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Detection of *VIM* and *NDM-1 metallo-beta-lactamase* genes in carbapenem-resistant *Pseudomonas aeruginosa* clinical strains in Bahrain

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Abstract:

INTRODUCTION: Carbapenem-resistant *Pseudomonas aeruginosa* has emerged as a life-threatening infectious agent worldwide. Carbapenemase genes are reported to be some of the most common mechanisms for carbapenem resistance in *P. aeruginosa*. No reports are available from the Kingdom of Bahrain about carbapenem resistance and the underlying cause. In this study, we determined to study the presence of the metallo-beta-lactamase *(MβL)* genes of *VIM* family and *NDM-1* in carbapenem-resistant *P. aeruginosa* strains.

METHODOLOGY: Fifty carbapenem-resistant *P. aeruginosa* isolates were obtained from three main hospitals of Bahrain. They were subjected to antimicrobial susceptibility testing by disc diffusion test. Subsequently, MβL was detected by imipenem-ethylene diamine tetraacetic acid (EDTA) combined disc test and conventional polymerase chain reaction.

RESULTS: Among 50 *P. aeruginosa* strains, 40 (80%) were imipenem resistant. Among the 40 imipenem-resistant strains, 35 (87.5%) strains were positive for the imipenem-EDTA combined disc test, and 21 (52%) were carrying MβL genes. Nineteen (47.5%) strains were positive for the *VIM* gene; one (2.5%) strain was carrying the *NDM-1* gene, while one strain was carrying both the *VIM* and *NDM-1* genes. None of the imipenem sensitive strains carried the *VIM* or *NDM-1* gene.

CONCLUSION: This is the first study to report the presence of the *VIM* family gene and *NDM-1* genes in imipenem-resistant *P. aeruginosa* isolates in the Kingdom of Bahrain. The study also confirms the multiple drug resistance by the MβL strains, attention should therefore from now on, be focused on prevention of further spread of such isolates by firm infection control measures, and to reduce its threat to public health.

Key words:

Carbapenem resistance, NDM-1 gene, *Pseudomonas aeruginosa*, VIM gene

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Introduction

Carbapenem resistance among *Pseudomonas aeruginosa* is a global health threat and has led to therapeutic limitations.[1] Carbapenemase genes which code for carbapenemases is reported to be an important mechanism in carbapenem resistance of *P. aeruginosa*.[2]

Carbapenemases are assigned to three classes of β lactamases: Ambler A, B, and D.[3] Class B metallo-beta-lactamases (MβL) include the enzymes that belong to *VIM, IMP, SPM, GIM,* and *NDM* families.[1] They hydrolyze all β-lactams, except aztreonam, and this activity can be inhibited by ethylene diamine tetraacetic acid (EDTA).[4] Nothing is known about *P. aeruginosa* MβL producers in the Kingdom of Bahrain.

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The most relevant epidemiologically and clinically important MβL types are VIM (Verona integrin-encoded MβL), IMP (imipenemase), NDM (New Delhi MβL), and SPM (Sao Paulo MβL).[5,6]

Here, we studied the *VIM* family and *NDM-1* genes among MβL-producing *P. aeruginosa* isolates by phenotypic test and polymerase chain reaction (PCR).

Methodology

Strain collection

The study was conducted following ethical approval from the Ethical Review Board of Arabian Gulf University (E014-PI-11/16). The study was conducted on 50 nonduplicate *P. aeruginosa* strains isolated from clinical samples from 50 patients (included community, wards, and ICU patients) attending the Salmaniya Medical Complex, King Hamad University Hospital, and Bahrain Defense Force Hospital, located in the Kingdom of Bahrain. The isolates were preserved in 20% skimmed milk with glycerol solution and stored in a freezer at −80°C until further processing.

Inclusion criteria

This study included all the *P. aeruginosa* strains isolated from patients of all the age groups and both the sexes. This included both the outpatients and the inpatients attending all the three hospitals in Bahrain.

Exclusion criteria

Repeat isolates from the same patients were excluded from the study.

The samples were collected under complete aseptic conditions and included wound swabs, sputum, deep tracheal aspirates, endotracheal tube, urine, blood, and tissue.

