Chapter 2

Genes Encoding Production of Metallo-β-Lactamases in Most Important Gram-Negative Pathogenes

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ABSTRACT

Since about twenty years, following the introduction into therapeutic of news β-lactam antibiotics (broad-spectrum cephalosporins, monobactams and carbapenems), a very significant number of new β-lactamases appeared. The genetic events involved in this evolution are two types: evolution of old enzymes by mutation and especially appearance of new genes coming from bacteria of the environment.

Numerous mechanisms of enzymatic resistance to the carbapenems have been described in some Gram-negative bacteria. The important mechanism of inactivation carbapenems is production metallo-β-lactamases (IMP, VIM, SPM and GIM types). They have reported worldwide but mostly from South East Asia and Europe. Gram-negative bacteria posses metallo-β-lactamases and seem to have acquired them through transmissible genetic elements (plasmids or transposons associated with integron) and can be transmission between different species. Antibiotic resistance genes have become highly mobile since development of antibiotic chemotherapy. The considerable diversity of bacteria and mobile elements in cell has meant that the spread of resistance genes has occurred by all currently known mechanisms for bacterial gene transfer. The dissemination of metallo-β-lactamases genes is thought to be driven by regional consumption of extended-spectrum antibiotics (e.g. carbapenems and/or cephalosporins).

The current situation of antibiotic resistance may announce the end of the antibiotic era.

Keywords: metallo-β-lactamases, genes, Gram-negative bacteria

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imperfect inverted repeat located at the 3' end of the gene called a '59-base element' (59-be) or *attC*, a diverse family of sequences which function as recognition sites for the site-specific integrase. The 59-be varies from 57 bp to 141 bp in length, and their nucleotide sequence similarities are primarily restricted to their boundaries which correspond to the inverse core site (ICS), with the sequence RYYYAAC, and the CS. Furthermore, the ICS of an integrated cassette is always perfectly complementary to the CS located upstream of the gene. Recombination between the bordering CS partners the complementary ICS and CS sequences within the 59-be of the circularized cassette.

The integron platforms are defective for self-transposition but they are often found associated with insertion sequences (ISs), transposons, and/or conjugative plasmids which can serve as vehicles for the intra- and interspecies transmission of genetic material. Integrons (Fig. 1) contain an *intI* gene encoding a site-specific recombinase belonging to the integrase family and a recombination site *attI*. A gene cassette includes an open reading frame (ORF) and, at the 3'-end, a recombination site *attC*. Integration or excision of cassettes occurs by a site-specific recombination mechanism catalyzed by the integrase. The integron integrase is responsible for the integration of circular-resistance gene cassettes. It does this by catalyzing site-specific recombination between the *att* sites on the gene cassette and the plasmid. The gene cassette is mobile and can also exist in free circular form. However, these cassettes do not include all functions required for their mobility. The mobility of cassettes is mediated by the *intI* gene, encoded *IntI*.

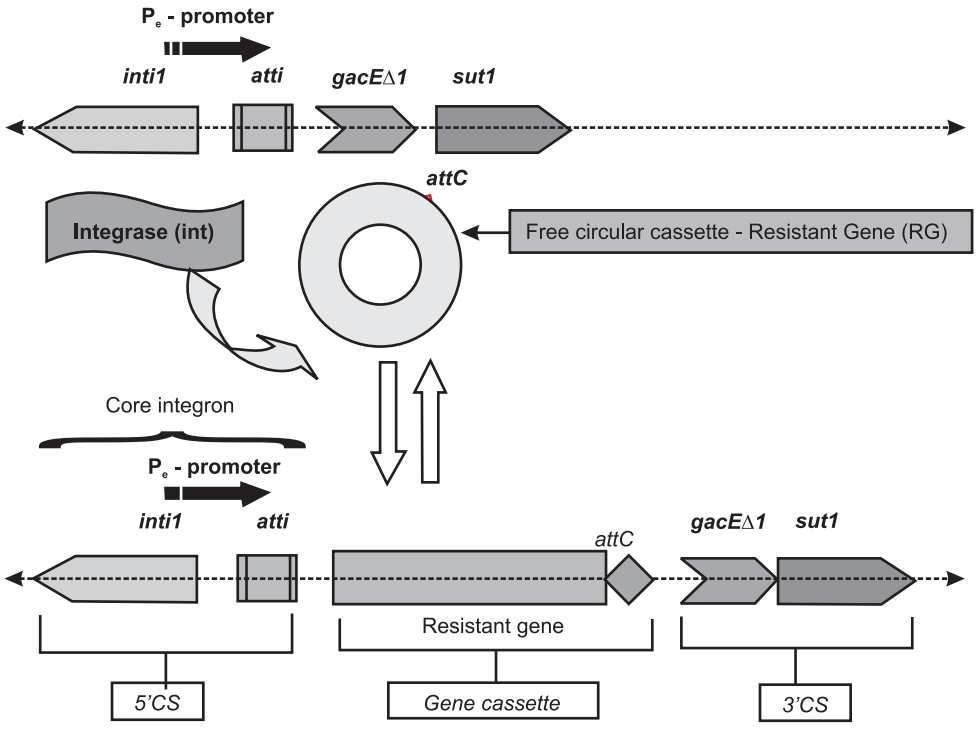

Fig. 1. P_e, promoter for gene cassette expression; *inti1*, integrase 1; *atti*, attachment site (integron); *attC*, attachment site (cassette); *qacEΔ1*, quaternary ammonium compund resistance; *sul1*, sulphonamide resistance.

Cassettes are only formally part of the integron when they are integrated at the integron-receptor site. Insertion can rarely occur, at non-specific sites leading to a stable situation for the cassette. Cassettes are transcribed from a common promoter located in the 5'-conserved segment and expression of distal genes is reduced by the presence of upstream cassettes. Most gene cassettes encode antibiotic resistant determinants (Weldhagen, 2004; Chandler, 2006).

Mobile DNA elements called integron cassettes are major vehicles in such gene exchange (Kovalevskaya, 2002). Integrons are considered to be well-organized vehicles for the transfer of resistance markers in unrelated bacterial populations (Recchia and Hall, 1995; Hall, 1997). Integron structures are naturally occurring gene expression systems that can potentially take into functionally expressed genes (Hall and Collis, 1995). It is these gene cassettes that encode the resistance to several antimicrobial agents (Fluit and Schmitz, 2004). Integrons seem to have a major role in the spread of multidrug resistance in Gram-negative bacteria.

Three broad groups of integrons can be defined based on phylogenetic considerations, which also agree with other characteristics (genetic structure, environmental distribution and/or taxonomic affiliation of the host): (i) the soil and freshwater proteobacteria group, (ii) the inverted integrase group, and (iii) the marine γ-proteobacteria group (Mazel, 2006).

Class 1 integrons have also been identified on transposable elements such as mercury resistance transposon *Tn21* (Grinsted, de la Cruz, Schmitt, 1990). Class 2 integrons are present on transposon *Tn7* and contain a dihydrofolate reductase gene cassette (Fling and Richards, 1983; Stokes and Hall, 1989). Class 3 integrons have recently been characterized in *Serratia marcescens,* isolated in Japan, by the identification of the bla_{IMP} gene, which encodes broad-spectrum β-lactam antibiotic resistance. The bla_{IMP} gene is flanked by a 59-base element and an integrase-like gene and the *aac(6')-Ib* gene, which encodes aminoglycoside resistance (Arakawa *et al.*, 1995). Class 4 integrons have been characterized only in *Vibrio cholerae*. This novel class contains the *intI4* gene, which encodes a new integrase which makes tandem arrays of *Vibrio cholerae* repeated sequences similar to the arrays of antibiotic resistance gene cassettes found in class 1 integrons (Mazel, Dychinco, Webb, Davies, 1998). Class 1, 2 and 3 integrons are most commonly embedded in diverse and highly mobile elements (e.g. various plasmids and transposons) and, thereby, have become broadly distributed amongst the Gram-negative bacteria (Hall *et al.*, 1999) and even, in some cases, to Gram-positive bacteria (Nandi, Maurer, Hofacre, Summers, 2004; Shi *et al*., 2006).

Although integrons belonging to classes 1, 2 and 3 are not close evolutionary relatives, they nonetheless share several important features. They are contained in transposons and plasmids and found to include small in relative terms (containing little more than 100 distinct cassettes) and almost uniquely comprising antibiotic resistance determinants (Recchia and Hall, 1995).

Only one scheme has been openly discussed in the literature and refers to integrons as 'mobile' (also sometimes termed multi-resistant integrons; MR-integrons) or 'super' (also referred to as chromosomal integrons or SI) (Rowe-Magnus *et al*., 1999; Hall and Stokes, 2004; Mazel, 2006).

identified as being closely associated with spread of many antibiotic resistance genes (also for MBL enzymes). IS*CR* elements are powerful genetic tools that can mobilize any gene from any location, without the need for element duplication. IS*CR* elements are likely to be at last of equal importance to transposons and integrons in evolution of antibiotic-resistant bacteria from human and animals. The first IS*CR* element was discovered and reported in early 1990s as a sequence of DNA of 2154 bp, incorporating *orf513* (a putative gene of unknown function) inserted beside the *sul1* genes of class 1 integrons, In*6* and In*7* (Stokes, Tomaras, Parsons, Hall, 1993). They can be divided into two groups: IS*CRs*1 form complex class 1 integrons and IS*CRs*2 to 12 are those associated with other type integrons (Fig. 2 and Fig. 3).

