GENETIC DIVERSITY OF STRUCTURES SURROUNDING *BLA* GENES IDENTIFIED IN *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES FROM BUCHAREST, ROMANIA

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ABSTRACT

Introduction. *Pseudomonas aeruginosa* (Pae), an opportunistic human pathogen and one of the most important nosocomial pathogens, is responsible for various types of serious infections. This species has an extraordinary ability to become resistant to any of the antibiotics used to treat Gram-negative infections. Extensive studies have documented the role of mobile genetic elements in the horizontal dissemination of antibiotic resistance genes.

Objectives. The aim of this retrospective study was to characterize the genetic structures associated with *bla* genes harboured by 93 Pae ceftazidime (CAZ^R) and imipenem resistant (IMP^R) clinical isolates collected during 2000–2008.

Methods. PCR using bla_{PER-1}, bla_{OXA-2}, bla_{OXA-10}, bla_{VIM} specific primers, followed by DNA sequencing was performed. The occurrence of transposons (Tn*1213*, Tn*402*) and class 1 integrons, including their variable regions structure, was assessed. Clonal relationships of isolates were established by pulsed-field gel electrophoresis (PFGE) analysis and multilocus sequence typing (MLST).

Results. Seventy-three isolates harboured bla_{PER-1} gene residing within transposon genetic structure Tn*1213*-like embedded inside the IS*Pa14* element. The gene cassette arrays of two class 1 integrons, distributed in 67 bla_{PER-1} positive isolates, comprised resistance genes against five antibiotic classes. The genetic context of bla_{VIM-2} gene in five metallo-beta-lactamases producing isolates showed that this gene was located chromosomally, mainly in an unusual class 1 integron flanked by the *tni* module similar to Tn*402* instead of the normal 3'CS end.

Conclusion. Our report documents the need of enhanced surveillance including an attentive study of integrons from Pae isolates, given their capacity for intra- and interspecies spread of multidrug resistance.

Keywords: bla_{PER-1} gene, bla_{VIM-2} gene, class 1 integrons, *Pseudomonas aeruginosa*, gene cassettes.

REZUMAT

Introducere. *Pseudomonas aeruginosa* (Pae), patogen uman oportunist și unul dintre cei mai importanți agenți patogeni nosocomiali, este responsabil de numeroase tipuri de infecții severe. Această specie are o capacitate remarcabilă de a deveni rezistentă la orice antibiotic utilizat în terapia infecțiilor cu bacterii Gram negative. Studii ample au documentat rolul elementelor genetice mobile în diseminarea pe orizontală a genelor de rezistență la antibiotice.

Obiective. Scopul acestui studiu retrospectiv a fost caracterizarea structurilor genetice asociate genelor *bla* identificate într-o colecție de 93 de tulpini Pae rezistente la ceftazidim (CAZ^R) și imipenem (IMP^R) izolate în intervalul 2000-2008.

Metode. S-a utilizat tehnica PCR folosind primeri specifici pentru genele bla_{PER-1}, bla_{OXA-2}, bla_{OXA-10}, bla_{VIM}, urmată de secvențierea ampliconilor. A fost investigată prezența transpozonilor Tn*1213*, respectiv Tn*402* și a integronilor de clasă 1, inclusiv compoziția regiunilor variabile ale acestora. Înrudirea clonală a izolatelor s-a stabilit prin electroforeză în câmp pulsator (PFGE) și MLST (multilocus sequence typing).

Rezultate. Șaptezeci și trei de izolate conțin gena bla_{PER-1} localizată într-un transpozon compus Tn*1213*-like integrat în elementul genetic IS*Pa14*. In regiunile variabile a doi integroni de clasa 1 prezenți în 67 de izolate bla_{PER-1} pozitive au fost identificate gene de rezistență la cinci clase de antibiotice. Analiza contextului genetic al genei bla_{VIM-2} prezentă în cinci izolate Pae producătoare de metalo-beta-lactamaze a arătat că această genă are localizare cromozomală, preponderent într-un integron de clasă 1 atipic, lipsit de regiunea conservată 3'-CS, înlocuită de un modul *tni* similar celui din Tn*402*.

Concluzie. Studiul nostru documentează necesitatea unei supravegheri intensive, care să includă și un studiu atent al integronilor din izolatele Pae, din cauza capacității acestora de a răspândi multipla rezistență la antibiotice atât intra- cât și interspecii.

Cuvinte-cheie: gena bla_{PER-1}, gena bla_{VIM-2}, integroni de clasa 1, *Pseudomonas aeruginosa*, casete genice.

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INTRODUCTION

Pseudomonas aeruginosa (Pae), an opportunistic human pathogen and one of the most important nosocomial pathogens, is responsible for various types of infections, including surgical site infections, urinary tract infections, pneumonia and bloodstream infections.

These bacteria are intrinsically resistant to many antibiotics, generally due to decreased outer membrane permeability combined with constitutive expression of efflux pumps, and are able to produce different virulence factors.

