) of metallo- β -lactamase producers had *bla*_{VIM-1} gene and *bla*_{IMP-1} gene, respectively.

Key words: metallo- β -lactamases, *Pseudomonas aeruginosa*, prevalence, clinical isolates

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that causes serious infection, particularly in immunocompromised patients. They play an important role in hospital intensive care units where they cause a wide spectrum of nosocomial [1]. *P. aeruginosa* exhibits high rates of resistance to several antimicrobial drugs. The carbapenems are usually the drugs that provide the best coverage against this microorganism. However, the carbapenem resistance has increased among these strains [2]. The presence of metallo- β -lactamases (MBL) has been pointed out as a major mechanism of this resistance [3]. The mechanisms of bacterial resistance to carbapenems were known to be the combined action of the acquisition of β -lactamases that hydrolyze carbapenems, Ambler class B (metalloenzymes, MBLs) and class D (oxacillinases) enzymes, mutations in genes coding for penicillin-binding proteins and decreased outer

membrane permeability or overexpressed efflux [4]. The most important carbapenemases in *P. aeruginosa* are class B metallo- β -lactamases (MBL) such as VIM and IMP types [5]. Twenty-five IMP variants have been described; IMP-1 was the first MBL identified in *P. aeruginosa* and its national spread in Gram-negative bacilli was reported in Japan [6].

Among the VIM types, VIM-2 is the most dominant worldwide and was particularly detected in Europe [7]. Therefore, this study was conducted to evaluate the prevalence and types of MBLs resulting in carbapenem resistance in King Abdulaziz University Hospital in Jeddah.

MATERIALS AND METHODS

Clinical isolates: A total of 75 non-repetitive isolates of *P. aeruginosa* were obtained from microbiology lab at King Abdulaziz University Hospital, Jeddah, KSA. These isolates were

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previously isolated from clinical specimens from patients with underlying upper and lower respiratory tract illness from King Abdulaziz University hospital, Jeddah, Kingdom of Saudi Arabia in the period from September 2014 to December 2014. Identification of the obtained isolates were carried out by using API 20E kit, (bioMérieux, France), then preserved in brain heart infusion broth with 15% glycerol at -86°C. For sensitivity testing, bacterial suspension was prepared from 18 h culture after adjusting its turbidity to match that of 0.5 on McFarland Scale (1×10^8 CFU/mL).

Antibiotic susceptibility testing: The antibacterial susceptibility testing of the isolates was done using the Kirby Bauer disk diffusion method following the definition of the Clinical and Laboratory Standards Institute [8]. Briefly, 20 ml of Mueller-Hinton agar (MHA, Difco) were prepared and poured into sterile petri dishes. The agar medium was allowed to solidify at room temperature on a flat bench. Then, Aliquots containing 100- μ L bacterial culture (1×10^7 CFU/mL) were homogeneously spread plated on the surface of the agar plates. Antibiotic discs (Oxoid, Wesel, Germany) were gently and firmly placed on the inoculated agar plates, left at room temperature for 1 h, then incubated at 35 - 37°C for 24 h. According to the size of inhibition zones, the tested isolates were classified as either resistant, intermediate sensitive or sensitive.

Screening MBL-producing isolates: A 10 μ g imipenem disc was placed on MHA plates surface inoculated with the tested isolates. Another imipenem disc containing 10 μ l aliquot of 0.5 M EDTA was placed 10 mm apart from edge to edge on surface of the agar plate, then the plate was incubated at 37°C for 18 h. The presence of an enlarged inhibition zone around imipenem-EDTA disc was interpreted as EDTA-synergy test positive [9].

MBL gene detection by PCR: Plasmid extraction was done using miniprep plasmid DNA purification kit (Sigma-Aldrich, USA). Multiplex PCR amplification of MBL genes including *bla*_{IMP-1}, and *bla*_{VIM-1} were performed using the following primers: *bla*_{IMP-1}-F (5'-ATGAGCAAGTTATCAGTATTC-3'), *bla*_{IMP-1}-R (5'-GCTGCAACGACTTGTAG-3') and *bla*_{VIM-1}-F (5'-CAGATTGCCGATGGTGTGG-3'), *bla*_{VIM-1}-R (5'-AGGTGGG CCATTCAGCCAGA-3') [10]. The amplification conditions were: initial denaturation at 95°C for 5 minutes, 30 cycles of 95°C for 60 seconds, 55°C for 60 seconds, 72°C for 60 seconds, and a final elongation at 72°C for 7 minutes [11]. Reaction products were separated by

horizontal electrophoresis (Topac Inc, USA) for 25 min at 100 V and varying agarose gel density within 1.2–1.7% in dependence of amplicon size. Agarose gel was prepared using Tris-acetate buffer (50 mM Tris-HCl, pH 8.3, 50 mM CH₃ COONa, 5 mM EDTA). Visualization of the bands was carried out after staining with ethidium bromide (0.5 μ g/ml) using an ultraviolet transilluminator and gel documentation system (G:Box, Syngene, UK).

