VIM-2 beta-lactamase in Pseudomonas aeruginosa isolates from Zagreb, Croatia

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

2 CROATIA

3

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18   ABSTRACT

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

20   Zagreb, Croatia.

21   100 *P. aeruginosa* isolates with reduced susceptibility to either imipenem or meropenem were

22   tested for the production of MBLs by E test MBL. The susceptibility to a wide range of

23   antibiotics was determined by broth microdilution method. The presence of bla_{MBL} genes was

24   detected by PCR. Hydrolysis of 0.1 mM imipenem by crude enzyme preparations of β-

25   lactamases was monitored by UV spectrophotometer.. Outer membrane proteins were

26   prepared and analysed by SDS-PAGE.

27   Six out of 100 isolates were positive for MBLs by E test. All strains were resistant to

28   gentamicin, ceftazidime and cefotaxime and all except of one to imipenem.

29   Six strains positive for MBLs in E test were identified as VIM MBLs producers by PCR.

30   Sequencing of bla_{VIM} genes revealed the production of VIM-2 β -lactamase in all six strains.

31   This investigation proved the occurence of VIM-2 β-lactamase among *P. aeruginosa* strains

32   from Zagreb, Croatia. VIM-2 β -lactamase with similar properties was previously described in

33   another region of Croatia, Italy, France, Spain, Greece, Taiwan and South Korea suggesting

34   that this type of enzyme is widespread in Mediteranean region of Europe and in Far East.

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

36   *aeruginosa*

37   INTRODUCTION

38   Carbapenemases may be defined as β-lactamases that significantly hydrolyse at least

39   imipenem or/and meropenem [1]. The most clinically significant carbapenemases belong to

40   class B [2]. They are metallo- enzymes which require zinc as a cofactor and comprise four

41   families: IMP, VIM, SPM, GIM and SIM [3-6]. The VIM-type enzymes appear to the most

42   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 5×10^5 CFU/ml. The plates were incubated for 18 h at 37

68 °C. The strains with MICs below the resistance breakpoint were tested with higher inoculum

69 of 10^8 CFU/ml as well. The test was performed in triplicate. *P. aeruginosa* ATCC 27853 was

70 used for quality control. Antibiotic powders were obtained from the following manufacturers:

71 ceftazidime, gentamicin and clavulanic acid-Pliva, Zagreb; cefotaxime-Belupo, Koprivnica;

72 cefepime-Bristol Myers Squibb, Zagreb; imipenem-MSD, Zagreb; meropenem-AstraZeneca,

73 Zagreb.

74 *Enzyme characterization*

75 The bacterial cells were pelleted from exponential phase cultures in Lurian-Bentoni (LB)

76 broth by centrifugation at 6000 g. The pellet was washed and resuspended in cation

77 supplemented phospate buffer (0.1 mM, pH 7). Enzymes were released by sonication in ice

78 bath. Cell debris was removed by centrifugation at 10 000 g and supernatant was stored at -

79 20°C as crude enzyme preparation. Hydrolysis of 0.1 mM imipenem by crude enzyme

80 preparations of β-lactamases was monitored by UV spectrophotometer at 298 nm. The change

81 of absorbance was recorded. Inhibition of enzyme activity was determined by 2 mM EDTA.

82 Enzyme activity was expressed as nmol of substrate hydrolyzed per minute expressed

83 relatively to the total protein content of the sample. Concentration of the protein in the

84 samples was determined with a commercial method (BioRad).

85 *Analytical isoelectric focusing (IEF)*

86 IEF was performed according to Matthew et al [9].The organisms were grown overnight in

87 BHI broth, collected by centrifugation and β-lactamases were extracted from bacteria by

88 sonication. Cell debris was removed by centrifugation at 14 000 g. Supernatant was used as

89 crude enzyme. Isoelectric focusing was performed on polyacrylamide gels (acrylamide 7%,

90 bis-acrylamide 0.2%) containing ampholines with a pH range of 3.5 to 10. The β-lactamases

91 were detected by staining of the gel with nitrocefin, following IEF. β-lactamases of known

4

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$_2$0. 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.

117   This step was repeated twice. The supernatant was transferred to the clean tube without

118   touching the water /chlorophorm interface or the precipitate in this region and 0.6 volumes of

119   isopropanol were added.   The samples were incubated 30 min at room temperature and

120   centrifuged at 14 000 rpm for 20 min. The supernatant was discarded and the pellet dried in

121   the vacuum centrifuge. The pellet was washed once with 70% ethanol and resuspended in TE

122   buffer. Samples were subjected to electrophoresis in 0.7 % agarose gel in TBE buffer. After

123   staining with ethidium bromide, the DNA was visualised by ultraviolet light.

124

125   *Genotyping of strains by pulsed-field gel electrophoresis*

126   Isolation of chromosomal DNA was performed as described by Kaufman et al [11]. For each

127   isolate 1,0 ml (optical suspension density 0,6-0,7 at 540 nm) of an overnight culture grown in

128   BHI broth was pelleted by centrifugation at 10 000 rpm for 2 min. After being washed in 1 ml

129   SE buffer (75mM NaCl;25mM EDTA, *Sigma*), bacteria were resuspended in 500µl SE buffer

130   with 10 µl lysosime (*Boehringer Mannheim GmbH*). Next, 500 µl of this bacterial suspension

131   was mixed with 500 µl 2,0% low- melting-temperature agarose (InCert agarose; FMC

132   Bioproducts) and left to solidify. Solid agarose plugs were then incubated for 24h at 56^0 C in

133   2ml of ESP buffer (1% N-lauril sarcosine; 0,5 M EDTANa2, pH 9,5; 500 µg/ml proteinase K,

134   *Sigma*). After 24h, the plugs were incubated at room temperature for 2 h in PMSF