This species has an extraordinary ability to become resistant to any of the antibiotics used to treat Gram-negative infections, as well as the potential to mutate to high-level resistance, mainly through accumulation of mutations affecting multidrug efflux systems, cell permeability, antimicrobial target sites, and expression of the natural β-lactamase AmpC [1-3].

This high-level resistance might emerge even during anti-pseudomonal chemotherapy. On the other hand, resistance to antibiotics such as β-lactams, aminoglycosides, macrolides, trimethoprim and chloramphenicol occurs because of many acquired genes [4].

In particular, resistance to extended-spectrum β-lactams and carbapenems in Pae strains occurs by several mechanisms in concert, being associated, beside non-enzymatic mechanisms, with the presence of several determinants encoding extended-spectrum AmpC β-lactamases (ESAC), class A or class D extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) [5].

Horizontal and vertical transfer of antibiotic resistance genes among the members of the *Enterobacteriaceae* family and *Pseudomonas* genus is mostly due to large broad-host-range plasmids and to the transposons they carry and share with other replicons.

Moreover, unrelated resistance determinants and mechanisms are gathered in single genetic structures, usually mobile elements-borne, that explains the co-selection of resistance phenotypes. A substantial proportion of these genetic determinants is part of small mobile elements known as gene cassettes, normally found integrated at a specific location in a complex genetic element called integron. Integrons are not mobile by their own abilities and structural features. Therefore they are transmitted mainly vertically. Nevertheless integrons are genetic systems for the mobilization of single resistance genes by site-specific recombination. Although integrons belonging to all classes have been found in clinical and environmental isolates of multiresistant bacteria, class 1 integrons have had a major role in the spread of antibiotic resistance in clinical contexts [6].

Class A ESBLs are typically identified in Pae isolates showing resistance to ceftazidime. The extended-spectrum β-lactamase PER-1 was first detected in Turkey (1993) and was found to be widespread mainly among Pae and *Acinetobacter* spp. Since then, PER-type ESBLs have been among the predominant ones reported in a number of studies from several European countries (France, Belgium, Italy, Hungary, Serbia, and Poland) and in Japan, suggesting their ongoing dissemination [7-11]. In Europe, the bla_{PER-1} gene resides within transposon genetic structures Tn*1213*/Tn*4176* or Tn*2345* and have not been identified inside integron structures so far [7, 12-14]. The presence of PER-1-producing Pae isolates in Romania has been already noticed since 2005 (Surdeanu M, personal communication).

The emergence of MBLs in major clinical pathogens was first described in the early 1990s in Japan and is now a matter of global concern (Europe, USA, Brazil, and Australia). The most common and widespread acquired MBLs are still those belonging to IMP and VIM types, which exhibit a worldwide distribution and for which several allelic variants are known [1, 15]. VIM-type enzymes are the most prevalent in Europe. Although originally confined to the Mediterranean countries, VIM-producing Pae strains have lately been detected in northern and eastern Europe, too [16].

The presence of MBLs producing Pae clinical isolates has been monitored in our laboratory since 2001. In 2007 and 2008 were identified the first five VIM-2 producing strains and our aim was to characterise these isolates retrospectively by molecular tools. This retrospective study had also a second aim: to characterise the genetic structures associated with any *bla* genes harboured by 93 Pae ceftazidime (CAZ^R) and imipenem resistant (IMP^R) clinical isolates collected during 2000–2008 and to establish any clonal relationships between the isolates.

MATERIALS AND METHODS

Bacterial strains

The Pae clinical isolates were collected from ten hospitals in Bucharest, during 2000 – 2008. Ninety-three non-duplicate ceftazidime resistant isolates were included in this study. Seventy-four isolates originated from respiratory tract specimens. The remaining 19 isolates originated from blood (4), wound (6), fistula (4), urine (2), urethral secretion (1) and unknown source (2).

Antimicrobial susceptibility testing

Susceptibility testing against 12 antimicrobial agents (ticarcillin, piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, imipenem, meropenem, aztreonam, gentamicin, tobramycin, amikacin, and ciprofloxacin) was previously performed with the disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI). Antimicrobial susceptibility test discs were purchased from Oxoid (Basingstoke, UK). Minimal inhibitory concentrations (MICs) for ceftazidime, imipenem and meropenem were determined with E test (AB Biodisk, Sweden), according to the manufacturer's instructions. The MICs results were interpreted according to guidelines of European Committee for Antimicrobial Susceptibility Testing (EUCAST v.2.0, www.eucast.org/clinical_breakpoints).

Serotyping

The O-antigen serotypes of Pae isolates were determined using slide agglutination method with polyvalent antisera and serotype specific monovalent antisera (Bio-Rad, Marnes-la-Coquette, France), according to manufacturer's instructions.

Detection of the beta-lactamase genes and their adjacent genetic structures

DNA extraction was performed according to the manufacturer's recommendations using High Pure PCR Template Preparation Kit (Roche).

PCR using $bla_{\text{PER-1}}$, $bla_{\text{OXA-2}}$, $bla_{\text{OXA-10}}$ and bla_{VIM} specific primers, followed by DNA sequencing, was performed in order to identify beta-lactamase genes present in Pae isolates [17-21] (Table 1).