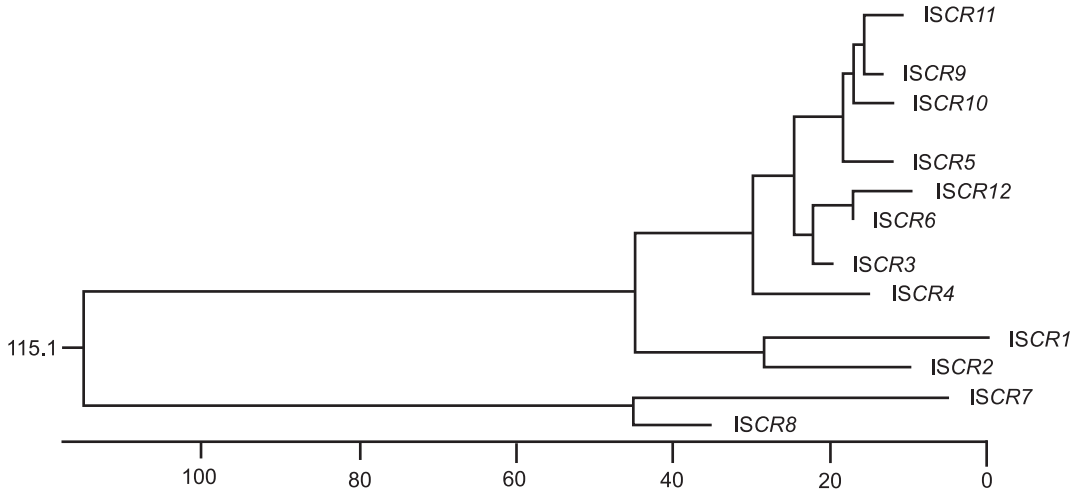

Fig. 2: Nucleotide substitution.

ISCR1	ISCR2	ISCR3	ISCR4	ISCR5	ISCR6	ISCR7	ISCR8	ISCR9	ISCR10	ISCR11	ISCR12	
	62.8	51.0	51.5	50.6	42.3	21.8	26.4	51.5	51.0	49.4	40.2	ISCR1
		55.4	57.1	55.8	44.6	21.9	27.9	54.1	55.4	52.8	46.8	ISCR2
			76.1	88.9	69.1	23.5	28.0	86.8	87.7	83.1	70.0	ISCR3
				76.4	58.2	23.6	27.0	75.5	77.2	74,3	61.2	ISCR4
					64.2	22.6	28.0	94.7	92.2	88.9	64.2	ISCR5
						25.3	29.7	84.6	85.2	81.3	72.0	ISCR6
							62.5	23.4	23.0	21.8	17.7	ISCR7
								24.9	23.8	22.8	18.1	ISCR8
									95.9	92.6	63.2	ISCR9
										90.1	64.9	ISCR10
											66.8	ISCR11
												ISCR12

Fig. 3: Per cent of sequence identity.

IS*CR* elements were detected in *Pseudomonas aeruginosa* and many other strains of bacteria (Toleman, Bennett, Walsh, 2006b). For example, IS*CR2* was discovered in a *P. aeruginosa* isolated in Brazil that harbored the MBL gene bla_{IMP-1} and IS*CR3* was discovered in two of *P. aeruginosa* strains isolated in Italy that harboured the MBL gene bla_{VIM-1}. The gene encoding other metallo-β-lactamase SPM-1 is associated with two different types of IS*CR* elements. The gene bla_{SPM-1} is not part of a gene cassette, nor is it found in the vicinity of class 1 integron as found in others metallo-β-lactamases genes. This element was first detected by its transposase, gene previously named *orf495* (Poirel, Magalhaes, Lopes, Nordmann, 2004a). The gene is located beside the IS*CR* variant IS*CR4*. The strain of *Pseudomonas aeruginosa* that produces the MBL SPM-1 carries two IS*CR* elements, IS*CR4* and a new element, IS*CR12* (Toleman *et al.*, 2002). Other IS*CR* element, IS*CR11* was discovered in two *Acinetobacter baumannii* isolates from Germany that have the MBL bla_{VIM-2} and in *Pseudomonas aeruginosa* isolate from Greece that produces the MBL VIM-1 (Toleman, Bennett, Jones, Walsh, 2004).

Clinically, the most worrying aspect of IS*CR* elements is that they are increasingly being linked with more with more potent examples of resistance, i.e., metallo-β-lactamases in *Pseudomonas aeruginosa* and other Gram-negative specimens.

2. METALLO-β-LACTAMASES – BIOCHEMICAL CHARACTERIZATION

Metallo-β-lactamases, based on functional characteristics, are classified as group 3 enzymes (Bush, Jacoby, Medeiros, 1995) because they can hydrolyze all β-lactam antibiotics (penicillins, cephalosporins and carbapenems) except monobactams. These enzymes are not susceptible to the commercially available β-lactam inhibitors. MBLs also require divalent cation(s), primarily zinc, for activity and are thus inhibited by metal chelators such as EDTA. According to Ambler (1980), they are classified as molecular class B enzymes. The metallo-enzymes are further categorized into three functional subgroups, B1, B2 and B3, based on metal requirements (Galleni *et al*., 2001; Hall, Salipante, Barlow, 2004).

In subclass B1, which includes most metallo-β-lactamases, Zn1 is tightly coordinated and Zn2 is loosely coordinated (Fabiane *et al*., 1998). The B2 functional subgroup possesses two zinc sites each with similar binding affinity (Crowder, Wang, Franklin, Zovinka, Benkovic, 1996). In contrast, the B3 subgroup has a Zn1 site that tightly binds zinc and is sufficient for maximal enzymatic activity; however, the binding of Zn2 drastically reduces the activity of the enzymes in this group (Hernandez, 1997; González, Martín, Costello, Tierney, Vila, 2007). Although there is structural homology between subclass B1 + B2 and subclass B3 (Hall, Salipante, Barlow, 2003), there is no detectable sequence homology between members of subclass B1+B2 and members of subclass B3.

2.1. Structural Components and Catalytic Mechanism

Despite low sequence similarity between various metallo-β-lactamases, the general tertiary structure of these enzymes is very similar (Fig. 4). They all possess a αbeta/betaβ sandwich structure composed of two beta-sheets at the core and five α-helices on the external faces (Wang, Fast, Valentine, Benkovic, 1999b).

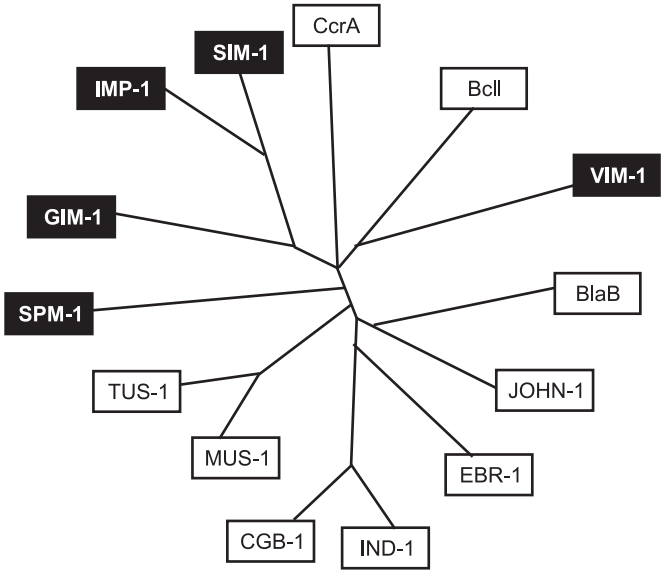

Fig. 5:

protein from *Chryseobacterium meningosepticum*, and the FEZ-1 enzyme from *Legionella gormanii* (Galleni *et al.*, 2001).

The genes coding for class B enzymes from *Bacillus cereus*, *Bacteroides fragilis*, *Stenotrophomonas (Xantomonas) maltophilia*, *Chryseobacterium meningosepticum* and *Aeromonas hydrophila* are chromosomal. The bla_{IMP} and bla_{VIM} genes can be plasmid-borne or chromosome-borne and are often found on integron elements that facilitate dissemination by horizontal transfer (Laraki *et al.*, 1999b; Lauretti *et al.*, 1999). The production of these enzymes is primarily constitutive, but it may also be inducible. For example, Alksne and Rasmussen (1997) showed in *Aeromonas jandaei* AER14 that an increase in imipenem hydrolysis, due to increased activation of the AsbM1 class B enzyme expression, occurred in the presence of the gene *blrA*. The *blrA* gene product, a potential response regulator of a two-component system, is believed to play a role in the induction of β-lactamase synthesis in *Aeromonas jandaei* (Alksne and Rasmussen, 1997). The production of *Aeromonas hydrophila* CphA is also induced by imipenem (Segatore, Massidda, Satta, Setacci, Amicosante, 1993).

3. IMP TYPE METALLO-β-LACTAMASES

Production of metallo-β-lactamases represents an important mechanism of resistance to carbapenems and other β-lactam antibiotics. The IMP enzymes (active on **IMiP**enem) are enough well get to known and described groups of MBL family. Currently have been described many variants of these enzymes (Table 1).