Identification of *Pseudomonas aeruginosa*

The isolates were identified as *P. aeruginosa* strains by standard laboratory techniques such as Gram staining, colony morphology, cetrimide test, catalase test, oxidase reaction, citrate utilization, TSI reaction, oxidation-fermentation test, gelatin hydrolysis test, polymyxin B sensitivity testing, and sugar fermentation tests.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disc diffusion method on Mueller-Hinton agar (MHA) plates and interpreted according to Clinical Laboratory Standards Institute recommendations (CLSI 2016). The antibiotic discs used were as follows: imipenem (10 μg), meropenem (10 μg), amikacin (30 μg), gentamycin (10 μg), ceftazidime (30 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), piperacillin + tazobactum (100/10 μg), tigecycline (15 μg), and colistin (10 μg).

Phenotypic detection of metallo-beta-lactamase activity

All the isolates resistant to imipenem (zone size ≤15 mm as per the CLSI guidelines 2016) by disc diffusion method on MHA were screened for MβL activity by imipenem-EDTA combined disc test (IMP-EDTA CDT) as described by Yong *et al*.[7] In brief, an overnight culture of the test organism was compared with 0.5 McFarland which is comparable to the density of bacterial suspension 1.5 × 10^8 CFU/ml and was inoculated on an MHA plate. Two imipenem discs (10 μg) were placed on the inoculated plate at a distance of 5 cm from each other, and 10 μl of 0.5M EDTA solution was added to one of the discs. The plates were incubated at 35°C for 16–18 h. The inhibition zones of each disc were compared, and the test isolates which showed a zone size of ≥7 mm for IMI-EDTA disc as compared to imipenem disc alone were considered as MβL positive [Figure 1]. *P. aeruginosa* ATCC 27853 strain was used as the control strain.

Polymerase chain reaction assay for detection of metallo-beta-lactamase genes of VIM family and NDM-1

Conventional PCR testing of all the isolates for the presence of the VIM family and NDM-1 genes was done as per manufacturer's instructions. The sequence of primers specific for VIM family and NDM-1 used in this study is listed in Table 1.[8] Total DNA of all the bacterial isolates was extracted by the boiling method.[9] The extracted DNA was then stored at −20°C until further processing. Amplification was done using GoTaq Green PCR Master Mix (Promega). The PCR mix consisted of 25 μl master mix, 1 μl each of forward and reverse primer, 5 μl template DNA and nuclease-free water to make a

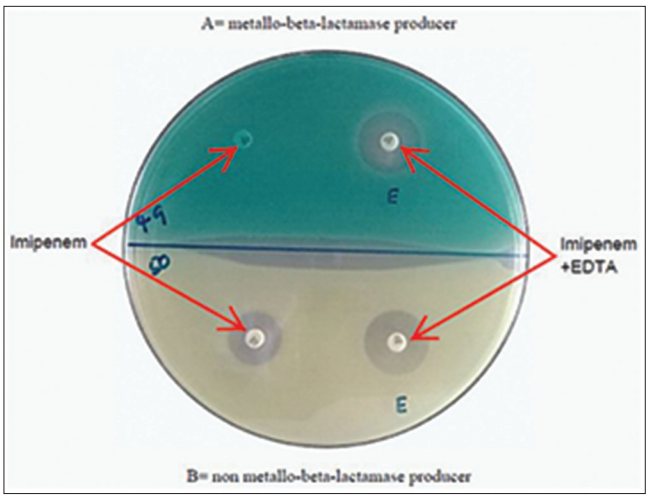

Figure 1: Photo of a Combined Disc Test showing strain A as metallo-beta-lactamase producer and strain B as non metallo-beta-lactamase producer

final volume of 50 µl. The thermal cycler program was as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s, followed by final extension at 72°C for 10 min. Agarose gel electrophoresis for the detection of amplicons was done by separating 10 µl of each amplicon and 100 bp ladder on a 1.5% agarose gel. Amplicons were visualized using ultraviolet transilluminator and subsequently analyzed.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Science (SPSS) version 20 (IBM, Armonk, NY, United States of America) to obtain descriptive data. Kappa test was used to measure the level of agreement between the phenotypic and genotypic test.