The presence of $bla_{\text{PER-1}}$ genes within Tn*1213* was detected by sequential PCR tests for two overlapping regions of this genetic element, using appropriate combinations of specific primers for IS*Pa12*, IS*Pa13*, IS*Pa14* and $bla_{\text{PER-1}}$ gene [7, 12] (Table 1).

PCR amplification of class 1 integrons

Class 1 integrons were detected by PCR using 5'CS and 3'CS primers as described by Levesque *et al.* [22] and genetically mapped using the primers listed in Table 2 and Table 3. Expand High Fidelity PCR System (Roche Diagnostics) was used for all PCR amplifications. PCR with a combination of the 5'-CS primer and a primer designed to detect the *tniC* gene of transposon Tn*5090*/Tn*402* was performed for class 1 integrons that lacked the normal 3'CS end [23]. All PCR products were sequenced to identify gene cassettes and their order in the variable region of the integrons [24, 25] (Table 3). For the sequencing reactions of the long size amplicons custom-designed primers obtained with Promo primer design tool were used [26].

RFLP of class 1 integron 5'CS – 3'CS amplicons

Amplicons were further analysed by RFLP (restriction fragment length polymorphism) technique using several combinations of restriction endonucleases (*Hae*III, *Hin*cII digestion, *Xba*I + *Eco*RI double digestion).

I-*Ceu*I technique: search for the chromosomal location of $bla_{\text{VIM-2}}$ gene

DNA plugs containing total genomic DNA from VIM-producing Pae isolates were digested overnight with I-*Ceu*I restriction enzyme (New England Biolabs), which digests a 26-bp sequence in the *rrn* genes for the 23S large-subunit rRNA [27]. DNA fragments were separated by PFGE. Southern blot hybridization was performed using DIG DNA Labelling Kit and DIG Luminescent Detection Kit for Nucleic Acids (Roche) according to manufacturer's instruction. PFGE profiles of I-*Ceu*I digestion were probed sequentially with a mix of DNA probes for 16S rRNA and 23S rRNA genes, and afterwards with a DNA probe internal to $bla_{\text{VIM-2}}$ gene. Previous attempts to demonstrate the presence of plasmids failed.

Molecular typing

Clonal relationships of the studied isolates were established by pulsed-field

Table 1. PCR primers used to analyze β-lactamase genes

Primer designation	Target gene or region	Sequence (5' - 3')	Purpose(s)	Expected size(s) (bp) of amplicon(s) (corresponding primer in the pair)	Reference
PER 1	bla_{PER-1}	ATGAATGTCATTATAAAAGC	PCR, sequencing	925 (PER 2)	17
PER 2	bla_{PER-1}	AATTTGGGCTTAGGGCAGAA	PCR, sequencing		17
PER-1E	bla_{PER-1}	GCACTGGAACACTAAACTCG	PCR		7
PERC	bla_{PER-1}	ACACAGCTGTCTGAAACCTC	PCR		7
ISPa12.B	ISPa12	GATCTCGCTTTACATTTACC	PCR	1,678 (PER-1E)	12
ISPa14.B	ISPa14	GCCTAATTCGATGCCTTAT	PCR	1,993 (PER-1E)	12
ISPa13.A	ISPa13	TAACCATATGCACTCAACGG	PCR	1,883 (PERC)	12
ISPa14.A	ISPa14	AATCAAATGTCCAACCTGCC	PCR	2,528 (PERC)	12
OPR1	bla_{OXA-10} type (bla_{OXA-74})	GTCTTTCGAGTACGGCATTA	PCR, sequencing	720 (OPR 2)	18
OPR 2	bla_{OXA-10} type (bla_{OXA-74})	ATTTTCTTAGCGGCAACTTAC	PCR, sequencing		18
OXA-2	bla_{OXA-2}	GCCAAAGGCACGATAGTTGT	PCR	701 (OXA-2)	19
OXA-2	bla_{OXA-2}	GCGTCCGAGTTGACTGCCGG	PCR		19
C-oxa2F	bla_{OXA-2}	TTCAAGCCAAAGGCACGATAG	sequencing		20
C-oxa2R	bla_{OXA-2}	TCCGAGTTGACTGCCGGGTTG	sequencing		20
VIMDIA-f	bla_{VIM}-type	CAGATTGCCGATGGTGTTTGG	PCR		21
VIMDIA-r	bla_{VIM}-type	AGGTGGGCCATTCAGCCAGA	PCR		21

gel electrophoresis (PFGE) analysis of macrorestriction patterns of genomic DNA. Genomic DNA was prepared in agarose plugs and digested with *Spe*I restriction enzyme (Promega) as previously described [28]. Electrophoresis was carried out in 1.2% in a CHEF Mapper System (BioRad) in 0.5X Tris-borate EDTA (TBE) running buffer at 12 °C and 6V/cm for 30 hours, with pulse time ranging from 1 to 50s. Computer-assisted analysis of PFGE profiles was performed using Fingerprinting II software (BioRad). A dendrogram was generated by the unweighted-pair group method using arithmetic average linkages (UPGMA) with the band position tolerance set to 1.5%. A cut-off value of ≥80% level of similarity, corresponding to a maximum six-band difference, was used to determine the genetic relatedness among isolates [29].