Table I. Origin, bacterial hosts of the IMP-type metallo-β-lactamases and co-occurring genes of resistance

Enzyme	Organism	Accession number	Country (year)*	Co-occurring genes of resistance **	Genes location	Reference
IMP-1	*Serratia marcescens*	S71932	Japan (1988)	aac(6')-Ib, bla_{TEM-1}	class 3 integron (chromosome)	(Osano *et al*., 1994; Arakawa *et al*., 1995)
	Pseudomonas aeruginosa	-	Japan (1991)	[??]	(plasmid)	(Watanabe *et al*., 1991)
	Pseudomonas aeruginosa	-	Japan (1991-1996)	[??]	[??]	(Hirakata *et al*., 1998)
	Serratia marcescens	-		[??]		
	Citrobacter freundii	-		[??]		
	Pseudomonas stutzeri	-		[??]		
	Alcaligenes (Achromobacter) xylosoxidans	-		[??]		
	Pseudomonas aeruginosa	-	Japan (1992-1994)	aac(6')-Ib	(plasmid)	(Senda *et al*., 1996b)
	Klebsiella pneumoniae	-	Singapore (1996)	[??]	(plasmid)	(Koh *et al*., 1999)
	Pseudomonas aeruginosa	AJ223604	Japan (1998)	aacA4, catB6, qacE2, qacED1, sul1	class 1 integron (In101; pPAM-101)	(Laraki *et al.,* 1999
	Acinetobacter baumannii	-	Italy (1999)	[??]	[??]	(Cornaglia *et al*., 1999)
	Serratia marcescens	-	Australia (2001)	[??]	class 3 integron	(Collis *et al*., 2002)
	Serratia marcescens	AB070224	Japan (2001)	aac(6')-Ib, bla_{TEM}	class 3 integron	(**)
	Pseudomonas aeruginosa	-	China (2001)	[??]	[??]	(Wang & Mi, 2004)
IMP-1	*Acinetobacter junii*	AY055216	United Kingdom (2002)	[??]	[??]	(Tysall *et al*., 2002)
	Pseudomonas aeruginosa	AY168635	Singapore (2002)	[??]	class 1 integron	(Koh *et al*., 2004a)

Table I. Contd.

Enzyme	Organism	Accession number	Country (year)*	Co-occurring genes of resistance **	Genes location	Reference
	Pseudomonas aeruginosa	-	South Korea (2008)	[??]	class 1 integron (plasmid)	(Nho *et al.*, 2008)
IMP-2	*Acinetobacter baumannii*	AJ243491	Italy (2000)	aacA4, aadA1	class 1 integron (In42) (chromosome)	(Riccio *et al.*, 2000)
	Serratia marcescens	AB182996	Japan (2004)	[??]	[??]	(**)
IMP-3	*Shigella flexnerii*	AB010417	Japan (1998)	[??]	(plasmid pMS390)	(O'Hara *et al.*, 1998)
IMP-4	*Acinetobacter baumanniii*	AF244145	Hong Kong (2000)	[??]	[??]	(Chu *et al.*, 2001)
	Acinetobacter baumanniii	AF445082	Honk Kong (2001)	qacG, aacA4, catB3	class 1 integron	(Woodford & Livermore, 2001); (Houang *et al.*, 2003)
	Citrobacter youngae	AF288045	China (2001)	[??]	(plasmid pOZ172)	(Hawkey *et al.*, 2001)
	Pseudomonas aeruginosa	DQ297664	China (2005)	[??]	[??]	(**)
IMP-4	*Klebsiella pneumoniae*	AJ609296	Australia (2005)	qacG2, aacA4, catB3	class 1 integron (plasmid pJIBE401)	(Thomas *et al.*, 2005)
	Serratia marcescens *Klebsiella pneumoniae* *Escherichia coli* *Enterobacter cloacae*	- - - -	Australia (2005)	[??] [??] [??] [??]	class 1 integron	(Peleg *et al.*, 2005)
	Klebsiella pneumoniae	AJ609296	Australia (2005)	qacG2, aacA4, catB3, qnrB2, qacEΔ1, sul1	(plasmid pJIBE401; IS26)	(Thomas *et al.*, 2005); (Espedido *et al.*, 2008)
	Acinetobacter calcoaceticus	DQ307573	Malaysia (2005)	[??]	[??]	(**)
	Acinetobacter junii	-	Australia (2006)	bla_{OXA-58}	[??]	(Peleg *et al.*, 2006b)

Table I. Contd.

Enzyme	Organism	Accession number	Country (year)*	Co-occurring genes of resistance **	Genes location	Reference
	Klebsiella pneumoniae	AY762325	Australia (2006)	bla_{OXA-73}	(plasmid pJIBE401)	(**)
	Acinetobacter baumanniii	DQ532122		qacG2, aacA4, catB3, bla_{OXA-58}		
	Acinetobacter baumanniii	AY795963	Singapore	[??]	class 1 integron	(Koh *et al*., 2007)
	Acinetobacter baumanniii	AY590475	(2007)	[??]		
	Klebsiella pneumoniae	EU368858		[??]	[??]	
	Citrobacter freundii	EU368857	China (2007)	[??]	[??]	(**)
IMP-5	*Acinetobacter baumannii*	AF290912	Portugal (2002)	[??]	[??]	(Da Silva *et al*., 2002)
	Pseudomonas aeruginosa	-	Portugal (2006)	[??]	class 1 integron (In76)	(Brízio *et al*., 2006)
IMP-6	*Serratia marcescens*	AB040994	Japan (1996)	[??]	(plasmid pKU502)	(Yano *et al*., 2001)
	Pseudomonas aeruginosa	AB188812	Japan (2004)	aacA4, aadA1, bla_{OXA-2}	class 1 integron	(**)
	Pseudomonas aeruginosa	EU117233	South Korea (2007)	aacA4, bla_{OXA-1}, aadA,qacED1, su11	class 1 integron (plasmid pKM0509)	(**)
	Pseudomonas aeruginosa	EU541448	South Korea (2008)	[??]	(plasmid pKMP0701)	(**)
IMP-7	*Pseudomonas aeruginosa*	AF318077	Canada (2002)	aacC4, aacC1	class 1 integron (transposon; InAB1)	(Gibb *et al*., 2002)
	Pseudomonas aeruginosa	AF416736	Malaysia (2002)	[??]	(chromosome?)	(Ho *et al*., 2002)
	Pseudomonas aeruginosa	AY625685	Singapore (2004)	accC1	class 1 integron	(Koh *et al*., 2004a)
	Pseudomonas aeruginosa	EF601914	Slovak Republic (2007)	[??]	class 1 integron	(Ohlasova *et al*., 2007)

Table I. Contd.

Enzyme	Organism	Accession number	Country (year)*	Co-occurring genes of resistance **	Genes location	Reference
	Pseudomonas aeruginosa	AJ628135	Italy (2005)	aacA4	class 1 integron (Transposon; Tn5051) chromosome?	(Pagani *et al*., 2005)
IMP-14	*Pseudomonas aeruginosa* *Pseudomonas aeruginosa*	AY553333 AY553332	Thailand (2004)	dhfr, aac(6')	class 1 integron	(**)
	Pseudomonas aeruginosa	-		aacA4, aadA1	class 1 integron	(Mendes *et al*., 2003)
IMP-15	*Pseudomonas aeruginosa*	AY553333	Thailand (2004)	dhfr, aac(6')	class 1 integron	(**)
	Pseudomonas aeruginosa	EF184217	Mexico (2006)	aadA6, qacEΔ1	class 1 integron (In97)	(**)
	Pseudomonas aeruginosa	EF184216	Mexico (2006)	aacA7, aadA7, qacH	class 1 integron (In95)	(Garza-Ramos *et al*., 2008a)
IMP-16	*Pseudomonas aeruginosa*	AJ584652	Brazil (2002)	aac(6')-30/aac(6')-Ib', aacA4, aadA1, qacEΔ1, sul1	class 1 integron (chromosome)	(Mendes *et al*., 2004b)
IMP-17	-	Assigned	-	-	-	-
IMP-18	*Pseudomonas aeruginosa*	AY780674	United States (2006)	[??]	[??]	(Hanson *et al*., 2006)
	Pseudomonas aeruginosa	EF184215	Mexico (2008)	aacA1	class 1 integron (In96)	(Garza-Ramos *et al*., 2008b)
IMP-19	*Acinetobacter baumannii*	AB184977		[??]		
	Pseudomonas aeruginosa	AB184976	Japan (2003)	[??]	class 3 integron	(Shibata *et al*., 2003)
	Pseudomonas putida	AB201265		[??]	[??]	
	Enterobacter cloacae	AB201264	Japan (2005)	[??]	[??]	(**)
	Achromobacter xylosoxidans	AB201263		[??]	[??]	
	Aeromonas punctata (*Aeromonas caviae*)	EF118171	France (2007)	aacA4	class 1 integron (plasmid pJDB2)	(Neuwirth *et al*., 2007)
IMP-20	*Pseudomonas aeruginosa*	AB196988	Japan (2004)	[??]	[??]	(**)

Table I. Contd.