DNA sequencing: After initial screening for the amplification of metallo β -lactamase genes, the plasmid borne genes were subjected to nucleic acid sequencing. The initial PCR amplified products were purified and treated with QIAquick PCR Purification Kit (QIAGEN Inc., Valencia CA, USA).

Direct sequencing of each amplicon was carried out using the Sanger dideoxynucleotide chain termination method with the ABI Prism Big Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Inc., Foster City, CA, USA) on an ABI Prism 3500 Automated Sequencer. Using data collection software version 2.0, and sequencing analysis software 5.1.1. For each sequencing reaction, 2 μ l purified PCR product were added to a final reaction volume of 10 μ l containing 1 \times of sequencing buffer; 4 μ l BigDye reaction mix; and 3.2 pM of each of the Forward and Reverse primer. The sequencing cycle was composed of two stages; stage one is denaturing at 96°C for 1 min, while stage two is composed of 25 cycles of denaturing at 96°C for 10 sec, annealing at 50°C for 5 sec, and extension at 60°C for 4 min [12]. Each cycle sequence product was purified by BigDye XTerminator Purification Kit. The purified PCR product was then placed in the DNA analyzer. The DNA sequences obtained were compared with those in the GenBank using the BLAST program (<http://blast.ncbi.nlm.nih.gov/>).

RESULTS

According to the obtained results, the percentages of resistance isolates were as follows: 80% to ceftizoxime, 40% to tobramycin, 16% to netilmicin, 13.3% to gentamicin, and 9.3% to piperacillin (Table 1). Of these isolates, 27 were positive for the EDTA-disc synergy test (Fig. 1). Alleles of *bla*_{VIM-1} or *bla*_{IMP-1} were detected in all 27 EDTA-disc synergy test -positive isolates (Fig. 2, 3). Overall, the proportion of *bla*_{VIM-1} allele-positive isolates (n=18, 66.7%) was greater than *bla*_{IMP-1} allele-positive isolates (n=9, 33.3%).

Among the studied 380 nucleotide bases compromising for *bla*_{IMP-1} gene, 377 bases were conserved while only 3 sites were variable in 2 isolates. Two out of the three base substitutions

were transitional changes, from G → A (12 and 70) arginine → asparagines and cysteine → tyrosine respectively. Only one base substitution was transversional change from A → C (78) histidine → proline (Fig. 4).

Among the studied 520 nucleotide bases compromising for *bla*_{VIM-1} gene, 518 bases were conserved while only 2 sites were variable in 4 isolates and were transitional changes, from C → T (8) serine → Leucine and T → C (404) isoleucine → threonine (Fig. 5).

DISCUSSION

The present study proposes a combined phenotypic and genotypic approach for the specific diagnosis of antibiotic resistance mediated by metallo β-lactamases harboring *Pseudomonas aeruginosa* isolates.

Carbapenems such as imipenem and meropenem generally represent last resources for the treatment of nosocomial infections produced by multidrug-resistant Gram-negative bacteria due to their broad antimicrobial activity spectrum and stability against most common β-lactamases [13]. However, emergence of resistance to these drugs becomes a threatening for the treatment of Gram-negative bacterial infection worldwide [14]. As the mechanisms of the carbapenem resistance have been studied, they are due to specific reductions in outer membrane permeability, efflux pump, alteration of penicillin-binding proteins and also presence of carbapenem-hydrolysing enzymes [15]. Production of carbapenemases, especially MBLs, is becoming a leading cause of the resistance and considered to be more important than other mechanisms due to the horizontal spread of plasmids [16].

The antimicrobial susceptibility testing of *P. aeruginosa* isolates revealed that 82.7% of the isolates were sensitive to netilmicin followed by piperacillin (73.4%), gentamicin (66.7%),

tobramycin (56%), and ceftizoxime (20%). In a previous hospital study resistance rate among *P. aeruginosa* was only 5–9% against aminoglycosides [17,18].

