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VIM-2 β-LACTAMASE IN *PSEUDOMONAS AERUGINOSA* ISOLATES FROM ZAGREB, CROATIA

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ABSTRACT

The aim of this investigation was to characterize MBLs in *P. aeruginosa* isolates from Zagreb, Croatia.

100 *P. aeruginosa* isolates with reduced susceptibility to either imipenem or meropenem were tested for the production of MBLs by E test MBL. The susceptibility to a wide range of antibiotics was determined by broth microdilution method. The presence of bla_{MBL} genes was detected by PCR. Hydrolysis of 0.1 mM imipenem by crude enzyme preparations of β-lactamases was monitored by UV spectrophotometer.. Outer membrane proteins were prepared and analysed by SDS-PAGE.

Six out of 100 isolates were positive for MBLs by E test. All strains were resistant to gentamicin, ceftazidime and cefotaxime and all except of one to imipenem.

Six strains positive for MBLs in E test were identified as VIM MBLs producers by PCR. Sequencing of bla_{VIM} genes revealed the production of VIM-2 β -lactamase in all six strains. This investigation proved the occurence of VIM-2 β-lactamase among *P. aeruginosa* strains from Zagreb, Croatia. VIM-2 β -lactamase with similar properties was previously described in another region of Croatia, Italy, France, Spain, Greece, Taiwan and South Korea suggesting that this type of enzyme is widespread in Mediteranean region of Europe and in Far East.

Key words: meropenem, imipenem, metallo-β-lactamases, resistance, *Pseudomonas aeruginosa*

INTRODUCTION

Carbapenemases may be defined as β-lactamases that significantly hydrolyse at least imipenem or/and meropenem [1]. The most clinically significant carbapenemases belong to class B [2]. They are metallo- enzymes which require zinc as a cofactor and comprise four families: IMP, VIM, SPM, GIM and SIM [3-6]. The VIM-type enzymes appear to the most prevalent in Europe and Korea, and at least 10 different variants have been described [4]. The

43 worldwide spread of acquired metallo-β-lactamases (MBLs) in gram-negative bacilli has
44 become a great concern. MBLs possess a broad hydrolysis profile that includes carbapenems
45 and almost all extended-spectrum β-lactams except of aztreonam. Carbapenem resistant
46 strains of *P.aeruginosa* have been detected with increasing frequency in hospitals in Zagreb
47 and pose serious therapeutic problem. The aim of this investigation was to characterize MBLs
48 in *P. aeruginosa* isolates from Zagreb, Croatia.
49
50 MATERIALS AND METHODS
51 *Bacteria*
52 100 *P. aeruginosa* isolates with reduced susceptibility to either imipenem or meropenem were
53 tested for the production of MBLs by E test MBL (Solna, Sweden). The strains were isolated
54 during 2002 to 2004 at the Clinical Hospital Center Zagreb and University Hospital Merkur
55 in Zagreb from various clinical specimens and wards (Table 1). Isolates were identified by
56 conventional biochemical methods.
57 *E test MBL test*
58 The strip (AB Biodisk, Solna, Sweden) contains a double-sided seven-dilution range of
59 imipenem (4 to 256 mg/L) and imipenem (1 to 64 mg/L) in combination with a fixed
60 concentration of EDTA. An overnight culture of the test strains in Mueller-Hinton (MH) broth
61 was diluted to match the turbidity of 0.5 McFarland, swabbed on the plates of MH agar and
62 the plates were incubated at 35°C. A decrease of imipenem MIC by ≥3 twofold dilutions in
63 the presence of EDTA was interpreted as being suggestive of MBL production [7].
64 *Susceptibility testing*
65 The susceptibility to a wide range of antibiotics was determined by twofold broth
66 microdilution method in cation supplemented MH broth in 96 well microtiter plates according
67 to CLSI [8]. The inoculum size was 5 x10^5 CFU/ml. The plates were incubated for 18 h at 37

°C. The strains with MICs below the resistance breakpoint were tested with higher inoculum of 10^8 CFU/ml as well. The test was performed in triplicate. *P. aeruginosa* ATCC 27853 was used for quality control. Antibiotic powders were obtained from the following manufacturers: ceftazidime, gentamicin and clavulanic acid-Pliva, Zagreb; cefotaxime-Belupo, Koprivnica; cefepime-Bristol Myers Squibb, Zagreb; imipenem-MSD, Zagreb; meropenem-AstraZeneca, Zagreb.