Enzyme	Organism	Accession number	Country (year)*	Co-occurring genes of resistance **	Genes location	Reference
IMP-21	*Pseudomonas aeruginosa*	AB204557	Japan (2005)	[??]	[??]	(**)
IMP-22	*Pseudomonas fluorescens*	DQ361087	Italy (2006)	[??]	class 1 integron	(Pellegrini *et al*., 2007)
IMP-23	-	Assigned	-	-	-	-
IMP-24	*Serratia marcescens*	EF192154	China (2006)	[??]	[??]	(**)
IMP-25	*Pseudomonas aeruginosa*	EU588392	China (2008)	aacA4, bla_{OXA-30}, catB3, qacEΔ1, sul1	class 1 integron (plasmid In786)	(**)

NOTE

* Year isolation or published date; ** according to reference or GenBank [Available at: *http://www.ncbi.nlm.nih.gov/Genbank/index.html*. Accessed June 2, 2008]; (**) data only in GenBank; [??] - no data; ? - no precise data.

aac, aac(6')-30/aac(6')-Ib, aac(6')-31, aac(6')-Iae, aac(6')-Ib, aacA4, aacA7, aacC1, aacC4 -aminoglicosyde acetyltrasnferase genes; *aadA, aadA1, aadA5, aadA6, aadA7, aadB* -aminoglycoside adenyltransferase genes; bla_{OXA-1}, bla_{OXA-2}, bla_{OXA-10}, bla_{OXA-30}, bla_{OXA-52}, bla_{OXA-58}, bla_{OXA-73} -OXA type β-lactamase genes; bla_{TEM-1} -TEM-1 β-lactamase gene; *catB3, catB4, catB6, catB8* -chloramphenicol acetylotransferase genes; *cm1A* -chloramphenicol transporter gene; *dfr23*-dihydrofolate reductase gene; *qacED1, qacE2, qacG, qacG2, qacH* -quaternary ammonium compound-resistance protein genes; *qnrB (qnrS), qnrB2* -quinolone resistant determinant group genes; *sul1* -dihydropteroate synthase (confers resistance to sulphonamides).

3.1. IMP-1

The IMP-1 enzymes was the first carbapenemase identified as source of acquired resistance to carbapenems in *Serratia marcescens* isolate in 1991, in Japan (Osano *et al*., 1994). The bla_{IMP} gene, which encodes the IMP-1 enzymes, was the first metallo-β-lactamase determinant to be identified as part of gene cassettes inserted into chromosomal or plasmid born integrons. The same bla_{IMP-1} gene has also been characterized from many species of bacteria in Japan and some countries in Europe (Tysall *et al*., 2002; Cornaglia *et al*., 1999) and America (Lincopan *et al*., 2005).

Genetic analysis of bla_{IMP-1} environment revealed typical features of integron-located genes, in particular cassette boundaries known as core sites and inverse core sites. The bla_{IMP-1} gene cassette was found to be inserted into different classes of integrons. Arakawa *et al*. (1995) found an integron-like element carrying the bla_{IMP} gene cassette which was quite different (61% amino acid identity) from class 1 integron.

This was the first description of a class 3 integron in which bla_{IMP} was associated with an *aac(6')-Ib* gene cassette encoding aminoglycoside resistance. Integron was localized on a large plasmid.

In another studies (Laraki *et al*., 1999b), bla_{IMP-1} gene was located on a 36-kb plasmid and was part of a gene cassette inserted into a class 1 integron, In*31*, in *Pseudomonas aeruginosa*. In*31* belongs to a group of defective transposon derivatives that originated from Tn*402*-like ancestors such as In*0*, In*2* and In*5* (Fig. 6). It comprises four additional gene cassettes (*aacA4, catB6, orfN,qacG*).

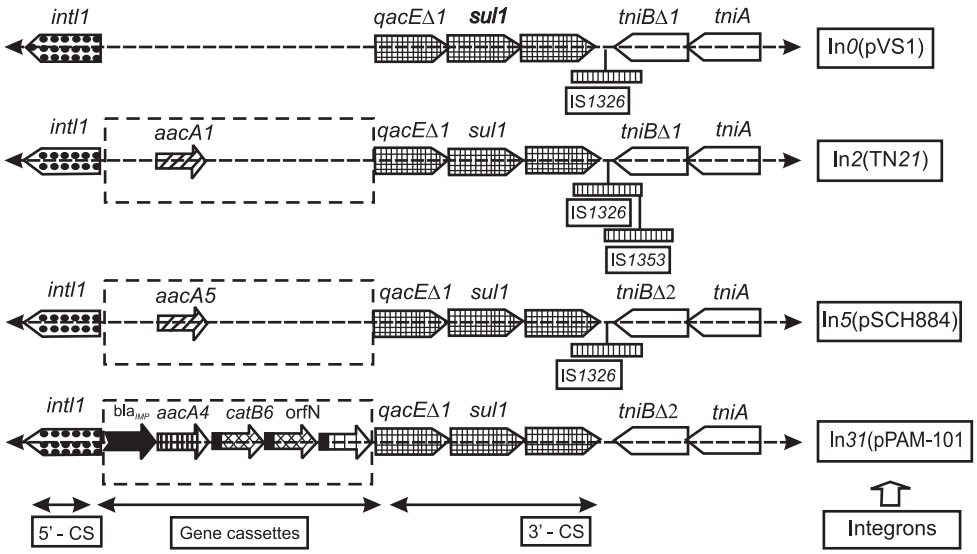

Fig. 6: *intI1*, intil1 DNA integrase: qacEΔ1, QacEdelta1 multidrug exposter (disinfectant resistance protein and ethidium bromide resistance protein); *sul1*, sul1 dihydropteroate synthase; *IS*, insertion element; *tniA*, TniA protein; *tniBΔ1*, TniBD1 protein; tnibD2, TniBD2 protein; *aacA1*, aminoglycoside 6'-N-acetyltransferase; *aacA4*, 6'-N-aminoglycoside acetyltransferase type II; *aacA5*, aminoglycoside acetyltransferase; *catB6*, chloramphenicol acetytransferase variant *orftl*, putative group 4 glycosyl transferase.

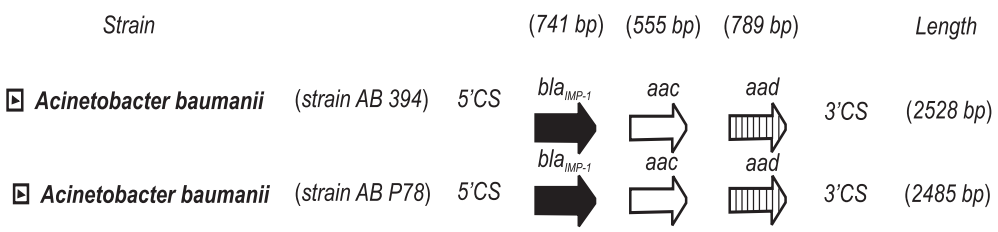

Fig. 7: *bla*$_{IMP-1}$, IMP-1 type of metallo-β-lactamase gene; *aac*, aminoglycoside acetyltransferse gene; *aad*, aminoglycoside adenylyltransferase gene

Shibata *et al*. (2003) was the first which reported metallo-β-lactamase producing *Providencia rettgeri* and *Morganella morganii* that produced the IMP-1 enzyme. In 2002, team of researchers from Toho University School of Medicine, Tokyo (Japan) isolated eight strains of *Providencia rettgeri* producing IMP-1 β-lactamase (Shiroto *et al*., 2005). The IMP-1 was encoded by a gene located in a class 1 integron (on conjugative plasmids) containing two gene cassettes, *bla*$_{IMP-1}$ and *aacA4*. It is interesting that strains of *Providencia rettgeri* were isolated from two different hospitals, which are separated by over 600 km.

Others, interesting cassette of genes, were described in two isolates *Acinetobacter baumanii*, in Taiwan (Liu *et al*., 2006). The structure of the sequence included three genes (*bla*$_{IMP-1}$, *aac* and *aadA5*) in class 1 integron (Fig. 7).

Koh *et al*., in 2004 year (Koh, Wang, Sng, 2004a), were described two clones of carbapenem resistant *Pseudomonas aeruginosa*. One clone carried a *bla*$_{IMP-1}$ gene was identical to the first reported in Japan and founded in *Klebsiella pneumoniae* in Singapore (Koh *et al*., 1999) and *Pseudomonas aeruginosa* in Singapore. The second clone (and subclones) carried a *bla*$_{IMP-1}$ variant containing sequences with four silent mutations at nucleotide positions 189 (C to T), 273 (C to T), 496 (T to G) and 702 (G to A) as described for *Pseudomonas fluorescens* from Singapore (Koh, Wang, Sng, 2004b).

The first report concerning with presence of the *bla*$_{IMP-1}$ in *Pseudomonas aeruginosa* (isolates recovered during 2001 – 2004), in China, was described by Wang and Mi, in 2004 year (Wang and Mi, 2004). In research findings performed through The Korean Nationwide Surveillance of Antimicrobial Resistance Group in 28 hospitals, isolated 11 strains of *Acinetobacter baumanii* which producing IMP-1 metallo-β-lactamase (Lee *et al*., 2003).

Next studies performed in Korea, during 2005-2006, demonstrated presence of the *bla*$_{IMP-1}$ genes also in *Pseudomonas aeruginosa*. The *bla*$_{IMP-1}$ carrying isolates of *Pseudomonas aeruginosa* were genetically unrelated. This gene was located in class 1 integron on plasmid (Nho *et al*., 2008).

In scientific literature, not too much, publications reported the presence of the *bla*$_{IMP-1}$ gene in different regions of the world. In Europe, IMP-1 was described only in Italy and United Kingdom (Cornaglia *et al*., 1999; Tysall *et al*., 2002). In Latin America, for the first time, IMP-1 was discovered in clinical strains *Klebsiella pneumoniae*. The gene responsible for resistance to carbapenems was found in a class 1 integron (Lincopan *et al*., 2005).

positive isolates contained detectable plasmids, and none could transfer carbapenem resistance by conjugation. It is therefore inferred that bla_{IMP-4} had become chromosomally integrated.