Multilocus sequence typing (MLST) was performed according to the protocol of Curran *et al*. [30] and the resulting PCR products were sequenced using BigDye Terminator v3.1 Cycle sequencing Kit on ABI 3130 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were determined for both strands and compared to existing sequences in the MLST database (www.pubmlst.org/paeruginosa) for assignment of allelic numbers and sequence types (STs). Clonal complexes were defined as a group of isolates with either identical STs or STs that varied at one or two loci (single- or double-locus variants).

RESULTS AND DISCUSSION

The ninety-three CAZ^R IMP^R Pae clinical isolates comprised 92 isolates resistant to multiple drugs (according to criteria for defining MDR in Pae) including β-lactams, aminoglycosides and ciprofloxacin [31].

Seventy-three out of the 93 CAZ^R IMP^R isolates examined harboured bla_{PER-1} gene, a class A beta-lactamase. The bla_{PER-1} amplification products (925 bp) from all positive isolates were cleaved into four fragments (451/231/158/85 bp) by *Pvu*II restriction enzyme. DNA sequence analysis of the 925 bp amplicon, originated from all 73 isolates, confirmed 100% homology with bla_{PER-1} gene (GenBank acc. no. AY779042.1). Single PCRs detecting class A beta-lactamase encoding genes bla_{VEB} and bla_{GES} were negative for all Pae isolates.

Table 2. Primers designed in this study in order to identify the variable region of class 1 integrons (3300 bp and 3900 bp)

Primer ID	Alias	Sequence (5' - 3')	Target sequence
PSEQ 1	187pae-F	ATGTCAAACGTTGGGCG	*cmlA* gene cassette
PSEQ 2	330pae-R	AACCGAAAAATGAAGGTTGC	*cmlA* gene cassette
PSEQ 3	553pae-F	ATTCAGCTTACGCTGACAAC	*cmlA* gene cassette
PSEQ 4	694pae-R	GAGCGAGGCCCATTGAC	*cmlA* gene cassette
PSEQ 5	919pae-F	GCGTTTCTAGGTTTGGGC	*cmlA* gene cassette
PSEQ 6	1046pae-R	AAGTTCAGGCACTTAACGGG	*cmlA* gene cassette
PSEQ 7	1279pae-F	GCAGTATTGCTTGCCATCAC	*cmlA* gene cassette
PSEQ 8	1405pae-R	CTCGAAGAGCGCCATTG	*cmlA* gene cassette
PSEQ 9	1pae-F	AAACAAAGTTAGGCCGCATG	*aadB* gene cassette
PSEQ 10	305pae-F	GTATGAAATCGCGGAGGC	*aadB* gene cassette
PSEQ 11	482pae-F	ATGCACCTCACTCGGGG	*aadB* gene cassette
PSEQ 12	485pae-R	GCATGCGAGCCTGTAGG	*aadB* gene cassette
PSEQ 13	663pae-F	GTTGGATGAATGCACTTTTG	*aphA*gene cassette
PSEQ 14	1024pae-F	CTGCGACTGAAGCTGGC	*aphA*gene cassette
PSEQ 15	1390pae-F	GATGAGTTCTTCTAAGCGCG	*aphA*gene cassette
PSEQ 16	851pae-R	GAGATCACTTCCGGTACGAG	*aphA*gene cassette
PSEQ 17	1214pae-R	GCATTACCTTGGAAGATGAAG	*aphA*gene cassette
PSEQ 18	1751paeF	TTCACAGTGCAAGTGCGG	unknown ORF
PSEQ 19	1574pae-R	GCGAGTACCGCCCATATC	unknown ORF
PSEQ 20	1933pae-R	CCGCCCTGTTGCTGTAC	unknown ORF
PSEQ 21	2481pae-F	TTACTGGATAGAAGGCAGCC	*oxa2* gene cassette
PSEQ 22	2298pae-R	TCGAAAAATCTGGAACTCATC	*oxa2* gene cassette
PSEQ 23	2660pae-R	ATACGGCCTTCCCAGCC	*oxa2* gene cassette
PSEQ 24	2847pae-F	AGCGCCGGTTACTTCAAC	*gcuD* (ORF D) gene cassette
PSEQ 25	3022pae-R	CTACACAAGGGCCAGAGGC	*gcuD* (ORF D) gene cassette
PSEQ 26	183qac	AAATCCAAGCAATAGCTGCC	*qacF* gene cassette
PSEQ 27	288qac	CGTTCTATTTTCTTGTCTCTCG	*qacF* gene cassette
PSEQ 28	105qac	CTATCCAAGGTCAGCGCAC	*qacF* gene cassette