Enzyme characterization

The bacterial cells were pelleted from exponential phase cultures in Lurian-Bentoni (LB) broth by centrifugation at 6000 g. The pellet was washed and resuspended in cation supplemented phospate buffer (0.1 mM, pH 7). Enzymes were released by sonication in ice bath. Cell debris was removed by centrifugation at 10 000 g and supernatant was stored at -20°C as crude enzyme preparation. Hydrolysis of 0.1 mM imipenem by crude enzyme preparations of β-lactamases was monitored by UV spectrophotometer at 298 nm. The change of absorbance was recorded. Inhibition of enzyme activity was determined by 2 mM EDTA. Enzyme activity was expressed as nmol of substrate hydrolyzed per minute expressed relatively to the total protein content of the sample. Concentration of the protein in the samples was determined with a commercial method (BioRad).

Analytical isoelectric focusing (IEF)

IEF was performed according to Matthew et al [9].The organisms were grown overnight in BHI broth, collected by centrifugation and β-lactamases were extracted from bacteria by sonication. Cell debris was removed by centrifugation at 14 000 g. Supernatant was used as crude enzyme. Isoelectric focusing was performed on polyacrylamide gels (acrylamide 7%, bis-acrylamide 0.2%) containing ampholines with a pH range of 3.5 to 10. The β-lactamases were detected by staining of the gel with nitrocefin, following IEF. β-lactamases of known

92 pI (isoelectric point) were used as standards: TEM-1, TEM-2, SHV-1, SHV-2, SHV-4 and
93 SHV-5.
94
95 *Polymerase chain reaction and sequencing of* bla$_{VIM}$ *genes*
96 The presence of *bla*$_{VIM}$ and *bla*$_{IMP}$ genes was tested by PCR. Primers: VIM1F (5'-CAG-ATT-
97 GCC-GAT-GGT-GGT-TGG-3') and VIM1R (5'-AGG-TGG-GCC-ATT-CAG-CCA-GA-3')
98 were used to amplify *bla*$_{VIM}$ genes. whereas primers IMP-A (5-GAA-GGY-GTT-TAT-GTT-
99 CAT-AC-3') and IMP-B (5'-GTA-MGT-TTC-AAG-AGT-GAT-GC-3') [1] were used for
100 detection of *bla*$_{IMP}$ genes. The cycling conditions were as follows: 94°C-5min, and then 30
101 cycles-94°C-1 min, 55°C-1 min, 72°C-1-min and final extension at 72°C for 5 min. PCR
102 products were subjected to electrophoresis in agarose gel at 100 V. PCR products were
103 detected under UV light after staining with etidium bromide.
104 The amplicons were sequenced from both sides.
105 *Plasmid analyis*
106 Plasmids were extracted by alkaline lysis method as described previously [10]. Overnight
107 cultures of the test strains in LB broth (12 ml) were centrifuged at 14 000 rpm and the pellet
108 was resuspended in Solution I (25 mM Tris Hcl (pH 8), 50 mM Glucose, 10 mM EDTA (pH
109 8) containing 5 mg/ml lysozyme and 10 mg/ml RNA-se. The suspensions were mixed and
110 incubated on ice for 10 min before addition of the Solution II (0.2 N NaOH, 1% SDS). The
111 samples were then mixed genty by inversion of the tubes. After incubation on ice for 15 min,
112 Solution III was added (3 M K-Ac, pH 4.8). Solution III contained 120 ml 5 M K-Ac, 23 ml
113 gliacil acetic acid and 57 ml H_20. Samples were again mixed by inversion of the tubes,
114 incubated on ice for 15 min and centrifuged for 15 min at 14 000 rpm at room temperature.
115 The supernatant was transferred to the clean tube avoiding any floating precipitate. Equal
116 volume of phenol/chlorophorm was added, samples were mixed and centrifuged for 5 min.