Next, transferable bla_{IMP-4} gene on conjugative plasmid was described by Hawkey *et al*., in a strain *Citrobacter youngae* (B38) in China (Hawkey, Xi, Ye, Li, M'Zali, 2001).

Another strain, *Acinetobacter baumanii*, producing IMP-4 was isolated in Hong Kong. The bla_{IMP-4} gene was borne on a cassette arraying with three other resistance gene cassettes, namely *qacG2*, *aacA4*, and *catB3*, in class 1 integron. The *qacG2* cassette downstream of bla_{IMP-4} including a long untranslated leading sequence is identical to the *qacG* cassette described for In*31* except for the 59-be (88% nucleotide sequence identity). The long untranslated leading sequence is believed to be a secondary promoter containing two putative strength-related hexameric motifs reported also as existing in *qacG* (encoding a small protein that confers resistance to quaternary ammonium) compounds in In31 (Laraki *et al*., 1999b). The *aacA4* gene cassette contains the aminoglycoside acetyltransferase AAC(6')-Ib7 gene and the *catB3* gene cassette encoding a chloramphenicol acetyltransferase (Bunny, Hall, Stokes, 1995). The bla_{IMP-4} gene had 93% nucleotide sequence identity than to that of bla_{IMP} (86% sequence identity), *bla*IMP-2 (44% sequence identity), and bla_{IMP-8} (27% sequence identity) (Houang, Chu, Lo, Chu, Cheng, 2003).

The first enzyme of IMP family that emerged on the Australian continent was IMP-4 reported in 2004 in *Pseudomonas aeruginosa* (Peleg, Franklin, Bell, Spelman, 2004) and *Klebsiella pneumoniae* and *Escherichia coli* isolates (Poirel *et al*., 2004b). In other study, in Australia, Peleg *et al*. (2005) described 16 isolates of *Enterobacteriaceae* and 3 of *Pseudomonas aeruginosa* with the bla_{IMP-4} genes. PCR and sequence analysis identified the bla_{IMP-4} gene was present in a class 1 integron in all isolates. Only 5 (26%) isolates were found to be carbapenem-resistant.

The carbapenem susceptibility can be explained by number of factors. Firstly, phenotypic expression of resistance is more likely when other resistance mechanisms are present. Other explanations include suppressed MBL gene expression by secondary regulatory system, leading to a silent or cryptic bla_{IMP} gene (Senda *et al*., 1996a), and varied carbapenem hydrolysis depending on the MBL gene dosage effect, which relates to the plasmid copy number (Hirakata *et al*., 1998). Recently, an *Aeromonas junii* isolate from Australia was found to have two (OXA-58 and IMP-4) carbapenemases (Peleg, Franklin, Walters, Bell, Spelman, 2006b).

In 2007, in Singapore, Koh *et al*., discovered four isolates *Acinetobacter baumanii* with bla_{IMP-4} and bla_{OXA-58} genes. In their study, the integrons containing bla_{IMP-4} had identical nucleotide sequences despite the fact that the strains had three different PFGE patterns The Hong Kong and Australian integron sequences are essentially identical and differ from the Singapore sequences by a few base pairs (Koh, Sng, Wang, Hsu, Zhao, 2007).

IMP-4 has also been found in *Acinetobacter calcoaceticus* in Malaysia though this only known from an entry in GenBank (accession number DQ307573). This suggests the possibility of international spread of the resistant determinant in these regions of the world.

3.5. IMP-5

Acinetobacter baumanii (65FFC) isolated at the Coimbra University Hospital, Portugal, in 1998, produced a new allelic variant of bla_{IMP} genes. The bla_{IMP} gene was 741 bp long and encoded a 246 amino acid polypeptide. Its gene sequence was identical to that of bla_{IMP-1} (Osano *et al*., 1994) except for 51 nucleotide substitutions, and was designated bla_{IMP-5}. Nineteen nucleotidic substitutions caused 17 amino acid changes between IMP-1 and IMP-5 β-lactamases. Six amino acid substitutions were never observed in the other IMP enzymes. IMP-5 β-lactamase showed a greater homology with IMP-1, IMP-3 and IMP-4 (identified in Southeast Asia), than with IMP-2, found in Italy (93%, 92%, 91% and 87% of amino acid identity, respectively). The bla_{IMP-5} was the only gene cassette inserted into a class 1 integron, named In*76* (Da Silva *et al*., 2002).

In 2006, again in Portugal, described eight strains of *Pseudomonas aeruginosa* producing IMP-5 carbapenemases. The bla_{IMP-5} gene was inserted into a class 1 integron previously reported in *Acinetobacter baumanii* (65FFC). Expression of the bla_{IMP-5} gene in *Pseudomonas aeruginosa* has been shown to be driven by the P_1 promoter [TTGATA] in which the cytosine was replaced by thymine, which caused an increase in transcription of bla_{IMP-5} that was confirmed by site-directed mutagenesis. Inserted genes are expressed primarily via a common promoter, P_1, located in the 5′ conserved segment. Different versions of P_1 are known to exist, with different combinations of -35 and -10 sequences compared with the consensus sequence (Lévesque, Brassard, Lapointe, Roy, 1994; Fluit and Schmitz, 1999). Sequence changes are a crude mechanism of control of gene expression and may influence the level of expression of a particular antibiotic resistance determinant.

In studies Brízio *et al.* (2006), the 'mutated' P_1 promoter (-35 [TTGATA] and -10 [TAAACT]), which drives expression of the *bla*IMP-5 gene in *Pseudomonas aeruginosa*, was stronger than the 'classical' P_1 (-35 [TTGACA] and -10 [TAAACT]) which found in integron In*76* from *Acinetobacter baumannii* (65FFC) (Da Silva *et al*., 2002). The secondary P_2 promoter was in its inactive form, suggesting that it was not contributing to transcription of bla_{IMP-5} gene. *Pseudomonas aeruginosa* isolates showed a unique gene cassette without internal promoters. Analysis of restriction fragments (with using PFGE) showed that these strains were not genetically related.

3.6. IMP-6

In 2001, Yano *et al*. (2001), examining isolate of *Serratia marcescens* (KU 3838) which was isolated in 1996, demonstrated the presence of the gene encoding a new MBL. The DNA sequence of MBL gene in plasmid (pKU503) shows that this metallo-β-lactamase gene was differed from gene encoding IMP-1 by one point mutation, leading to one amino acid substitution: 640-A in the base sequence of the IMP-1 gene was replaced by G, and Ser-196 was replaced by Gly in the mature enzyme. These results support the hypothesis that the hydroxyl group of Ser-196 plays an important role in meropenem hydrolysis. This enzyme was designated IMP-6.

3.7. IMP-7

IMP-7 MBL was described for the first time in *Pseudomonas aeruginosa* in Canada (Gibb *et al*., 2002). The encoding bla_{IMP-7} gene was found on class 1 integron containing three other cassettes of gene. The first sequence was an unknown open reading frame (ORF1). The second and third cassettes contained aacC4 and aacC1, both of which encode aminoglycoside acetyltransferases. The third cassette, $bla_{IMP-7,}$ encoded MBL that shares >86% amino acid identity with IMP known enzymes.

In Europe IMP-7 enzyme for the first time was described in *Pseudomonas aeruginosa* in Slovakia (Ohlasova, Kmet, Niks, 2007). Their results were different from other Europe countries. In Europe different types of metallo-β-lactamases already have been described, with dominance of the VIM type (Nordmann and Poirel, 2002).

Only Italy, United Kingdom, Portugal and France (and recently also in Poland) were European country where Gram-negative bacteria (*Acinetobacter baumanii* and *Pseudomonas aeruginosa*) producing IMP-type enzymes have been reported (Riccio *et al*., 2000; Da Silva *et al*., 2002; Nordmann and Poirel, 2002; Tysall *et al*., 2002; Toleman *et al*., 2003; Brízio *et al*., 2006; Sacha *et al*., 2007).

In Malaysia, Ho *et al.* (2002), described multidrug resistant isolate of *Pseudomonas aeruginosa* (Imp699) which the nucleotide sequence obtained revealed a 100% homology to the bla_{IMP-7} nucleotide of *Pseudomonas aeruginosa* from Canada (GenBank accession no. AF318077).

3.8. IMP-8

A multidrug-resistant plasmid encoded TEM-1, SHV-12, and variant of IMP-2 MBL was identified in 2001, in Taiwan, from a clinical isolate of *Klebsiella pneumoniae*. The sequence of the gene was identical to that bla_{IMP-2}, except for four nucleotide substitutions which resulted in two amino acid changes. Substitutions of a G for a C and a C for a G at nucleotide positions 61 and 62, respectively, and substitution of a G for a T at nucleotide position 617 resulted in the replacements of an Arg by an Ala and a Val by a Gly at amino acid positions 21 and 206, respectively, in the mature metallo-β-lactamase. Enzyme encoded by this gene was named IMP-8. The bla_{IMP-8} gene was found be flanked by identical sequence to partial sequence of the intII and aac(6′)-Ib genes.

This results indicating that bla_{IMP-8} is carried on an integron-borne gene cassette and containing an aminoglycoside resistance gene, aac(6′)-Ib (Yan, Ko, Wu, 2001c).