Sixty-five out of the 73 bla_{PER-1} positive isolates harboured both class D beta-lactamase genes bla_{OXA-2} (amplicon size 701 bp) and $bla_{OXA-10\ like}$ (amplicon size 720 bp). Two isolates out of the 73 PER-1 producing isolates harboured only $bla_{OXA-10\ like}$ gene. RFLP analysis was performed on the amplification products obtained with bla_{OXA-10} specific primers, using restriction enzymes *Hha*I and *Hae*III. The amplicons were cleaved into three fragments (284/240/196 bp) by *Hae*III and other three fragments (534/120/66 bp) by *Hha*I, suggesting the presence of an ESBL-type oxacillinase gene [18]. DNA sequencing of both strands of PCR product identified the presence of bla_{OXA-74} gene, that correlated with appearance of an additional *Hha*I restriction site if compared with *Hha*I restriction profile of bla_{OXA-17}. One of the major concerns for controlling the spread of class D β-lactamase producers was the absence of appropriate phenotypic tests that could contribute to their easy recognition. Unlike for class D β-lactamase producers, there are plenty available tools for the screening and detection of class A ESBLs (clavulanic acid-based synergy tests), class B MBLs (EDTA-based approaches) and class C AmpC enzymes (cloxacillin-based tests).

Table 3. PCR primers for variable regions of integrons harboured by VIM-2 producing Pae isolates

Primer designation	Sequence (5' - 3')	Target sequence	Reference
tniCF	CGATCTCTGCGAAGAACTCG		23
aacA7F	AATGGATAGTTCGCCGCTCG		23
aacA7R	TTCCGGAAGCAGCGTACTTG		23
VIM2-F	ATGTTCAAACTTTTGAGTAAG		24
VIM2-R	CTACTCAACGACTGAGCG		24
vim-1010R	GCGACCAAACACCATCG		this study
aadA2-F	TTCGAACCAACTATCAGAGGTGCTAA		25
aadA2-R	AAAGCGAATAAATTCTTCCAAGTGATCT		25
INPA537F	CAAGAAATAACCCAAAAAATTGG	*oxa4* gene cassette	this study
INPA875F	AAGCAAATCAGGACATAAATATG	idem	this study
INPA1409F	GTTATCCGGCTAAGCGC	*aadA2* gene cassette	this study
INPA1771F	CAGCCCGTCTTACTTGAAGC	*idem*	this study
INPA1375R	TGACTTCTATAGCGCGGAGC	*idem*	this study
TniAF738	AAAACTGCGAGTGCCGG	*tni* complete module	this study
TniAF1592	ACTACTACGCCGATGCGC	idem	this study
TniBF2242	CGACCAACAATGGCAAG	idem	this study
TniBF2609	CTACCTAGCCATCCGCTCC	idem	this study
ORF 6F3288	GATCCATCACGAGCTGGC	idem	this study
ORF 6F3970	ACACAACCCCGAGACGG	idem	this study

Five out of 20 PER-1 negative isolates harboured bla_{VIM-2} gene. These five strains were designated PAE 8.0, PAE 3.3, PAE 5.0, PAE 5.9, and PAE 6.0, respectively. PAE 5.0 harboured also an Ambler class D bla_{OXA-4} gene. This is a retrospective study including strains collected in a period when despite the significant increase in carbapenem resistance in Pae isolates from Romanian hospitals and the significant association of carbapenem resistance with MDR, the prevalence of MBL-producing strains was still very low. Over the last years, completely different results were reported from several regions of Romania, concerning mainly isolates originating from hospitals [32–39].

In all the PER-1 positive isolates, bla_{PER-1} gene was located on a composite transposon Tn*1213*-like, bracketed by two different insertion sequences, IS*Pa12* and IS*Pa13*, embedded inside the IS*Pa14* element, representing its genetic environment (Figs. 1A and 1B).

Another genetic structure surrounding *bla* genes belonged to integron elements. Class 1 integrons, with sizes in the range of 1300-4200 bp, were present in 81 Pae isolates, namely 67 PER-1 positive, 5 VIM-2 positive PER-1 negative, and 9 PER-1 VIM-2 negative Pae isolates.

Among the 73 isolates carrying bla_{PER-1} gene, PCR with class 1 integron conserved sequence (CS) primers 5'-CS and 3'-CS revealed the presence of two integrons of ~ 3300 bp (InA) and ~ 3900 bp (InB) in 65 isolates; the other 2 PER-1 positive isolates harboured only the InA integron. Identical digestion profiles (RFLP) obtained when integron amplicons of the same size were cut with *Hae*III, *Hin*cII restriction enzymes or *Xba*I + *Eco*RI double digested, suggested the presence of the same gene cassettes, and in the same order, within the integron variable region. The variable region of InA class 1 integron harboured a cassette structure consisting of a tandem array of bla_{OXA-74} gene (encoding an extended-spectrum oxacillinase; size – 800 bp**)**, *aac(6')-Ib*-cr like gene (encoding a ciprofloxacin-modifying enzyme, still remaining an aminoglycoside acetyltransferase as well**,** that conferred reduced susceptibility to kanamycin, tobramycin and amikacin; size - 555 bp) and *cml*A7-like gene (encoding an inner

