Identification and genetic characterization of metallo-beta-lactamase-producing strains of *Pseudomonas aeruginosa* in Tehran, Iran

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SUMMARY

Metallo-beta-lactamases (MBLs) are being reported with increasing frequency worldwide. The aim of this study was to investigate the prevalence of $bla_{IMP-1,}$ $bla_{VIM-1,2}$ and bla_{SPM-1} genes encoding metallo–beta-lactamases (MBLs) among a collection of *Pseudomonas aeruginosa* strains isolated from patients at different hospitals in Tehran and to trace the disseminated clones at these hospitals by pulsed field gel electrophoresis (PFGE). Susceptibility of 610 *P. aeruginosa* to 14 different antibiotics was determined using disc diffusion method. Isolates showing resistance to imipenem and ceftazidime were subjected to micro broth dilution assay to determine their MIC values. The bla_{IMP-1}, bla_{VIM-1}, $bla_{VIM-2,}$ and bla_{SPM-1} genes were amplified by PCR. Isolates containing bla_{VIM-1} were analyzed by PFGE.
Sixty-eight isolates were resistant to imipenem (MIC ≥4 µg/ml) of which 16 isolates carried bla_{VIM-1} gene using PCR assay. No other MBL genes were detected in this study. Three different unrelated patterns were found for isolates containing bla_{VIM-1} gene by PFGE of which pattern A was predominant. All isolates were susceptible to colistin and polymixin B. bla_{VIM-1} was the main gene encoding MBL among the isolates of *P. aeruginosa* in our study. Clonal spread of isolates containing bla_{VIM-1} had occurred at Tehran hospitals. However, heterogeneous clones also were involved in the outbreaks.

KEY WORDS: Pseudomonas aeruginosa, Metallo-β-lactamases, VIM, IMP, SPM

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen associated with a range of nosocomial infections. Carbapenems, including meropenem and imipenem, are the most effective antibiotic against this organism isolated from patients. However, resistance to carbapenems has emerged by different mechanisms such as impermeability to drug due to loss of OprD porin, the up-regulation of an active efflux pump system present in the cytoplasmic membrane of these organisms or production of metallo-beta-lactamases (MBLs) that hydrolyze all carbapenems (Kohler *et al.*, 1999; Livermore *et al.*, 1992; Nordmann and Poirel, 2002). Increases in prevalence of carbapenem resistance mediated by acquired MBLs including, IMP and VIM have been reported from several countries (Iseri *et al.*, 2008; Picao *et al.*, 2009; Kouda *et al.*, 2009; Mansour *et al.*, 2009). However, the prevalence of MBL producing isolates of *P. aeruginosa* varies in Asian countries (Walsh *et al.,* 2002).

The most common and widespread acquired MBLs are those of the IMP and VIM types, which exhibit a worldwide distribution and for which several allelic variants are known. Acquired drug resistance is frequent in nosocomial isolates of *P. aeruginosa* and often involves more than one antibiotic class (Poirel *et al.,* 2001; Rossolini and Mantengoli, 2005).

Previous studies showed the high prevalence of multidrug resistant *P. aeruginosa* isolated from

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different clinical specimens especially from burn units in Iran (Shahcheraghi *et al.,* 2003; Shahcheraghi *et al.,* 2009). However, little information is available on the distribution of MBL-producing isolates and colonal infections with these isolates in our country. The aims of this study were to determine the prevalence of $bla_{\text{IMP-1,}}$ $bla_{\text{,VIM-1,2}}$ and $bla_{\text{SPM-1}}$ genes encoding MLBs among *P. aeruginosa* isolated from clinical specimens at different hospitals in Tehran and to investigate possible clonal dissemination of these organisms by PFGE.