In next studies, Wu *et al.*, (Wu, Ko, Tsai, Yan, 2007) were be found occurrence of this gene at different isolates of Enterobacteriacae. Twenty isolates of *Enterobacter cloacae* were found to carry bla_{IMP-8}. Of the twenty IMP-8 producers, 17 isolates also harboured bla_{SHV-12}, which was on the same transferable plasmids. The all bla_{IMP-8} positive isolates appeared susceptible to imipenem and meropenem. This fact is showing on the difficulty in detection of metallo-β-lactamases in Enterobacteriaceae by routine susceptibility testing.

In 2007 (in Taiwan), for the first time among isolates of *Enterobacter cloacae* producing IMP-8 enzyme, described additional cassette of gene qrn in integron containing a bla_{IMP-8}

3.12. IMP-13

Another metallo-β-lactamase (named IMP-13) was described in *Pseudomonas aeruginosa*, also in Italy (Toleman *et al*., 2003). Molecular analysis of this MBL showed the presence of a $bla_{\text{IMP-13}}$ gene carried on a gene cassette inserted in a class 1 integron which also contained an *aacA4*-aminoglycoside resistance cassette encoding an AAC (6′)-Ib enzyme. The $bla_{\text{IMP-13}}$ gene was not transferable by conjugation and was apparently carried on the chromosome (Pagani *et al*., 2005).

3.13. IMP-14 and IMP-15

In 2003, Mendes *et al.* (2003), on the Interscience Conference on Antimicrobial Agents and Chemotherapy in Chicago presented new variant of MBL, IMP-14. The MBL from *Pseudomonas aeruginosa*, showed the highest identity to IMP-11 (92.4%) and IMP-8 (87.7%). The $bla_{\text{IMP-14}}$ gene was located in integron, together with the other cassette of genes (*aacA4* and *aadA1*) conditioning the resistance to aminoglycoside antibiotics. Information concerning the cassette of gene $bla_{\text{IMP-14,}}$ identified in isolates of *Pseudomonas aeruginosa*, in Thailand is available only in the GenBank base (GenBank accession number AY553333 and AY553332), similarly as isolates of *Pseudomonas aeruginosa* producing IMP-15 from Mexico (GenBank accession number EF184216 and EF184217) and Thailand (GenBank accession number AY553333).

3.14. IMP-16

The $bla_{\text{IMP-16}}$ is a very interesting variant bla_{IMP} gene. The $bla_{\text{IMP-16}}$ was found on the chromosome of *Pseudomonas aeruginosa* and was associated with a class 1 integron, which also encoded aminoglycoside-modifying enzymes. Downstream of $bla_{\text{IMP-16}}$ resided an open reading frame, which consisted of a new aminoglycoside-modifying gene, namely, *aac(6′)-30*, which was fused with *aac(6′)-Ib*′(Fig. 8). The fourth gene cassette constituted *aadA1*. An open reading frame (ORF) of 984 bp consisted of a novel gene cassette, namely *aac(6′)-30* fused with the *aac(6′)-Ib*′ gene. *aac(6′)-30* was also flanked by typical features, but it presented a shortened 59-be of 19 bp, including the core and inverse core sites. *aac(6′)-Ib* had a core site with a 1-bp mismatch, an A residue (in boldface) instead of the usual G residue (ATTAGGC) and an inverse core site (GCCTAAC), and the translation could start at the GTG codon located 19 bp downstream from its core site or at either one of the ATG codons located farther (Nobuta, Tolmasky, Crosa, Crosa, 1988; Galimand, Lambert, Gerbaud, Courvalin, 1993).

3.15. IMP-18

The $bla_{\text{IMP-18}}$ gene was detected in *Pseudomonas aeruginosa* in United States, in 2006 year (Hanson, Hossain, Buck, Moland, Thomson, 2006). IMP-8 and IMP-14 were the metallo-β-lactamases most identity to IMP-18. IMP-18 differed from IMP-14 by 21 amino acids

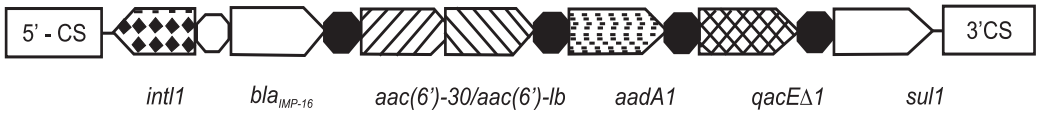

Fig. 8. *intI1*, Integrase; *bla*$_{IMP-16}$. IMP-16 gene; *aac(6')-30/aac(6')-Ib*. 6'-N-aminoglycoside acetyltransferase type I; *aadA1*, 3'-(9)-O-adenylyltransferase; *qacE*Δ*1*, QacEdelta1 multidrug exporter (disinfectant resistance protein and ethidium bromide resistance protein).

(91% identity), while IMP-8 differed by 29 amino acid (88% identity). The same gene was identified and described in 2008, in Mexico (Garza-Ramos *et al*., 2008b). The *bla*$_{IMP-18}$ gene was located in class 1 integron (In*96*) and contained additional gene cassette *aaA1*, which encoded resistance to streptomycin and spectinomycin.

3.16. IMP-19

In 2007, the group of scientists from France (Neuwirth, Siebor, Robin, Bonnet, 2007) isolated from *Aeromonas caviae* a new variant of metallo-β-lactamase, named IMP-19. The amino acid sequence revealed IMP-19 was similar to IMP-2 (Arg for IMP-2 and Ala for IMP-19 at position 38) and IMP-8 (Gly for IMP-8 and Val for IMP-19 at position 254). It is the first report of IMP producer in France and the first occurrence of a *bla*$_{IMP}$ gene in *Aeromonas caviae*.

3.17. IMP-22

The *bla*$_{IMP-22}$ gene was isolated from *Pseudomonas fluorescens*, in 2006 year, in Italy (Pellegrini *et al.*, 2007). The IMP-22 enzyme is quite divergent from other IMP variants (94% identity to IMP-16 and 85% to IMP-1).

Information about new variants of IMP (IMP-11, IMP-20, IMP-21, IMP-24 and IMP-25) enzymes weren't still published in science literature and they are available only in GenBank. The information referring them was presented in the table (Table I). Figure 9 presenting a genetic relationship (resemblance and differences) of some IMP enzymes (IMP1 to 13) and others to others metallo-β-lactamases (VIM, GIM and SPM).

4. VIM TYPE METALLO-β-LACTAMASES

Another prevalent group of metallo-β-lactamases (MBLs) is composed of the VIM enzymes. The VIM family (**V**erona **IM**ipenemase) currently consists of 22 members (*http://www.lahey.org/Studies/other.asp#table1.* [Accessed July 1, 2008] (Table 2). Genes encoding these MBLs can be plasmid-mediated or chromosomal mediated and are by usually located within integrons (Nordmann and Poirel, 2002). The dissemination of

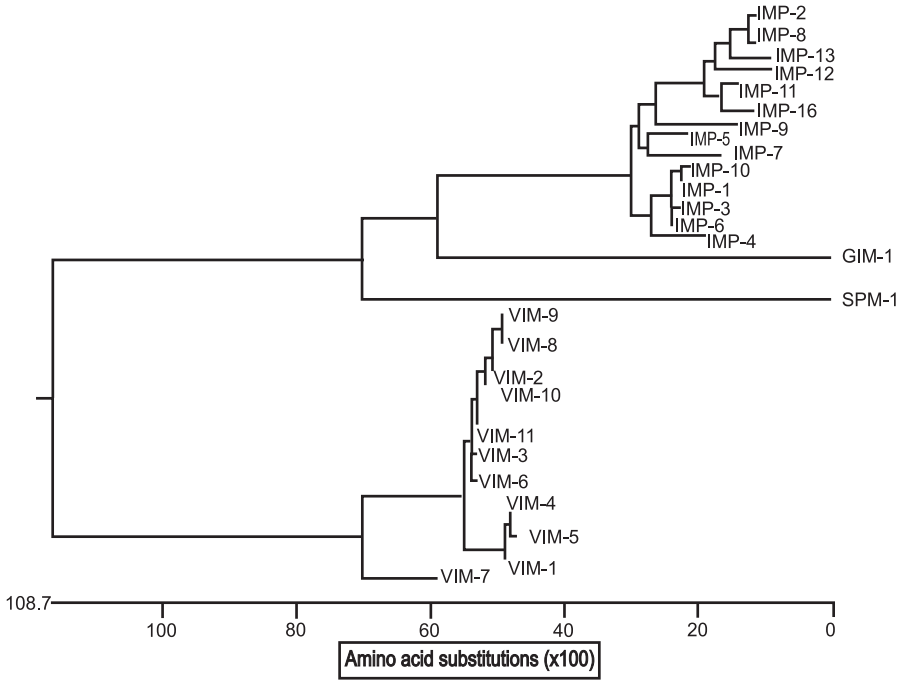

Fig. 9:

Table 2: VIM-type metallo-β-lactamases*

Enzyme	Accession number	Organism	Date	Reference
VIM-1	Y18050	*Pseudomonas aeruginosa*	1999	(Lauretti *et al*., 1999)
VIM-2	AF191564	*Pseudomonas aeruginosa*	2000	(Poirel *et al*., 2000b)
VIM-3	AF300454	*Pseudomonas aeruginosa*	2001	(Yan *et al*., 2001a)
VIM-4	AY135661	*Pseudomonas aeruginosa*	2002	(Pournaras *et al*., 2002)
VIM-5	AY144612	*Pseudomonas aeruginosa*	2004	(Bahar *et al*., 2004)
VIM-6	AY165025	*Pseudomonas putida*	2004	(Koh *et al*., 2004b)
VIM-7	AJ536835	*Pseudomonas aeruginosa*	2004	(Toleman *et al*., 2004b)
VIM-8	AY524987	*Pseudomonas aeruginosa*	2004	(Crespo *et al*., 2004)
VIM-9	AY524988	*Pseudomonas aeruginosa*	2004	(**)
VIM-10	AY524989	*Pseudomonas aeruginosa*	2004	(**)
VIM-11	AY605049	*Pseudomonas aeruginosa*	2005	(Pasteran *et al*., 2005)
VIM-12	DQ143913	*Klebsiella pneumoniae*	2005	(Pournaras *et al*., 2005)
VIM-13	DQ365886	*Pseudomonas aeruginosa*	2006	(**)
VIM-14	AY635904	*Pseudomonas aeruginosa*	2004	(**)
VIM-15	Assigned	-	-	-
VIM-16	Assigned	-	-	-
VIM-17	Assigned	-	-	-
VIM-18	AM778091	*Pseudomonas aeruginosa*	2007	(**)
VIM-19	Assigned	-	-	-
VIM-20	Assigned	-	-	-
VIM-21	Assigned	-	-	-
VIM-22	Assigned	-	-	-

NOTE

* According to Lahey Clinic [Available at: *http://www.lahey.org/Studies/other.asp#table1*. Accessed July 1, 2008]; (**) Data only in GenBank [Available at: *http://www.ncbi.nlm.nih.gov/Genbank/index.html*. Accessed June 6, 2008]

In *70.2* integron was originally located on the chromosome and was apparently present in a single copy in *Pseudomonas aeruginosa* isolate (Lauretti *et al*., 1999; Riccio *et al*., 2005). The variable region contains four gene cassettes, including the bla_{VIM-1} cassette followed by three aminoglycoside-resistance gene cassettes, an *aacA4* cassette encoding aminoglycoside acetyltransferase, an *aphA15* cassette encoding aminoglycoside phosphotransferase, and an *aadA1* cassette encoding aminoglycoside adenyltransferase. The last cassette exhibits a partially deleted *attC* recombination site and is followed by a *qacEÄ1* allele, typical of the 3′-conserved segment (3′-CS) of *sul1*-associated class 1 integrons (Fig. 10); (Hall and Collis, 1995).

The bla_{VIM-1} open reading frame (ORF) encodes a 266-amino-acid polypeptide. The G+C content of bla_{VIM} cassette was 56%, being considerably higher than that of the bla_{IMP} cassette (40%) (Lauretti *et al*., 1999). VIM-1 enzyme is distantly related to other metallo-β-lactamases. It is most closely related to Bc-II enzyme from *Bacillus cereus*, sharing only 39% amino acid identity (Walsh *et al*., 2005a; Lauretti *et al*., 1999). All the six invariant residues shared by the other class B enzymes (His-88, Asp-90, Gly-93, His-149, Gly-179, and His-210, with the numbering for the Bc-II enzyme of *Bacillus cereus* 569/H [Carfi *et al*., 1995]) were also retained in the VIM-1 sequence. At positions 86 and 168, VIM-1 contained His and Cys residues, respectively, similar to most other enzymes of this family. Of the additional residues known to be in or close to the active site of Bc-II (Carfi *et al*., 1995), five (Asn-42, Trp-59, Leu-114, Asn-180, and Asp-183) were found to be conserved in the VIM-1 protein, whereas two (Thr-150 and Lys-171) were conservatively substituted, and eight (Leu-110, Lys-117, Asn-118, Lys-147, Glu-151, Asp-177, Tyr-185, and Glu-214) were non-conservatively substituted (Carfi *et al*., 1995). VIM-1 is typical of class B enzymes, with very broad substrate specificity, hydrolyzing most β-lactams except aztreonam (Lauretti *et al*., 1999;Walsh *et al*., 2005a), similarly to the IMP-1 (Osano *et al*., 1994; Laraki et al., 1999a), CcrA (Yang, Rasmussen, Bush, 1992), BlaB (Rossolini *et al*., 1998), and L1 (Felici *et al*., 1993) metallo-β-lactamases. Resistance to the monobactams (aztreonam), in the original *Pseudomonas aeruginosa* was likely due to another resistance mechanism such as active efflux, cephalosporinases hyperproduction or a loss of OprD (Lauretti *et al*., 1999).

The bla_{VIM-1}-containing integrons carried by another two *Pseudomonas aeruginosa* isolates had an identical set of gene cassettes and were identical to In*70.2* (TS-832035 isolate) (Rossolini *et al*., 2000; Riccio *et al*., 2005) or different by a single nucleotide at the level of the P_c (P_1) integron promoter (G→C transversion at position 95 of the *intI1* gene) (PPV-108 isolate), suggesting that they share a common ancestry. The latter integron was named In*70.3* (Rossolini *et al*., 2000; Riccio *et al*., 2005). Both In*70.2* and In*70.3* were associated with a Tn*402* derivative inserted in proximity of the *res* site of a Tn*5051*-like backbone located in a conserved genomic context; however, the insertion sites of the Tn*402* derivatives containing In*70.2* and In*70.3* in the cognate transposon were different, likely reflecting independent insertional events. Overall, these findings suggest that the bla_{VIM-1}-positive *Pseudomonas aeruginosa* isolates that emerged in northern Italy in the late 1990s are probably derived from insertional events of Tn*402*-like elements associated with In*70*-like integrons in a conserved Tn*5051*-like backbone present in the chromosomes of members of an epidemic clonal complex circulating in that area and

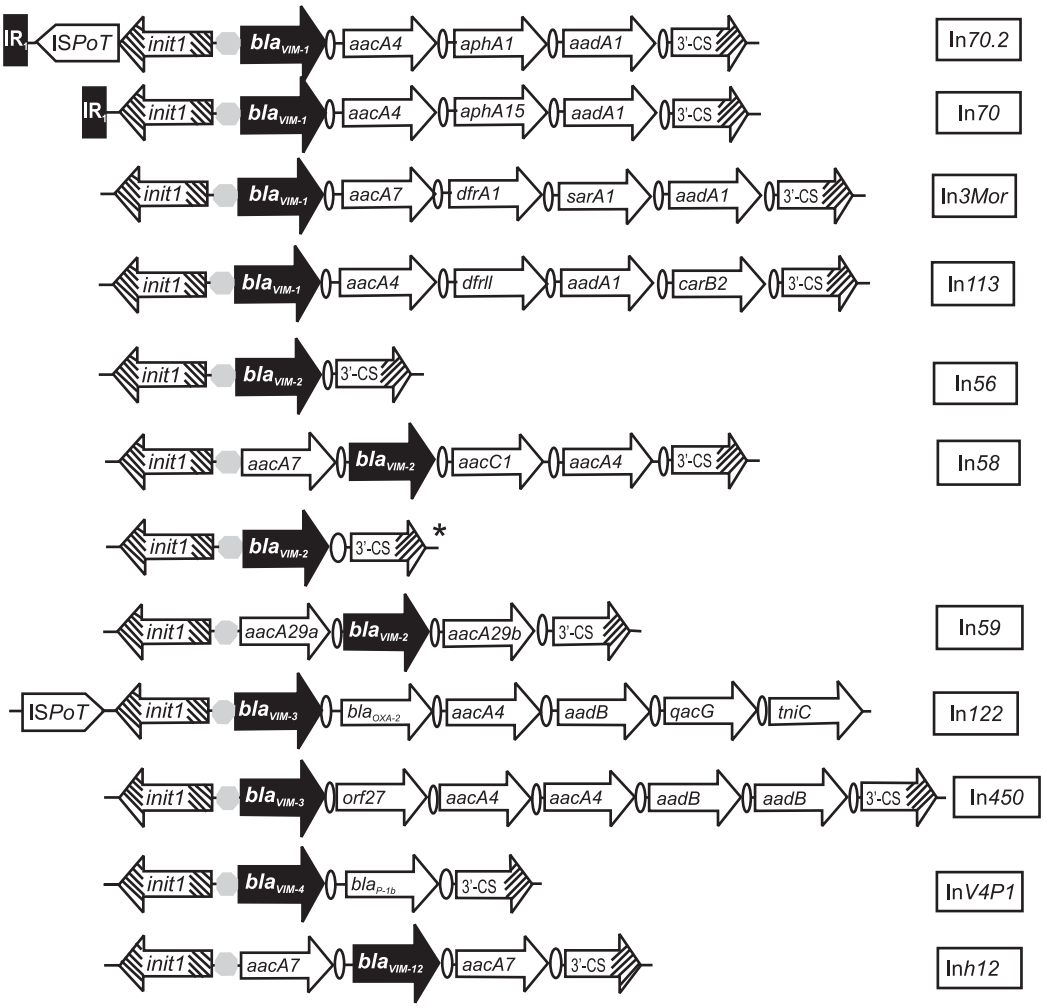

Fig. 10. Open reading frames (*orf*) are indicated by arrows; the *attC* recombination site (59-bp) of gene cassettes are indicated by ovals; the gray circles represent the recombination site *attI1*. The integrase gene is the part of 5'CS; the 3'-CS located downstream of the integrated gene cassettes include the sulphonamide resistance gene (*sul1*), the disinfectant resistance determinant (*qacEΔ1*) and open reading frame (*orf5*).

bla_{VIM-1}, metallo-beta-lactamase gene; bla_{VIM-2}, VIM-2 metallo-beta-lactamase gene; bla_{VIM-3}, VIM-3 metallo-beta-lactamase gene; bla_{VIM-4}, VIM-4 metallo-beta-lactamase gene bla_{VIM-12}, VIM-12 metallo-beta-lactamase gene; IR_1, terminal inverted repeat; IS*Pa7*, insertion element; *aacA4*, *aacA7*, *aacA29a*, *aacA29B*, aminoglycoside-6-N-acetyltransferase genes; *aacC1*, aminoglycoside-3'-N-acetyltransferase gene; *aphA15*, aminoglycoside phosphotransferase gene; *aadA1*, *aadB*, aminoglycoside 3'-acetyltransferase genes; *dfrA1*, *dfrII*, dihydrofolate reductase genes; *sat1*, streptothricin acetyltransferase 1 gene; *catB2*, chloramphenicol acetyltransferase gene; bla_{OXA-2}, OXA-2 beta-lactamase gene; *qacG*, quaternary ammonium compound resistance protein gene; *tniC*, TniC protein; *orf27*, open reading frame; bla_{P-1b}, PSE-1/CARB-2 beta-lactamase gene.