Results

The majority of the *Pseudomonas aeruginosa* strains are imipenem resistant

Fifty nonduplicate *P. aeruginosa* strains were isolated from clinical sources. The antimicrobial susceptibility test of these samples shows that out of 50 *P. aeruginosa* strains, 40 (80%) were imipenem resistant. All the isolates were resistant to ciprofloxacin (100%). 90% of the strains were resistant to norfloxacin, meropenem, and piperacillin/tazobactam which is shown in Table 2.

Most of the imipenem-resistant strains are positive for metallo-beta-lactamase

To determine whether imipenem resistance is caused by the production of MβL or by other mechanisms, the 40 imipenem-resistant strains were analyzed with the imipenem-EDTA combined disc test. An example of the imipenem-EDTA combined disc test is shown in Figure 1. This phenotyping revealed that 35 (88%) of these 40 strains were positive for MβL.

More than half of the imipenem resistance is due to *VIM* family and *NDM-1* genes

Genotyping of all 50 strains was performed to determine the presence of the *VIM* family and NDM-*1* genes. In Figures 2 and 3, the analyses of the PCR products are illustrated for the *VIM* and *NDM-1* genes, respectively. The results showed that of the 40 imipenem-resistant strains, 19 (47.5%) were positive for the *VIM* gene, one isolate (2.5%) for the *NDM-1* gene, and one (2.5%) was carrying both the *VIM* and *NDM-1* genes, as shown in Table 3. None of the imipenem sensitive strains were carrying these genes.

Many of the *VIM* and *NDM-1* gene-positive MβL producers were isolates obtained from endotracheal aspirate (seven of 19 VIM-positive strains [37%] and one of two NDM-1-positive strains [50%]) as can be seen in Table 4. These MβL producers were also resistant to most of the other antibiotics tested while they were all sensitive to colistin [Table 5].

Table 1: Polymerase chain reaction primers for amplification of *VIM* and *NDM-1* genes

Gene	Primer sequence (5'-3')	Amplicon size
VIM	Vim-F GAT GGT GTT TGG TCG CAT A	390 bp
	Vim-R CGA ATG CGC AGC ACC AG	
NDM-1	NDM-1 F CAT TAG CCG CTG CAT TGA TG	445 bp
	NDM-1 R GCG AAA GTC AGG CTG TGT TG	

Table 2: Antibiotic resistance pattern of fifty isolates to various antibiotics

Antibiotic	Number of resistant isolates (%)
Ciprofloxacin	50 (100)
Norfloxacin	45 (90)
Meropenem	45 (90)
Imipenem	40 (80)
Ceftazidime	43 (86)
Cefotaxime	43 (86)
Tigecycline	38 (76)
Piperacillin/tazobactam	45 (90)
Gentamicin	43 (86)
Amikacin	36 (72)
Colistin	0

Table 3: Distribution of *VIM* and *NDM-1* genes in 50 *Pseudomonas aeruginosa* strains by using polymerase chain reaction

	Imipenem sensitive, total isolates=10	Imipenem resistant, total isolates=40 (%)
VIM	0	19 (47.5)
NDM-1	0	1 (2.5)
VIM + NDM-1	0	1 (2.5)
Total	0	21 (52.5)

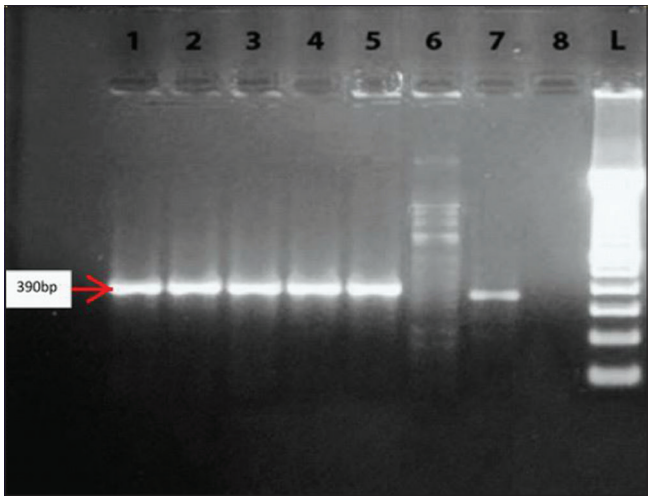

Figure 2: Polymerase chain reaction products after agarose gel electrophoresis. Lanes 1-5 and 7 show one band with molecular size 390 bp (*VIM* gene), lane 6 is a *VIM* negative strain, and lane 8 is water (negative control). Lane L contains a 100 bp ladder