135   (phenylmethanesulfonyl-fluoride, *Aldrich)* and then washed three times for 30 min at 4^0 C

136   with TE buffer (10mM Tris-Hcl,pH 8, 0,1 mM EDTA, *Sigma*) before macrorestriction with

137   10U / 1 µl *Xba*I for 3 h at 37^0 C. Restriction fragments of DNA were separated by PFGE with

138   a CHEF-DRIII apparatus (Bio-Rad Laboratories) through 1% pulsed-field certified agarose

139   (*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

140   TBE buffer with thiurea (50mM, *Sigma*). A lambda ladder (*Roche*) was used as the molecular

141   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

168   RESULTS

169   *E test*

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

171   *Prevalence*

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

173   *Susceptibility testing*

174   All strains were resistant to gentamicin, ceftazidime and cefotaxime. All except of strain

175   (132) were resistant to imipenem. Strains 6, 12, 132 and 135 were resistant to ciprofloxacin,

176   strains 12, 22, 35 and 135 to aztreonam, strains 6, 12, 22 and 135 to cefoperazon and strains

177   22, 35 and 135 to cefepime. Resistance to piperacillin alone and combined with tazobactam

178   was observed in only one strain (135).

179   *Enzyme characterization*

180   The enzyme activity ranged from6 to 420 nmol/imipenem/min/mg of protein. Carbapenemase

181   activity was almost completely inhibited by 2 mM EDTA. (Table 2) .

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

183   *Polymerase chain reaction and sequencing of* bla$_{VIM}$ *genes*

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

185   Sequencing of *bla*$_{VIM}$ genes revealed the production of VIM-2 β-lactamase in all six strains

186   (Fig. 1). No IMP MBLs producers were detected by PCR.

187   *Plasmid analysis*

188   No plasmid DNA was found.

189   *Pulsed field gel electrophoresis*

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

191   *Characterization of outer membrane proteins*

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

193

194   DISCUSSION

195   This investigation proved the occurence of VIM-2 β-lactamase among *P. aeruginosa* strains

196   from Zagreb, Croatia. VIM-2 β -lactamase was previously reported from another region in

197   Croatia [14] suggesting that there is a regional spread of this resistance determinant. VIM-2 β

198   -lactamase with similar properties was previously described in Italy [15], France [16], Spain

199   [17], Greece [18], Taiwan [19] and South Korea [20] suggesting that this type of enzyme is

200   widespread in Mediterreanean region of Europe and Far East. VIM-2 is the most prevalent

201   MBL in Taiwan [21].

202   The fact that all strains displayed similar enzyme activities, but one strain (132) showed

203   markedly lower carbapenem MIC than the others suggests that there are other resistance

204   mechanisms involved in the strains with high carbapenem MIC such as loss of outer

205   membrane proteins detected in four strains (6, 12, 22, 135) or efflux. The strain with low

206   carbapenem MIC probably had higher permeability coefficient or less efficient efflux pump.

207   However, the carbapenem MICs rose above the resistance breakpoint for the strain 132 when

208   a higher inoculum which is more likely to occur in *in vivo* situation, was applied. It is possible

209   that *in vivo* in the presence of a high inoculum, high level resistance arises. Little clinical

210   information is available on the outcome of patients infected with carbapenem-susceptible

211   MBL producing *P. aeruginosa*. There is no concensus on the interpretation and reporting of

212   the antibiograms for the MBL producers. Four strains were resistant to aztreonam contrary to

213   the results obtained from other authors [16-17,22]. Aztreonam is not hydrolyzed by MBLs [1]

214   but resistance could be due to other mechanisms like overproduction of AmpC β–lactamase,

215   coexistace of other β-lactamase or efflux as reported previously [23]. The macrorestriction of

216   the Xba digested chromosomal DNA showed distinct patterns indicating that strains

217   harbouring bla$_{VIM}$ genes are not clonally related. Thus it is more likely that the spread of MBL

218   genes is due to the genetic exchange between different clones. The occurence of MBLs was

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

220 period.

221 Since all three attempts to transfer ceftazidime resistance to recipient strain and to isolate

222 plasmid DNA were unsuccessful we can conclude that gene cassettes harbouring bla_{VIM} genes

223 were located in the integrons [20] which are inserted in the chromosome and that their

224 dissemination in Croatia is due to the mobilization of the resistance genes.

225 Our strains harbouring VIM-2 β -lactamase were resistant to all β -lactam antibiotics,

226 aminoglycosides and fluoroquinolones and pose a serious therapeutic problem in our

227 hospitals. The fact that all strains were resistant to gentamicin suggests that bla_{VIM} genes are

228 located on integrons containing gene casettes with resistance determinants for

229 aminoglycosides. Since most of the strains were resistant to aminoglycosides and

230 fluoroquinolones as well only toxic compounds such as colistine are left to be administered as

231 a therapeutic agent. The prevalence of MBL positive *P. aeruginosa* among our carbapenem

232 resistant strains of this species is still low (6%) but there is a possibility of horizontal spread

233 of bla_{VIM} genes to *Enterobacteriaceae* which are more frequent pathogens. MBLs have

234 already been found in the members of the family *Enterobacteriaceae* [18-19].The difficulties

235 in detecting MBLs producers in the routine laboratories together with the mobile nature of the

236 gene cassettes carrying $\underline{bla}_{VIM}$ genes facilitates their dissemination. Meropenem has been

237 extensively used in our hospitals and the presence of MBLs among carbapenem resistant

238 *P. aeruginosa* of different genotypes, isolated from different hospitals, although in low

239 frequency, underscores the need for restricted use of carbapenems and their systematic

240 surveillance. Constant and consistent surveillance of the MBL producing strains will be the

241 prime measure to prevent their further dissemination.

242

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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}