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ORIGINAL ARTICLE

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

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Abstract

The aim of this investigation was to characterize metallo-β-lactamases (MBLs) in Pseudomonas aeruginosa isolates from Zagreb, Croatia. One hundred P. aeruginosa isolates with reduced susceptibility to either imipenem or meropenem were tested for the production of MBLs by MBL-Etest. The susceptibility to a wide range of antibiotics was determined by broth microdilution method. The presence of bla_{MBL} genes was detected by polymerase chain reaction (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 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Six out of 100 isolates were positive for MBLs by Etest. All strains were resistant to gentamicin, ceftazidime and cefotaxime, and all except 1 were resistant to imipenem. Six strains positive for MBLs by Etest were identified as VIM MBL-producers by PCR. Sequencing of bla_{VIM} genes revealed the production of VIM-2 β-lactamase in all 6 strains. This investigation proved the occurrence of VIM-2 β-lactamase among P. aeruginosa strains from Zagreb, Croatia. VIM-2 β-lactamase with similar properties has previously been described in another region of Croatia and in Italy, France, Spain, Greece, Taiwan and South Korea, suggesting that this type of enzyme is widespread in the Mediterranean region of Europe and in the Far East.

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 metalloenzymes that require zinc as a cofactor and comprise 4 families: IMP, VIM, SPM, GIM and SIM [3–6]. The VIM-type enzymes appear to be the most prevalent in Europe and Korea, and at least 10 different variants have been described [4]. The worldwide spread of acquired metallo-β-lactamases (MBLs) in Gram-negative bacilli has become a great concern. MBLs possess a broad hydrolysis profile that includes carbapenems and almost all extended-spectrum β-lactams with the exception of aztreonam. Carbapenem-resistant strains of Pseudomonas aeruginosa have been detected with increasing frequency in hospitals in Zagreb and pose a serious therapeutic problem. The aim of this investigation was to characterize MBLs in P. aeruginosa isolates from Zagreb, Croatia.

Materials and methods

Bacteria

One hundred P. aeruginosa isolates with reduced susceptibility to either imipenem or meropenem were tested for the production of MBLs by MBL-Etest (Solna, Sweden). The strains were isolated during 2002–2004 at the Clinical Hospital Centre Zagreb and University Hospital Merkur in Zagreb from various clinical specimens and wards (Table I). Isolates were identified by conventional biochemical methods.

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Table I. Epidemiologic characteristics, minimum inhibitory concentrations of various antibiotics against MBL-positive Pseudomonas aeruginosa strains, and alteration of outer membrane proteins.

Strain No.	Epidemiologic characteristics of P. aeruginosa strains					MIC (mg/l)											Porins
	Specimen	Hospital[a]	Unit	PFGE type	Date of isolation (D/M/Y)	IMI	MEM	CAZ	CAZ/CL	CTX	CFP	FEP	AMT	PIP	TZP	GM	CIP	OmpD2
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	Bronchial aspirate	B	Internal ICU	5	07/01/2002	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	–

MIC, minimum inhibitory concentration; MBL, metallo-β-lactamase; PFGE, pulsed-field gel electrophoresis; 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; ICU, intensive care unit.
[a]A = University Hospital Centre, Zagreb; B = University Hospital Merkur, Zagreb.

MBL-Etest

The strip (AB Biodisk, Solna, Sweden) contains a double-sided 7-dilution range of imipenem (4 to 256 mg/l) and imipenem (1 to 64 mg/l) in combination with a fixed concentration of ethylenediamine tetraacetic acid (EDTA). A decrease of imipenem minimum inhibitory concentration (MIC) by ≥3 2-fold dilutions in the presence of EDTA was interpreted as being suggestive of MBL production [7].

Susceptibility testing

The susceptibility to a wide range of antibiotics was determined by 2-fold broth microdilution method in cation-supplemented Mueller–Hinton broth in 96-well microtitre plates, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [8]. The inoculum size was 5×10^5 CFU/ml. The plates were incubated for 18 h at 37°C. The strains with MICs below the resistance breakpoint were also tested with a higher inoculum of 10^8 CFU/ml. The test was performed in triplicate. P. aeruginosa ATCC 27853 was used for quality control.

Enzyme characterization

The bacterial cells were pelleted from exponential phase cultures in Luria–Bertani broth by centrifugation at 6000 *g*. The pellet was washed and resuspended in cation-supplemented phosphate buffer (0.1 mM, pH 7). Enzymes were released by sonication in an ice bath. Cell debris was removed by centrifugation at 10,000 *g* and the supernatant was stored at −20°C as a 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 min expressed relative to the total protein content of the sample. The concentration of protein in the samples was determined with a commercial method (BioRad).

Analytical isoelectric focusing (IEF)

IEF was performed according to Matthew et al. [9].