Correlation of the phenotypic test results with polymerase chain reaction

When comparing the phenotypic test results with genotypic test results, we found that the strength of agreement was fair between the two tests (kappa value = 0.47) as shown in Table 6. The sensitivity and specificity of the combined disc test in relation to PCR was 100% and 51.72%, respectively.

Discussion

P. aeruginosa is a multidrug-resistant organism causing nosocomial infections.[10] Over the past decades, the resistance to carbapenems is increasing and has become a major health threat.[11] Early detection of MβL producers is therefore very crucial for optimal treatment of infections, and this will further reduce the resistance rate and prevent nosocomial spread. In the present study, out of 50 *P. aeruginosa* isolates, 40 (80%) were found to be resistant to imipenem which is in agreement with the study by Polotto *et al*.[12] in Brazil where they reported 96.4% of their strains as imipenem resistant. Another study, in India, by Arunagiri *et al*.[13] reported 62.7% of the isolates as resistant to imipenem. In Egypt, EL-Mosallamy *et al*.[8] conducted a study on 100 strains, wherein they found 25 (25%) strains imipenem resistant. In Saudi Arabia, Mohamed *et al*.[14] reported that imipenem resistance rate was 38.6% in 2011, while 5 years later, another study reported that 91% of 33 *P. aeruginosa* isolates were resistant to imipenem.[15]

There are no official standard guidelines for MβL detection. PCR analysis is the gold standard, but it is not practiced in routine microbiology laboratories. We therefore first used IMI-EDTA CDT phenotyping for MβL screening and compared the results with the genotyping results. By IMI-EDTA CDT phenotyping, we observed that out of 40 imipenem-resistant strains, 35 (88%) produced MβL whereas Pitout *et al*.[16] from Canada found that 110/241 (46%) imipenem-resistant strains were MβL positive while in Iran, Saderi *et al*.[17] reported that 65/100 (65%) of their imipenem-resistant strains were MβL positive using phenotypic methods. Another study by Panchal *et al*. compared different phenotypic tests for MβL detection and found that 19/30 (63.33%) were positive by IMI-EDTA combined disc test.[18]

The PCR results revealed that out of 40 imipenem-resistant strains, 19 (47.5%) strains were positive for the *VIM* gene which is similar to a study from Egypt by Essa and Afif where they found 40% of their imipenem-resistant strains carrying the *VIM* gene.[18] Al-Agamy *et al*. from Saudi Arabia reported 20.6% of the imipenem-resistant strains as MβL producers and all the MβL strains were found to carry the *VIM* gene.[19] Furthermore, in a study by Tawfik *et al*. from Saudi Arabia, *VIM* was found in all the

Table 4: Clinical source of the *VIM* and *NDM-1* positive samples

Clinical isolate	*VIM* positive, total=19 (%)	*NDM*-1 positive, total=2 (%)
Endotracheal aspirate	7 (37)	1 (50)
Swab*	5 (26)	1 (50)
Blood	1 (5)	
Respiratory secretions	4 (21)	
Urine	1 (5)	
Tissue	1 (5)	

*One sample was positive for both *VIM* and *NDM-1*

Table 5: Antibiotic susceptibility pattern of isolated metallo-beta-lactamase producing *Pseudomonas aeruginosa* strains

Antibiotic	MβL producers (total isolates=21)		
	Resistant (%)	Intermediate	Sensitive (%)
Imipenem	21 (100)	-	-
Meropenem	18 (86)	-	3 (14)
Amikacin	14 (67)	-	7 (33)
Gentamycin	17 (81)	-	4 (19)
Ceftazidime	18 (86)	-	3 (14)
Cefotaxime	17 (81)	-	4 (19)
Ciprofloxacin	21 (100)	-	
Norfloxacin	18 (86)	-	3 (14)
Piperacillin + tazobactam	17 (81)	-	4 (19)
Tigecycline	16 (76)	-	5 (24)
Colistin	-	-	21 (100)