Table 4. Molecular features of the VIM-2 producing Pae isolates

ID isolate	ST	Serotype	bla_{VIM-2} gene	Location	5'CS - 3'CS amplicons	5'CS – *tniC* amplicons
PAE 8.0	233	O11	2 copies	chromosome	4200 bp (InPa8.2)	2200bp (InPa8.1)
PAE 3.3	233	O6	2 copies	chromosome	absent	2200bp (InPa8.1) plus 2 amplicons of ~2600bp;~3000bp
PAE 5.0	233	O6	2 copies	chromosome	~3500 bp (InPa5.1)	2200bp (InPa8.1) plus 2 amplicons of ~2600bp;~3000bp
PAE 5.9	233	O6	2 copies	chromosome	~3500 bp	2200bp (InPa8.1) plus 2 amplicons of ~2600bp;~3000bp
PAE 6.0	235	polyagglutinable	at least one copy	ND	2300 bp (InPa6.1) plus 2 amplicons (~1300bp;~2400bp)	absent

membrane protein that confers resistance to chloramphenicol via active efflux; size – 1260 bp) cassettes, based on PCR mapping and DNA sequencing results (Fig. 2a). The variable region of InB class 1 integron harboured a cassette structure consisting of a tandem array of the following genes: *aadB* (encoding the AAD (2'') enzyme that confers gentamicin, kanamycin and tobramycin resistance; size - 534 bp); *qacF* (encoding a multidrug exporter that mediates quaternary ammonium compounds resistance; size - 333 bp); *aphA* (encoding the APH (3') enzyme that confers kanamycin and netilmicin resistance; size - 795 bp); *oxa-2* (encoding a narrow-spectrum oxacillinase; size - 830 bp); *orfD* with unknown function; size - 291 bp) (Fig. 2b). The remaining six PER-1 positive isolates harboured no class 1 5'CS – 3'CS integrons.

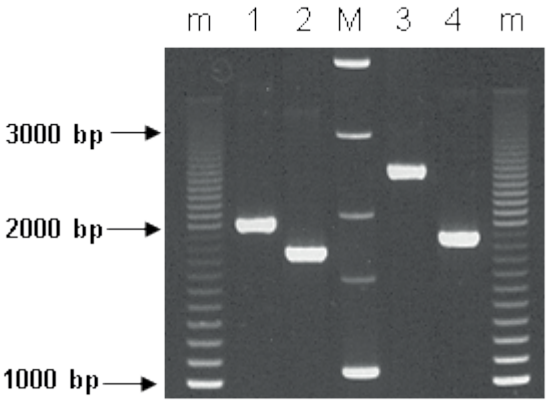

Fig. 1A. Electrophoresis of PCR amplicons obtained with specific primers for Tn*1213* and bla_{PER-1} gene. m, 100 bp DNA Step Ladder (Promega); M, MassRuler Express DNA Ladder Mix (Fermentas)

Among the five VIM-2 positive PER-1 negative isolates, VIM-2 expression was confirmed by analytical isoelectric focusing (data not shown).

Molecular features of the five strains carrying bla_{VIM-2} gene are summarized in Table 4. Four strains carried two bla_{VIM-2} gene copies, chromosomally located. One copy was identified on an unusual class 1 integron, designated InPa8.1, containing the *tni*C gene encoding the resolvase of Tn*5090*/Tn*402*, at the 3'end, adjacent to the variable region of

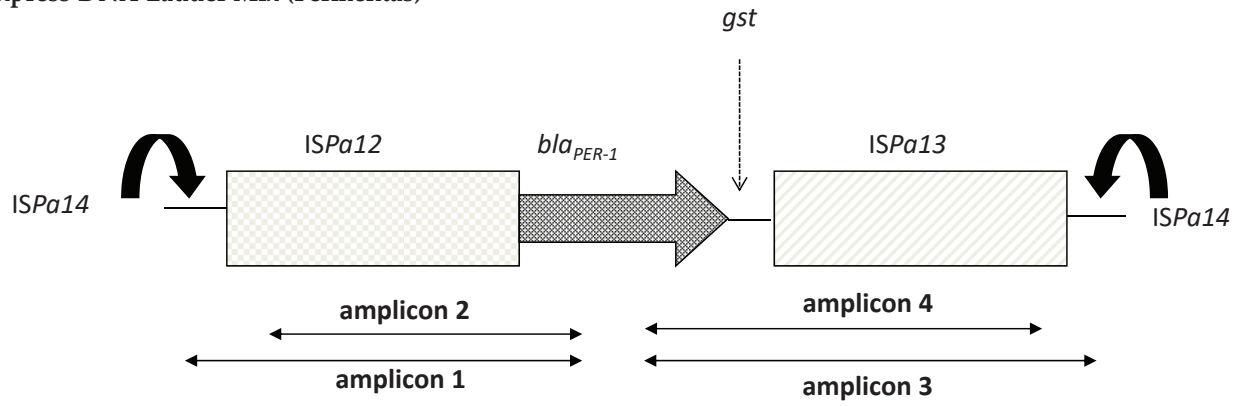

Fig. 1B. Schematic representation of composite transposon Tn*1213* and the corresponding amplicons 1, 2, 3, and 4 (*gst* – glutathione-S-transferase gene)