This step was repeated twice. The supernatant was transferred to the clean tube without touching the water /chlorophorm interface or the precipitate in this region and 0.6 volumes of isopropanol were added. The samples were incubated 30 min at room temperature and centrifuged at 14 000 rpm for 20 min. The supernatant was discarded and the pellet dried in the vacuum centrifuge. The pellet was washed once with 70% ethanol and resuspended in TE buffer. Samples were subjected to electrophoresis in 0.7 % agarose gel in TBE buffer. After staining with ethidium bromide, the DNA was visualised by ultraviolet light.

Genotyping of strains by pulsed-field gel electrophoresis

Isolation of chromosomal DNA was performed as described by Kaufman et al [11]. For each isolate 1,0 ml (optical suspension density 0,6-0,7 at 540 nm) of an overnight culture grown in BHI broth was pelleted by centrifugation at 10 000 rpm for 2 min. After being washed in 1 ml SE buffer (75mM NaCl;25mM EDTA, *Sigma*), bacteria were resuspended in 500µl SE buffer with 10 µl lysosime (*Boehringer Mannheim GmbH*). Next, 500 µl of this bacterial suspension was mixed with 500 µl 2,0% low- melting-temperature agarose (InCert agarose; FMC Bioproducts) and left to solidify. Solid agarose plugs were then incubated for 24h at 56^0 C in 2ml of ESP buffer (1% N-lauril sarcosine; 0,5 M EDTANa2, pH 9,5; 500 µg/ml proteinase K, *Sigma*). After 24h, the plugs were incubated at room temperature for 2 h in PMSF (phenylmethanesulfonyl-fluoride, *Aldrich)* and then washed three times for 30 min at 4^0 C with TE buffer (10mM Tris-Hcl,pH 8, 0,1 mM EDTA, *Sigma*) before macrorestriction with 10U / 1 µl *Xba*I for 3 h at 37^0 C. Restriction fragments of DNA were separated by PFGE with a CHEF-DRIII apparatus (Bio-Rad Laboratories) through 1% pulsed-field certified agarose (*Bio-Rad*) at a field strength of 6 V/cm for 20 h at 11^0 C**;** with pulses from 5 to 50 -s in 0,5 TBE buffer with thiurea (50mM, *Sigma*). A lambda ladder (*Roche*) was used as the molecular size marker. After electrophoresis, gels were stained with ethidium bromide, rinsed, and

142 photographed under UV light. The PFGE patterns were compared following the criteria of
143 Tenover and coleagues for bacterial strain typing [12] and analysed by computer software
144 (*GelComparII*). The patterns obtained were compared by clustering methods (unweighted
145 pairgroup method with arithmetic averages) using the *Dice* coefficient. An optimization of
146 0,50% and position tolerance of 3,00% were applied during the comparison of PFGE
147 fingerprinting patterns.
148
149 *Characterization of outer membrane proteins*
150 Outer membrane proteins of six MBL positive *P. aeruginosa* strains were prepared as
151 described previously [13]. Cells were harvested from overnight cultures in LB broth and
152 resuspended in phosphate buffer. Proteins were released from the cells by sonication in ice
153 bath and collected by centrifugation at 10 000 g. After solubilization in 10 mM Tris HCl , 5
154 mM $MgCl_2$ and to 2% sodium lauroyl sarconisate for for 1 h at 37°C the insoluble OMPs
155 were recovered at 14 000 g. A second solubilization step was performed and the OMPs were
156 again pelleted as above. Prior to SDS PAGE, the OMPs were denatured by the addition of
157 3% SDS-5% β-mercaptoethanol and boiled for 10 min. Laemmli's sample buffer (62.5 mM
158 Tris/Hcl, 2% SDS, 10% glycerol, 5% mercaptoethanol) and electrode buffer (25 mM Tris (pH
159 8.3), 190 mM glycine, 0.1% SDS) were used.
160 Electrophoretic analysis of *P. aeruginosa* OMPs was performed in polyacrylamide gel. The
161 gel contained 11% acrylamide, 0.54% bisacrylamide plus 0.2% SDS in 0.375 M Tris/HCl (pH
162 8.8). Gel was polymerised with 0.2% TEMED and 0.25% ammonium persulfate. Stacking gel
163 contained 4% acrylamide, 0.1% bisacrylamide, 0.1% SDS in 0.125 M Tris /HCl (pH 6.8), and
164 was polymerised as above. Staining was performed with 0.125% Coomassie brilliant blue
165 R250 in 45% methanol, 10% acetic acid for about 30 min. Destaining was performed in 45%
166 methanol, 10% acetic acid.
167