MATERIALS AND METHODS

Bacterial strains

Six hundred and ten isolates of *P. aeruginosa* were collected from two hospitals in Tehran during June 2005–March 2007. The specimens included urine (n=153, 25%), blood (n= 27, 4%), wound (n=129, 22%), respiratory tract (n=176, 29%), Cerebral Spinal Fluid (n=4, 0.6%), ear (n=8, 1.3%), eye (n=16, 2.6%), stool (n=44, 7%) and other sites (n=53, 9%). These isolates were collected from different wards including ICU, urology, respiratory and surgery wards. Replicated isolates from the same patients were excluded from the study. All isolates were identified by conventional bacteriological tests.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) (M100-S16 CLSI, 2006) using disks containing ceftazidime (CAZ:30 µg), ceftriaxone (CRO:30 µg), cefotaxime (CTX:30 µg), ceftizoxime (ZOX:30 µg), piperacilin (PIP:100 µg), piperacilin/tazobactam (PT:110 µg), gentamicin (GM:10 µg), amikacin (AN:30 µg), imipenem (IMP:10 µg), ciprofloxacin (CIP:5 µg) collistin (COL:10 µg), polimixin B (PB:300 unit), meropenem (MEM:10 µg) and aztreonam (ATM: 30 µg) (MAST, Merseyside, U.K). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as controls for antibiotic resistance.
MICs for ceftazidime and imipenem were determined by micro broth dilution method.

Identification of metallo-beta lactamases

Double disk synergy test (DDST) was used for phenotypic detection of MBLs (Pitout *et al.,* 2005). In brief, disks containing 930 µg of EDTA plus 10 µg of imipenem were placed on the inoculated plates containing Muller Hinton agar. An increase of ≥7 mm in zone diameter in the presence of 930 µg of EDTA compared to imipenem tested alone was considered to be a positive test for the presence of an MBL.
DNA template from imipenem resistant isolates (MIC≥4 µg/ml) were extracted by boiling for 15 minutes and used in PCR to amplify $bla_{\text{VIM-1}}$, $bla_{\text{VIM-2}}$, $bla_{\text{SPM-1}}$ and $bla_{\text{IMP-1}}$ genes (Shibata *et al.,* 2003). PCR were carried out in solution containing 200 µM concentrations of dNTPs, 10 pM of each primer, 0.8 mM $MgCl_2$, 0.5 U Taq polymerase and 50 ng DNA templates in a final volume of 25 µL.
The mentioned genes were amplified under following condition: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 10 min. *Acinetobacter baumannii* AC54/97 producing bla_{IMP} gene (Gombac *et al.,* 2002), *P. aeruginosa* PO510 producing $bla_{\text{VIM-1}}$, *P. aeruginosa* COL-1 producing $bla_{\text{VIM-2}}$ and *P. aeruginosa* 16 producing $bla_{\text{SPM-1}}$ (Kindly provided by P. Nordmann) were used as controls.
The amplicons were electrophoresed in 1% agarose gel and visualized after staining with ethidium bromide. A 100 bp ladder (Fermentas, Vilnius, Lithuania) was used as molecular weight marker. The PCR products for $bla_{\text{VIM-1}}$ were purified on Qiaquick columns (QIAGEN) and the sequencing was carried out using the ABI capillary system (Macrogen Research, Seoul, Korea).

PFGE analysis

The intact chromosomal DNAs from VIM-1-producing and non-producing strains (MIC≥4 µg/ml) were extracted for PFGE as described previously (Nikbin *et al.,* 2007). The genomic DNA in agarose plugs were digested with *Xba*I restriction endonuclease (Fermentas, Vilnius, Lithuania) and the fragments were separated by CHEF-DR III apparatus (Bio-Rad Laboratories, Richmond, CA). The system was set up at 6 V/cm for 19 h with pulse time ramped from 5 to 35s. *Salmonella braenderup* H9812 used as molecular weight Marker. The gels were stained with ethidium bromide and DNA patterns were photographed with

UVP gel Documentation (UVP, UK) (Figure 1). The DNA banding patterns were interpreted as instructed by Tenover *et al.* (Tenover *et al.,* 1995).

Nucleotide Sequencing

The nucleotide sequences of amplicons have been assigned to the Gene Bank under accession numbers FJ226585 and FJ355956-355963. Aligning of the obtained sequences with those of reference strains in Gene Bank confirmed the correct identification of $bla_{\text{VIM-1}}$ by PCR.