the integron consisting of gene cassette array: *aacA7* (conferring resistance to tobramycin, netilmicin, amikacin; size - 459 bp) – bla_{VIM-2} (conferring resistance to all β-lactams except aztreonam; size - 801 bp) – *dhfrB* (conferring resistance to trimethoprim; size - 237 bp). PCR linking bla_{VIM-2} to *tniA*, *tniB* and *orf6* confirmed that the whole *tni* module was present, suggesting a functional transposon (Fig. 2c). The cassettes array, identified in our bla_{VIM-2} carrying integron, represents common open reading frames within all the related integrons described in different countries (USA, Russia, Norway, Taiwan, India) [23, 40, 41]. Strain PAE 8.0 harboured additionally a standard class 1 integron InPa8.2 that contained five antibiotic resistance genes, including the second copy of bla_{VIM-2} gene (Fig. 2d). The gene cassettes array consisted of: *aacA7* (conferring resistance to tobramycin, netilmicin, amikacin; size – 459 bp) - bla_{VIM-2} - *dhfrB* - *aacC5* (encoding an aminoglycoside (3) acetyltransferase; size – 477 bp) – *cmlA*.

A 3500 bp class 1 integron carried by the strain PAE 5.0 harboured a variable region consisting of a tandem array of bla_{OXA-4} (encoding a narrow-spectrum oxacillinase – 831 bp), *aadA2* (encoding an aminoglycoside (3'') adenylyl transferase, conferring streptomycin and spectinomycin resistance - 780 bp) and *cmlA6* gene cassettes (Fig. 2e).

The isolate PAE 6.0 was rather difficult to characterize. In this strain, an integron designated InPa6.1, closely related to integron In59 (Genbank acc. no. EU118149) previously reported from France and Greece [42, 43] was identified (Fig. 2f). Additionally, two amplicons of approx. 1300 bp, and 2400 bp were obtained when performing PCR using 5'CS & 3'CS specific primers.

This molecular characterization of genetic environment of bla_{VIM-2} genes, identified in Pae