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

The presence of bla_{VIM} and bla_{IMP} genes was tested by polymerase chain reaction (PCR). Primers VIM1F (5′-CAG-ATT-GCC-GAT-GGT-GGT-TGG-3′)

and VIM1R (5′-AGG-TGG-GCC-ATT-CAG-CCA-GA-3′) were used to amplify bla_{VIM} genes, whereas primers IMP-A (5′-GAA-GGY-GTT-TAT-GTT-CAT-AC-3′) and IMP-B (5′-GTA-MGT-TTC-AAG-AGT-GAT-GC-3′) [1] were used for the detection of bla_{IMP} genes. The cycling conditions were as follows: 94°C for 5 min, and then 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were subjected to electrophoresis in agarose gel at 100 V. PCR products were detected under UV light after staining with ethidium bromide.

The amplicons were sequenced from both sides.

Plasmid analysis

Plasmids were extracted by alkaline lysis method as described previously [10].

Genotyping of strains by pulsed-field gel electrophoresis

Isolation of chromosomal DNA was performed as described by Kaufman [11]. Pulsed-field gel electrophoresis (PFGE) genotyping of *Xba*I-digested genomic DNA was performed with a CHEF-DRIII system (BioRad); the images were processed using Gel-Compar software, and a dendrogram was computed after band intensity correlation using global alignment with 2% optimization and UPGMA (unweighted pair-group method using arithmetical averages) clustering [12]. The strains were considered to be clonally related if they showed more than 80% similarity of their PFGE patterns.

Characterization of outer membrane proteins

Outer membrane proteins of 6 MBL-positive P. aeruginosa strains were prepared as described previously [13]. Following sonication, membranes were collected by ultracentrifugation at 100,000 *g* for 35 min. Outer membrane proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and gels were stained with Coomassie blue.

Results

Six out of 100 Pseudomonas isolates with reduced susceptibility to carbapenems were positive for MBLs by Etest.

All strains were resistant to gentamicin, ceftazidime and cefotaxime. Four isolates were resistant to ciprofloxacin, 4 to aztreonam, 4 to cefoperazone and strains, 3 to cefepime and 3 to imipenem. Resistance to piperacillin alone and combined with tazobactam was observed in only 1 isolate.

The enzyme activity ranged from 6 to 420 nmol/imipenem/min/mg of protein. Carbapenemase activity was almost completely inhibited by 2 mM EDTA (Table II).

IEF revealed a band with the pI of 5.3 in all strains. 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 6 strains. No IMP MBL-producers were detected by PCR. No plasmid DNA was found. The strains showed distinct PFGE patterns and were not clonally related. Four of the tested strains lacked altered OmpD2 protein (Table I).

Discussion

This investigation proved the occurrence of VIM-2 β-lactamase among P. aeruginosa strains from Zagreb, Croatia. VIM-2 β-lactamase has previously been 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 has previously been 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 the Mediterranean region of Europe and the Far East. VIM-2 is the most prevalent MBL in Taiwan [21].

The fact that all strains displayed similar enzyme activities but 1 strain (132) showed a markedly lower carbapenem MIC than the others, suggests that there are other resistance mechanisms involved in the strains with high carbapenem MICs, such as loss of outer membrane proteins detected in 4 strains (6, 12, 22, 135) or efflux. The strain with a low carbapenem MIC probably had a higher permeability coefficient or less efficient efflux pump. However, the carbapenem MICs rose above the resistance breakpoint for strain 132 when a higher inoculum, which is more likely to occur in the in vivo situation, was applied. It is possible that in vivo, in the presence of a high inoculum, high level

Table II. Hydrolysis rates against imipenem of metallo-β-lactamases produced by Pseudomonas 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}

EDTA, ethylenediamine tetraacetic acid.

resistance arises. Little clinical information is available on the outcome of patients infected with carbapenem-susceptible MBL-producing P. aeruginosa. There is no consensus on the interpretation and reporting of the antibiograms for the MBL producers. Four strains were resistant to aztreonam, in contrast with the results obtained from other authors [16–17,22]. Aztreonam is not hydrolyzed by MBLs [1], but resistance was due to the hyperproduction of AmpC β-lactamase, the coexistence 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 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 occurrence of MBLs was sporadic. Heterogeneity could be explained by the fact that strains originated from 2 different hospitals, from various wards and were collected over a prolonged time period.

Since all 3 attempts to transfer ceftazidime resistance to a 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 cassettes with resistance determinants for aminoglycosides. Since most of the strains were resistant to aminoglycosides and fluoroquinolones as well, only toxic compounds such as colistin remain as possible therapeutic agents. The microbiological finding did not change the empiric therapy. In critically ill patients, empiric therapy is based on carbapenems. 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 MBL producers in 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.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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