RESULTS

E test

Six out of 100 isolates were positive for MBLs by E test.

Prevalence

The prevalence of MBLs among carbapenem resistant isolates of *P. aeruginosa* was 6%.

Susceptibility testing

All strains were resistant to gentamicin, ceftazidime and cefotaxime. All except of strain (132) were resistant to imipenem. Strains 6, 12, 132 and 135 were resistant to ciprofloxacin, strains 12, 22, 35 and 135 to aztreonam, strains 6, 12, 22 and 135 to cefoperazon and strains 22, 35 and 135 to cefepime. Resistance to piperacillin alone and combined with tazobactam was observed in only one strain (135).

Enzyme characterization

The enzyme activity ranged from6 to 420 nmol/imipenem/min/mg of protein. Carbapenemase activity was almost completely inhibited by 2 mM EDTA. (Table 2) .

IEF revealed a band with the pI of 5.3 in all strains.

Polymerase chain reaction and sequencing of bla_{VIM} *genes*

Six strains yielded an amplicon of 523 bp with primers specific for VIM β-lactamases.

Sequencing of bla_{VIM} genes revealed the production of VIM-2 β-lactamase in all six strains (Fig. 1). No IMP MBLs producers were detected by PCR.

Plasmid analysis

No plasmid DNA was found.

Pulsed field gel electrophoresis

The strains showed distinct PFGE patterns and were not clonally related (Fig. 2).

Characterization of outer membrane proteins

Four of the tested strains lacked altered OmpD2 protein (Table 1).

DISCUSSION

This investigation proved the occurence of VIM-2 β-lactamase among *P. aeruginosa* strains from Zagreb, Croatia. VIM-2 β -lactamase was previously reported from another region in Croatia [14] suggesting that there is a regional spread of this resistance determinant. VIM-2 β -lactamase with similar properties was previously described in Italy [15], France [16], Spain [17], Greece [18], Taiwan [19] and South Korea [20] suggesting that this type of enzyme is widespread in Mediterreanean region of Europe and Far East. VIM-2 is the most prevalent MBL in Taiwan [21].

The fact that all strains displayed similar enzyme activities, but one strain (132) showed markedly lower carbapenem MIC than the others suggests that there are other resistance mechanisms involved in the strains with high carbapenem MIC such as loss of outer membrane proteins detected in four strains (6, 12, 22, 135) or efflux. The strain with low carbapenem MIC probably had higher permeability coefficient or less efficient efflux pump. However, the carbapenem MICs rose above the resistance breakpoint for the strain 132 when a higher inoculum which is more likely to occur in *in vivo* situation, was applied. It is possible that *in vivo* in the presence of a high inoculum, high level resistance arises. Little clinical information is available on the outcome of patients infected with carbapenem-susceptible MBL producing *P. aeruginosa*. There is no concensus on the interpretation and reporting of the antibiograms for the MBL producers. Four strains were resistant to aztreonam contrary to the results obtained from other authors [16-17,22]. Aztreonam is not hydrolyzed by MBLs [1] but resistance could be due to other mechanisms like overproduction of AmpC β–lactamase, coexistace of other β-lactamase or efflux as reported previously [23]. The macrorestriction of the Xba digested chromosomal DNA showed distinct patterns indicating that strains harbouring $\underline{bla}_{VIM}$ genes are not clonally related. Thus it is more likely that the spread of MBL genes is due to the genetic exchange between different clones. The occurence of MBLs was

sporadic. The strains were obtained from various wards in two hospitals during a wide time period.