RESULTS

Of the total 610 *P. aeruginosa* collected in this study 76 isolates were found resistant to imipenem by disk diffusion test. The majority of these resistant isolates (n=65, 85.5%) belong to a general hospital and the remainder (n=11, 14.5%) to a children hospital. Of these 76 imipenem resistant isolates, 68 isolates showed MIC ≥4 µg/ml for imipenem. Based on the results of DDST, 72% of these isolates were positive for production of MBL and 28% of them were MBL negative. The rate of resistance in MBL positive/negative isolates to the antibiotic were as follows: amikacin (73/87), gentamicin (81/88), imipenem (100/100), ceftazidime (100/100), ceftriaxone (100/100), piperacilin (85/100), piperacilin/tazobactam (75/100) and ciprofloxacin (70/66). All imipenem resistant isolates in this study were susceptible to colistin and polymixin B.

The rate of resistance to antibiotics and MIC values for imipenem did not show significant difference among the isolates collected over the time of this research.

The results of PCR assay for 68 isolates showing MIC ≥4 µg/ml for imipenem showed that only sixteen isolates (32≥ MIC ≥8 µg/ml) harbored $bla_{\text{VIM-1}}$ gene from which four isolates were negative and twelve were positive in double disk synergy test for MBL. These 16 strains were isolated from patients in a general (15 strains) and a children hospital and were cultured from wound (n=5) and tracheal aspirates (n=11) (Table 1). No $bla_{\text{IMP-1,}}$ $bla_{\text{SPM-1}}$ and $bla_{\text{VIM-2}}$ genes were detected among imipenem resistant strains in this study.

All of the $bla_{\text{VIM-1}}$ positive isolates were multi resistant to aztreonam, ceftazidime, ceftriaxone, cefotaxime, ceftizoxime, piperacilin, piperacilin/tazobactam, gentamicin, amikacin, imipenem, ciprofloxacin. Only one isolate showed susceptibility to ciprofloxacin and gentamicin and one isolate was susceptible to meropenem (Table 1).

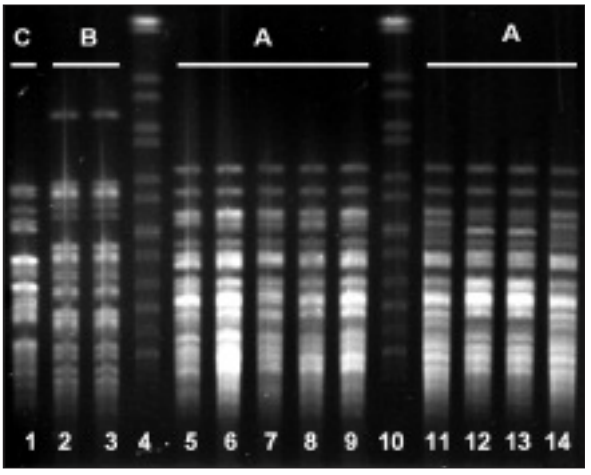

FIGURE 1 - *The PFGE patterns of VIM-1 producing isolates after digestion of genomic DNA with Xba I. Lane 4 and 10: S. braenderup H9812 as molecular weight Marker. lane 1: IM-596, lane 2 and 3: IM-699 and IM-653, lane 5: IM-562, lane 6,7,8,9: IM-527,546,684 and MT-1205. lane 11,12,14: IM-607,613,556. lane 13: IM-549.*

PFGE analysis

The sixteen VIM-1-producing *P. aeruginosa* isolates belong to three different unrelated patterns based on the criteria explained by Tenover *et al.,* (Tenover *et al.,* 1995) (Figure 1). These patterns were allocated as A to C (Table 1). With the exception of an isolate within pattern C (Isolate IM-596), the remaining 15 isolates were resistant to ciprofloxacine and gentamicin. Pattern A also found among at least five isolates lacking $bla_{\text{VIM-1}}$ gene.

DISCUSSION

During the last decade, several MBL enzymes have been identified in *P. aeruginosa*, beginning with IMP-1 and its derivatives, which are widespread in Japan and China (Senda *et al.,* 1996; Chu *et al.,* 2001).

They are also emerging in Europe and Canada (Nordmann *et al.,* 2002; Gibb *et al.,* 2002). The VIM-1 and VIM-2 enzymes are also highly prevalent in Korea (88%) and Greece (85%) (Oh *et al.,*

Table 1 - Phenotypic characteristics and PFGE patterns of 16 P. aeruginosa isolates carrying blaVIM-1 gene.