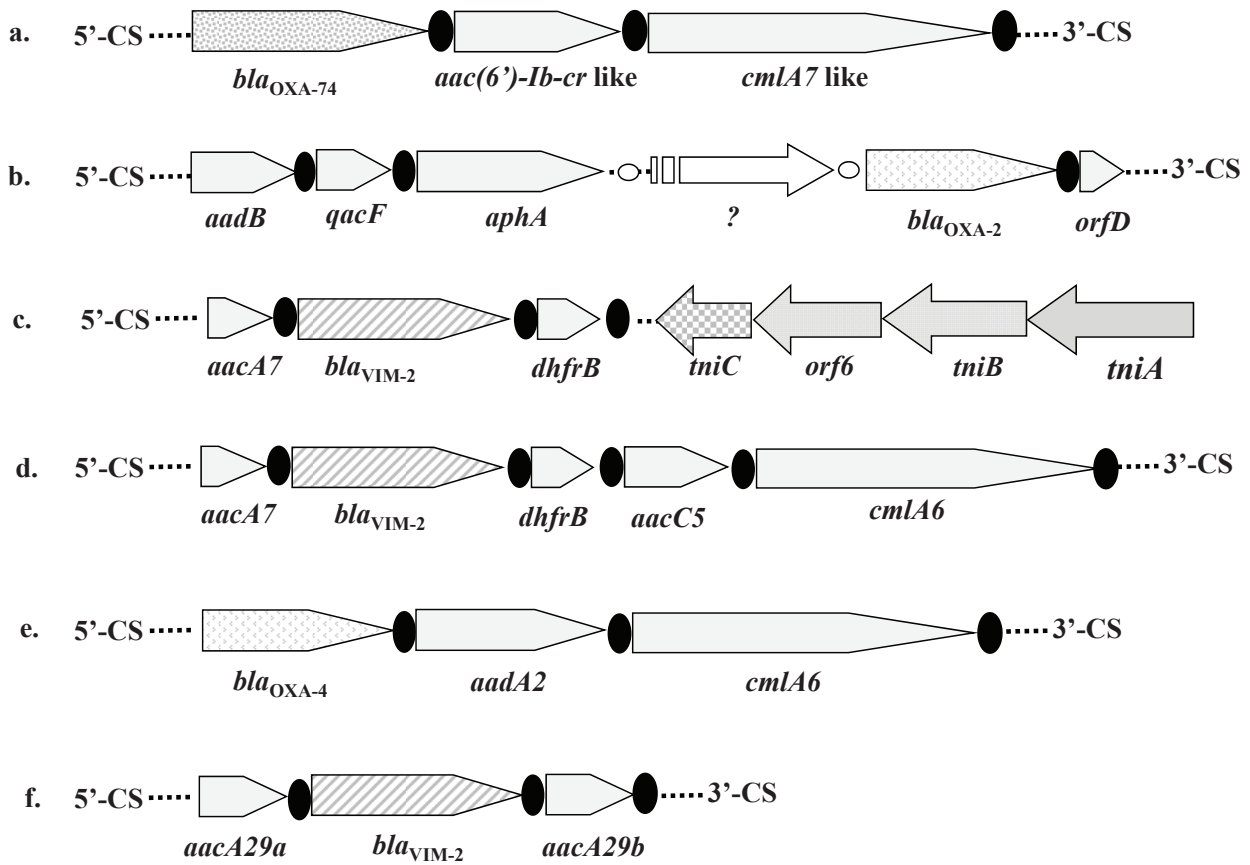

Fig. 2. Diversity of variable region arrangements within the main class 1 integrons found among the examined Pae strains. Schematic representation (not to scale) of: **a**. InA (3300 bp integron) carrying bla_{OXA-74} gene; **b**. InB (3900 bp integron) carrying bla_{OXA-2} gene; **c**. unusual class 1 integron InPa8.1 carrying bla_{VIM-2} gene; **d**. InPa8.2 carrying bla_{VIM-2} gene; **e**. InPa5.1 carrying bla_{OXA-4} gene; InPa6.1 integron related to In59 carrying bla_{VIM-2} gene. Open reading frames of the various resistance genes and the transcriptional orientation are represented by arrows; the *attC* sites (59-base elements) of each individual gene cassette are indicated by filled ovales.

clinical isolates from Bucharest, highlights the epidemiological importance of TniC-like integrons in connection with the global dissemination of bla_{VIM-2}. The finding of a number of class 1 integron structures without 3'CS draws attention to the fact that the frequency of class 1 integrons in clinically important bacterial pathogens is probably underestimated.

Using PFGE analysis, clonally related isolates (Dice coefficient ≥ 80%) could be identified within five clusters (A – E). Four related clusters (A – D) comprised the 67 bla_{PER-1} & class 1 integron (InA ± InB) positive isolates. Cluster E comprised the group of six exclusively bla_{PER-1} positive isolates and was distantly related to clusters A – D. As a general rule, bla_{PER-1} negative isolates exhibited a significant degree of diversity (cluster F). Interestingly, three out of five VIM-2 producing strains (PAE 3.3, PAE 5.0 and PAE 5.9) presented an identical *Spe*I PFGE profile, interspersed among cluster F isolates. For all five VIM-2 producing isolates, a correlation between PFGE analysis profiles and serotyping results was noticed.

The 67 isolates belonging to PFGE clusters A – D were also assessed as belonging to epidemic clone ST235. Within this study five new STs, representing new combinations of known alleles, were identified and submitted to MLST database. They were assigned ST712 (bla_{PER-1} positive isolates, PFGE cluster E) and ST713, ST1028, ST1030 (bla_{PER-1} negative isolates, PFGE cluster F). ST1029 was assigned to a CAZ susceptible IMP^R isolate that was not included in this study eventually. PFGE cluster F heterogeneity correlated with STs diversity of these isolates (*i.e.* ST111, ST235, ST266, ST274, ST621, ST713, ST1028, ST1030). Two international clonal complexes (CC), CC235 and CC111, usually reported as associated mainly with MBL-producing isolates, were dominant among Bucharest circulating strains that lack MBL genes.

The four bla_{VIM-2} and unusual class 1 integron positive isolates belong to ST233, as an isolate reported from Norway and supposed to have been imported from Ghana [44]. The remaining bla_{VIM-2} positive strain belongs to ST235, a sequence type shared by MBL positive and negative isolates from Central and Eastern Europe [16, 45-49].

A complete investigation of the carbapenem resistance in Pae isolates should include several targets: overproduction of chromosomal beta-lactamase AmpC, presence of extended-spectrum cephalosporinases, overexpression of drug efflux pumps, mutational inactivation of porin gene *oprD* and, last but not least, the increased expression of MBLs or ESBLs, with rare cases of co-expression of both A and B Ambler classes [50-53].

Increasing rates of integron-mediated antimicrobial resistance among non-fermenters are a constant threat toward the effectiveness of antibiotics used as last-line therapeutic options. Active surveillance is needed to detect MDR Pae epidemic clones, particularly those carrying integron-borne resistance determinants. As already mentioned, integrons are genetic elements able to capture and express gene cassettes that are usually promoterless, being transcribed from a common promoter (Pc) located within the integrase gene. The effects of differences in Pc strength and of cassette position toward Pc on the level of expression of antibiotic resistance genes are likely to be relevant in the clinical context. The expression level might be lowered because of either a weak promoter or the presence of upstream cassettes or by both. If cassette position within integron might be the factor, under selective pressure of the corresponding antibiotic, such silent genes could be revealed by integrase-mediated rearrangement of the cassettes array, in order to create variants in which the cassette is closer to Pc and thus expressed at higher levels [53].

Only fifteen years ago, the simple detection of the resistance genes associated to certain resistance phenotypes and, at most, their chromosomal or plasmid location represented enough additional information for a complete epidemiological study. Entering the new millennium, a lot of research groups started to examine intensively the genetic context or genetic environment of resistance genes in an attempt to decipher the increasing burden of successful antibiotic resistant clones disseminated worldwide. The diversity of results presented in this retrospective study emphasizes this request.

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