Since all three attempts to transfer ceftazidime resistance to recipient strain and to isolate plasmid DNA were unsuccessful we can conclude that gene cassettes harbouring *bla*$_{VIM}$ genes were located in the integrons [20] which are inserted in the chromosome and that their dissemination in Croatia is due to the mobilization of the resistance genes.

Our strains harbouring VIM-2 β -lactamase were resistant to all β -lactam antibiotics, aminoglycosides and fluoroquinolones and pose a serious therapeutic problem in our hospitals. The fact that all strains were resistant to gentamicin suggests that *bla*$_{VIM}$ genes are located on integrons containing gene casettes with resistance determinants for aminoglycosides. Since most of the strains were resistant to aminoglycosides and fluoroquinolones as well only toxic compounds such as colistine are left to be administered as a therapeutic agent. The prevalence of MBL positive *P. aeruginosa* among our carbapenem resistant strains of this species is still low (6%) but there is a possibility of horizontal spread of *bla*$_{VIM}$ genes to *Enterobacteriaceae* which are more frequent pathogens. MBLs have already been found in the members of the family *Enterobacteriaceae* [18-19].The difficulties in detecting MBLs producers in the routine laboratories together with the mobile nature of the gene cassettes carrying bla$_{VIM}$ genes facilitates their dissemination. Meropenem has been extensively used in our hospitals and the presence of MBLs among carbapenem resistant *P. aeruginosa* of different genotypes, isolated from different hospitals, although in low frequency, underscores the need for restricted use of carbapenems and their systematic surveillance. Constant and consistent surveillance of the MBL producing strains will be the prime measure to prevent their further dissemination.

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Table1. Epidemiologic characteristics, minimum inhibitory concentrations (MIC) of various antibiotics against MBL positive *P. aeruginosa* strains and alteration of outer membrane proteins.

	Epidemiologic chracteristics of P. aeruginosa strains					MIC (mg/L)[1]												Porins
Strain No	Specimen	Hospital[2]	Unit	PFGE type	Date of isolation (D/M/Y)	IMI	MEM	CAZ	CAZ/CL	CTX	CFP	FEP	AMT	PIP	TZP	GM	CIP	$OMPD_2$
6	urine	A	nephrology	1	15/01/2004	>128	64	16	>128	>128	>128	16	8	64	32	>128	16	-
12	stool	A	haematology	2	14/01/2003	64	32	>128	>128	32	>128	16	>128	64	32	>128	64	-
22	Tracheal aspirate	A	Paediatric ICU	3	15/02/2003	>128	32	32	64	>128	16	64	>128	32	16	64	0.5	-
35	Wound swab	A	oncology	4	25/02/2003	>128	32	16	32	32	4	32	>128	64	32	>128	4	+
132	Tracheal aspirate	B	Surgery ICU	4	09/08/2003	16	8	>128	>128	64	4	16	0.12	32	8	16	32	+
135	Bronchal aspirate	B	Internal ICU	5	07/01/2002	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	-

[1] Abbreviations-IMI-imipenem, MEM-meropenem, CAZ-ceftazidime, CAZ/CL-ceftazidime+clavulanic acid, CTX-cefotaxime, CFP-cefoperazone, FEP-cefepime, AMT-aztreonam, PIP-piperacillin, TZP-Tazobactam/piperacillin, GM-gentamicin, CIP-ciprofloxacin

[2] A- University Hospital Center-Zagreb, B-University Hospital Merkur

Table 2. Hydrolysis rates against imipenem of metallo-β-lactamases produced by *P. aeruginosa* strains.

Strain No	Hydrolysis rate (nmol/min/mg protein)	Hydrolysis rate in the presence of 2 mM EDTA (nmol/min/mg protein)
6	6×10^{-9}	1×10^{-13}
12	9×10^{-9}	1.8×10^{-11}
22	4.2×10^{-8}	9.4×10^{-12}
35	4×10^{-8}	3.8×10^{-12}
132	3.5×10^{-8}	2.7×10^{-10}
135	4×10^{-8}	1.2×10^{-9}