In present study, ambler class B carbapenemases, MBLs, such as *bla*_{VIM-1} or *bla*_{IMP-1} were detected in all imipenem-resistant isolates; this was in accordance with previous studies done in Korea where ambler class B carbapenemases, MBLs, such as IMP, VIM and SIM are frequently detected enzyme types in imipenem-resistant *P. aeruginosa* and *A. baumannii* isolates [19].

For class B carbapenem- hydrolysing enzymes, *bla*_{VIM-1} was found out to be more prevalent MBL type than *bla*_{IMP-1} type among imipenem-nonsusceptible *P. aeruginosa* in our study. The results were consistent with other reports in a study at a hospital in Seoul, where IMP-1-like-producing *P. aeruginosa* was detected in strains isolated in 2003-2004, but the number was only two of 76 isolates of *Pseudomonas* spp [20].

Early recognition of carbapenemase producers among pathogenic isolates and a preliminary characterization of the type of enzyme produced are considered to an essential step for infection control in the hospital. Guiding the use of antibiotics that may favor the spread of carbapenemase producing bacteria also plays an important role in infection-control measures [14].

CONCLUSION

The study figured out the most common genes responsible for the expression of metallo β-lactamase enzymes in *P. aeruginosa* isolates. The study also revealed that, isolates having microevolutionary changes in their nucleotide composition of the detected genes have higher resistance pattern towards antimicrobial agents than those were all bases are conserved.

Table 1: Antimicrobial susceptibility pattern among *P. aeruginosa* isolates.

Antimicrobial agents*	<i>P. aeruginosa</i> (n =75 isolates)					
	Sensitive		Intermediately sensitive isolates		Resistant	
	No.	%	No.	%	No.	%
Ceftizoxime	15	20	0	0	60	80
Gentamicin	50	66.7	15	20	10	13.3
Netilmicin	62	82.7	1	1.3	12	16
Piperacillin	55	73.4	13	17.3	7	9.3
Tobramycin	42	56	3	4	30	40

*antimicrobial agents were chosen according to the CLSI, 2009

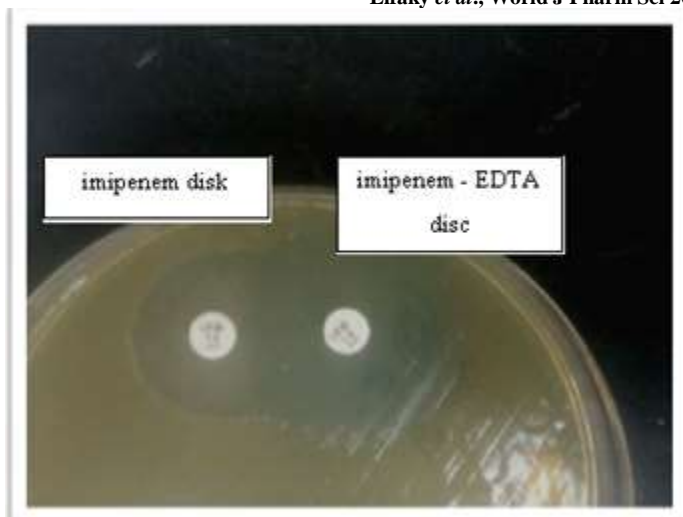


Figure 1: Ethylene diamine tetraacetic acid (EDTA) disc synergy test. An enhancement in the inhibition zone was appeared around the imipenem - EDTA disc.

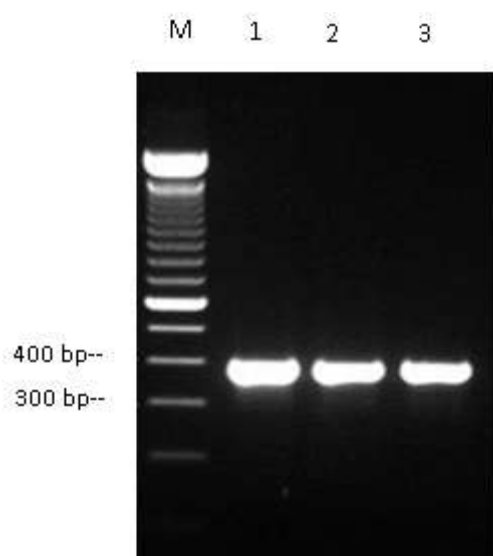


Figure 2: PCR amplification with *bla*_{IMP-1} primers. A 380-bp of *bla*_{IMP-1} gene. Lane M, 100bp DNA size marker, Lanes 1-3 isolates producing *bla*_{IMP-1} gene.