Name of isolate	*Source of isolate*	*MIC_{CAZ}*	*MIC_{IMP}*	*MEM*	*DDST (EDTA+IMP)*	*PFGE pattern*
IM-527	Tracheal	256	16	R	+	A
IM-546	Wound	256	16	R	+	A
IM-549	Wound	512	16	R	-	A
IM-556	Wound	512	16	R	+	A
IM-562	Wound	64	16	S	+	A
IM-607	Tracheal	16	16	R	+	A
IM-613	Tracheal	16	16	R	-	A
IM-684	Tracheal	16	16	R	-	A
IM-691	Sputum	256	16	R	+	A
IM-694	Tracheal	512	32	R	+	A
IM-695	Tracheal	256	8	R	+	A
IM-702	Tracheal	512	16	R	+	A
MT-1205	Tracheal	32	32	R	-	A
IM-653	Tracheal	32	32	R	+	B
IM-699	Wound	512	32	R	+	B
IM-596	Tracheal	16	32	R	+	C

2003; Giakkoupi *et al.,* 2003). Previous investigations indicated that IMP and VIM types of MBLs are also widespread in Asian countries (Yan *et al.,* 2001, Yatsuyanagi *et al.,* 2004). VIM type is also the most prevalent enzyme in our neighboring country, Turkey (Strateva and Yordanov 2009)

The emergence of multidrug resistant strains of *P. aeruginosa* is a serious concern especially in burns patients who are hospitalized in Tehran (Shahcheraghi *et al.,* 2003).

In this study, *P. aeruginosa* strains (n=610) showed a multidrug resistance phenotype though imipenem, ciprofloxacin and amikacin were most effective antibiotics respectively. Fortunately, in spite of increasing resistance to imipenem in *P. aeruginosa* isolated from other countries, the rate of resistance to imipenem appears not to be high in Tehran hospitals (11%).

This may be due to limitations in the prescription of this carbapenem since it is solely administered in hospitals. In a previous study in Iran (Khosravi and Mihani 2008), like our findings, only bla_{VIM} gene was detected among the isolates and no MBL-producing isolates were positive for bla_{IMP} gene by PCR.

However, the rate of bla_{VIM-1} gene among the imipenem resistant strains tested in this study and resistance of these strains to all tested antibiotics should be considered.

Importantly, these resistant strains showed low level of resistance to imipenem (MIC ≤32 µg/ml) but most of them showed a high level of resistance to ceftazidime (Table 1) and all of them were susceptible to collistin and polymixin B.

Metallo-β-lactamases can hydrolyze all beta-lactams except aztreonam but our study showed that all MBL-producing isolates were resistant to aztreonam.

This could be due to existence of other mechanisms such as ESBL production, efflux pumps

and cephalosporinase hyperproduction (Walsh *et al.,* 2002).

Molecular typing by PFGE disclosed three different unrelated patterns among VIM-1-producing strains.

It also showed that VIM negative *P. aeruginosa* strains may belong to pattern A that was the dominant pattern observed in VIM-1-producing strains (VIM negative data not shown). The results of PFGE also suggest that VIM-1 positive strains were likely derived from a clonal lineage that was already circulating in the nosocomial setting upon acquisition of the $bla_{\text{VIM-1}}$ determinant. As $bla_{\text{VIM-1}}$ is located on mobile elements (Lauretti *et al.,* 1999), dissemination of $bla_{\text{VIM-1}}$ could be due to both movement of resistance genes and clonal dissemination of the resistant strains.

As MBL genes are located on mobile genetic cassettes with other resistance determinants inserted into integrons (Nordmann *et al.,* 2002; Bush, 2001; Livermore and Woodford, 2000), the isolates of *P. aeruginosa* producing these enzymes often exhibit resistance to additional classes of drugs and behave as multi-resistant. Our data also demonstrated that most of VIM-1-producing strains are resistant to aminoglycosides and to fluoroquinolones.

In conclusion, the results obtained in the present study indicated that VIM-1 is the only MBL enzyme to emerge among clinical isolates of *P. aeruginosa* in our country to date. As patients with infections caused by MBL-producing organisms are at an increased risk of treatment failure, rapid identification of these organisms is essential and care should be taken to use imipenem just in critical conditions. So, it is recommended that a simple test based on CLSI recommendation should be adopted to confirm MBL-producing bacteria in clinical laboratories.

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