MβL = Metallo-beta-lactamase

Table 6: Comparison of the results of the combined disc test with polymerase chain reaction

CDT	PCR			κ
	Positive	Negative	Total	
Positive	21	14	35	0.47
Negative	0	15	15	
Total	21	29	50	

CDT = Combined disc test, PCR = Polymerase chain reaction

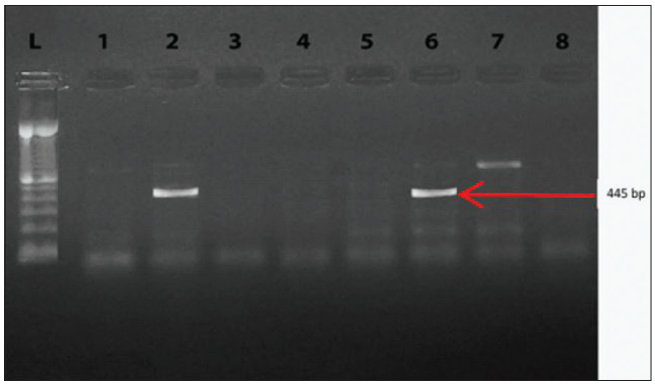

Figure 3: Polymerase chain reaction products after agarose gel electrophoresis. Lanes 2 and 6 show one band with molecular size 445 bp (*NDM-1* gene, lanes 1,3,4,5, and 7 are *NDM-1* negative strains and lane 8 is water (negative control). Lane L contains a 100 bp ladder

15 MβL-positive isolates (100%).[20] In a study in Canada, 43% of the strains were positive for the *VIM* gene.[16]

Resistance transferred by the *NDM-1* gene is also a growing public health problem. The main reservoir is the Indian subcontinent, and the secondary reservoirs are the Balkans regions and the Middle East.[21] Here, we observed only one isolate (2.5%) positive for *NDM-1* gene which is in corroboration with a study from Egypt by Zafer *et al*. which concluded that the prevalence of the *NDM-1* gene was only 4.2%.[22] Another study by Shanthi *et al*.[23] from India in 2014 reported that only four isolates out of 61 were positive for *NDM-1*. We observed only one isolate that carried both the *VIM* and *NDM-1* genes, whereas in Saudi Arabia, Shaaban *et al*.[24] reported 8 out of 16 imipenem-resistant strains carrying both *NDM-1* and *VIM* subtypes (*VIM 1* and *VIM 2*). A few previous studies have also reported the presence of multiple carbapenemase genes in *P. aeruginosa*, including the *KPC* and *VIM* in Colombia[25] and *SPM-1*, *KPC-2*, and *VIM-2* in Brazil.[2] The differences in the incidence and the types of genes seen in MβL producing strains are likely due to the geographical variations and differences in antibiotic usage.

The strength of agreement between the combined disc test and PCR is moderate. The sensitivity and specificity of IMI-EDTA CDT in relation to PCR is 100% and 51.72%, respectively, which was similar to the studies conducted by Pandya *et al*.[26] and Arunagiri *et al*.[13] where they reported sensitivity of IMI-EDTA CDT as 96.3% and 94%, respectively, while Picão *et al*.[27] reported a lower sensitivity of 80%.

Conclusion

This is the first study to report the presence of *VIM* and *NDM-1* in imipenem-resistant *P. aeruginosa* strains in the kingdom of Bahrain. The test results also showed that imipenem-EDTA combined disc test is a sensitive method for the detection of MβL producers. This test can, therefore, be used as an alternative to PCR in diagnostic laboratories. The study also identified the multiple drug resistance of the MβL producers. Attention should be focused on early detection of MβL producers to prevent further spread of such multidrug-resistant strains. The development of strong antimicrobial stewardship programs is essential, with emphasis on the importance of infection control measures to prevent further spread of these strains.

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Conflicts of interest
There are no conflicts of interest.

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