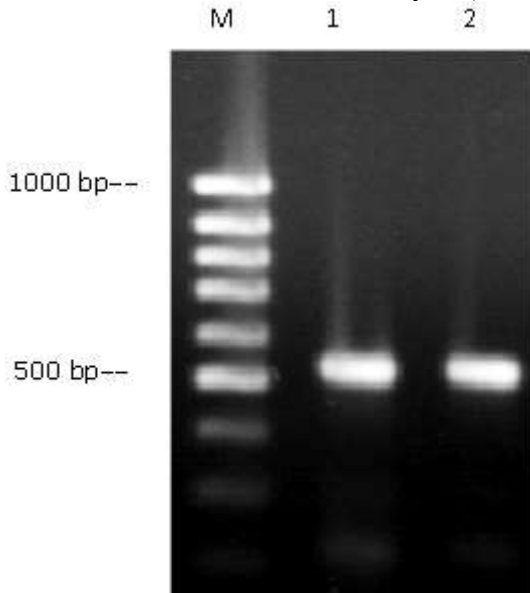


Figure 3: PCR amplification with *bla*_{VIM-1} primers. A 520-bp of *bla*_{VIM-1} gene. Lane M, 100bp DNA size marker, Lanes 1 and 2 isolates producing *bla*_{VIM-1} gene.

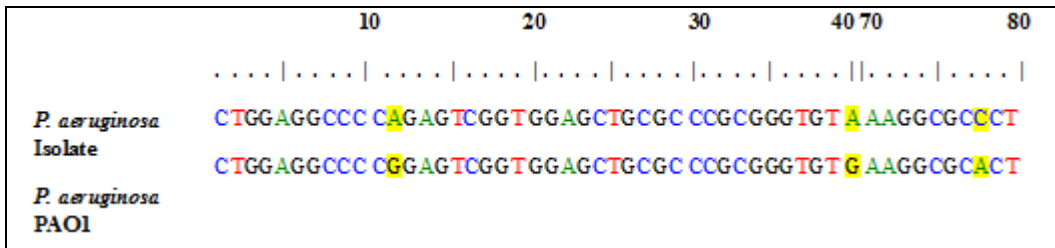


Figure 4: Multiple DNA sequence alignment of metallo beta lactamase *bla*_{IMP-1} gene isolated from *P. aeruginosa* tested isolates and retrieved sequences from Genbank.

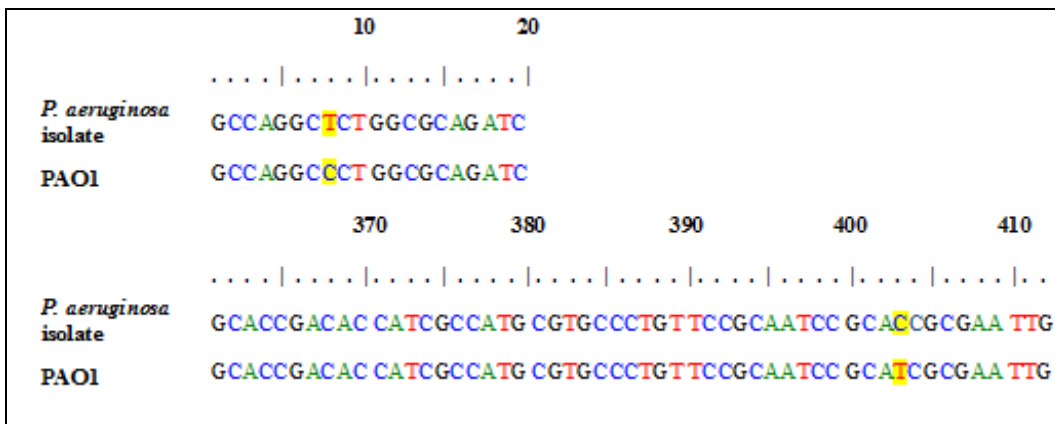


Figure 5: Multiple DNA sequence alignment of metallo beta lactamase encoding gene (*bla*_{VIM-1}) isolated from *P. aeruginosa* tested isolate and retrieved sequences from Genebank.

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