* the bla_{VIM-2} gene cassette with short 59-base element.

also suggest VR-143/97 and TS-832035 likely originated from the same ancestor after the occurrence of a similar insertional event, while PPV-108 probably results from a different insertional event that occurred in a member of the same clonal complex (Riccio *et al*., 2005).

In*70.2* and In*70.3* integrons have been reported among epidemiologically unrelated *Pseudomonas aeruginosa* strains from other Italian cities (Riccio *et al*., 2001; Toleman *et al*., 2003). VIM-1-producing *Pseudomonas aeruginosa* isolates carrying the In*70.2* integron have been reported from several geographic locations of Italy, including the northern region (Verona, Pavia, and Trieste), central Italy (Rome), and southern region of Italy (Sicily) (Rossolini *et al*., 2000; Toleman *et al*., 2005).

The both In*70.2* and In*70.3* integrons are structurally related to In*70* and In*110*, two plasmid-borne bla_{VIM-1}-containing integrons from *Achromobacter xylosoxidans* (Riccio *et al*., 2001) and *Pseudomonas putida* isolates (Lombardi *et al*., 2002), respectively, from the same geographic area (northern Italy). In*70* integron is another member of the group of class 1 integrons associated with defective transposon derivatives originating from Tn*402*-like elements. Among them, In*70* apparently shares the closest ancestry with members of the In*0*-In*2* lineage (Bissonnette and Roy, 1992; Brown, Stokes, Hall, 1996). However, the finding of an IR_i-flanking region which is different from that of either In*0* or In*2* but identical to those of integrons of the In*5*-In31 lineage (Laraki *et al*., 1999b) raises the question of the mobility of the defective transposon carrying the integron. This In*70* integron was first well characterized (before the first isolated In*70.2)* among In*70*-like members (In*70*, In*70.2*, and In*70.3*).

Characterization of this integron revealed an original array of four gene cassettes which were located on a 30-kb nonconjugative plasmid, named pAX22 (Riccio *et al*., 2001). The first cassette carries a bla_{VIM-1} determinant and is identical to the bla_{VIM-1} cassette from *Pseudomonas aeruginosa* VR-143/97, the VIM-1 index strain previously isolated in the same hospital (Lauretti *et al*., 1999). This finding suggests a common origin for the two determinants, although the original source remains unknown. It also indicates that bla_{VIM-1}, similar to bla_{VIM-2} (Poirel *et al*., 2000b), can even be found on plasmids. Three different aminoglycoside resistance determinants occur in next positions. There are cassettes containing an *aacA4* allele, *aph15* allele and *aadA1* allele (Fig. 10) (Riccio *et al*., 2001). The *aacA4* gene encodes an AAC(6′)-II aminoglycoside acetyltransferase identical to that encoded by the *aacA4* allele from plasmid pIP1855 of *Pseudomonas fluorescens* BM2687 (Lambert, Ploy, Courvalin, 1994). Both In*70* and In*70.2* differ between themselves by two point mutations in this gene cassette (Riccio *et al*., 2005). The third cassette of In*70* is original and contains a 795-bp ORF encoding a protein which exhibits the closest sequence similarities with an APH(3′)-IIb aminoglycoside phosphotransferase from *Pseudomonas aeruginosa* (Hächler, Santanam, Kayser, 1996) (38% identity) and with the APH(3′)-IIa enzyme encoded by Tn*5* (Beck, Ludwig, Auerswald, Reiss, Schaller, 1982) (36% identity). The gene was named *aphA15.* This gene is the first example of an *aph*-like gene carried on a mobile gene cassette (Riccio *et al*., 2001). The fourth cassette of In*70* contains an *aadA1* allele encoding an AAD(3′′) aminoglycoside adenylyl transferase (Shaw, Rather, Hare, Miller, 1993) and a partially deleted 59-be (Riccio *et al*., 2001).

resistant, VIM-1 producing *Klebsiella pneumoniae*. The proportion of imipenem-resistant *Klebsiella pneumoniae* has increased from less than 1% in 2001, to 20% in isolates from hospital wards and to 50% in isolates from ICUs in 2006 (Vatopoulos, 2008). Likewise, in 2002, these strains were identified in only three hospitals, whereas now they are isolated in at least 25 of the 40 hospitals participating in the Greek Surveillance System. This situation seems to be due to the spread of the $bla_{\text{VIM-1}}$ cassette among the rapidly evolving multiresistant plasmids and multiresistant or even panresistant strains of mainly *Klebsiella pneumoniae* and also other enterobacterial species. The frequency of the isolation of VIM-producing *Enterobacteriaceae* seems to be gradually increasing worldwide (mainly *Klebsiella pneumoniae*, *Enterobacter* spp., but also *Escherichia coli*, *Proteus mirabilis*, and other), and in Greece seems to be an important new chapter in the epidemiology of this resistance mechanism (Giakkoupi *et al*., 2003b; Vatopoulos, 2008). Recently, sporadic isolates of enteric bacteria producing VIM-1 have been described in other countries such as Italy (Castanheira *et al*., 2007; Aschbacher, Doumith, Livermore, Larcher, Woodford, 2008), Germany (Weile *et al*., 2007), France (Kassis-Chikhani *et al*., 2006), and Spain (Tórtola *et al*., 2005).

In Greece, the VIM-1 gene was generally found to be part of related type 1 integrons. The cassette region of these integrons typically contains (from 5′ to 3′) the $bla_{\text{VIM-1}}$, and the *aacA*, *dhfrI*, and *aadA* genes (Giakkoupi *et al*., 2003b; Miriagou *et al*., 2003; Vourli *et al*., 2006; Vatopoulos, 2008). This array of four gene cassettes constitute the structure of which was different from that of the previously described Italian VIM-encoding integrons (In*70.2*, In*70*) (Lauretti *et al*., 1999; Riccio *et al*., 2001), and compared to Italian strains a great diversification of the arrangement of the gene cassettes is being watched in various integrons (Table 3).

The first VIM-producing enteric bacterium in Greece was an *Escherichia coli* (VIM-1) isolated in 2001 (Miriagou *et al*., 2003). Since then VIM-producing *Escherichia coli* have been reported sporadically (Galani *et al*., 2007), and hospital outbreaks have also occurred (Scoulica, Neonakis, Gikas, Tselentis, 2004).

VIM-producing *Klebsiella pneumoniae* were first reported in 2002 in the intensive care unit of three teaching hospitals located in Athens. This organism has been reported as a reservoir of mobile elements carrying the $bla_{\text{VIM-1}}$ gene in Greek hospitals, until now successfully confined in the ICUs (Giakkoupi *et al*., 2003b). Spread of these elements via transferable plasmids to other species may facilitate their propagation out of the ICUs and into the community. The extent of the problem is unknown since the presence of MBL enzymes is underestimated in *Enterobacteriaceae* because of significant variation of carbapenem MICs among them (Galani *et al*., 2007). The fact that the MICs of imipenem and meropenem for most strains are below or near the proposed breakpoint definition for resistance create diagnostic and therapeutic problems and possibly obstruct the assessment of the real incidence of these strains (Scoulica *et al*., 2004; Vatopoulos, 2008). There is definitely an urgent need for accurate screening methods for MBL-producing isolates and prompt institution of infection control measures to effectively prevent their spread in the hospital.

The $bla_{\text{VIM-1}}$ cassettes (including the 81 nucleotides of the 59-base element) of *Escherichia coli* and *Klebsiella pneumoniae* were identical to that originally described in

Pseudomonas aeruginosa (Lauretti *et al*., 1999) and were located on the first position (Giakkoupi *et al*., 2003b; Miriagou *et al*., 2003). The integron of *Escherichia coli* contained bla_{VIM-1} gene, *aacA7* gene, *dhfrI* gene and *aadA* gene, and was located on the plasmid p541 (50-kb size). The initiation codon of the bla_{VIM-1} gene was preceded by a strong P_1 promoter ($TTGACAN_{17}TAAACT$) and the inactive form of P_2 ($TTGTTAN_{14}TACAGT$) located at the 5′ end of the integrase 1 gene (Miriagou *et al*., 2003). The putative promoter was a strong P_1 while the bla_{VIM-1} genes described previously in *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* were under the control of a weak P_1 promoter and a hybrid P_1 promoter, respectively (Lauretti *et al*., 1999; Riccio *et al*., 2001).

The strong version of the P_1 promoter is more effective than the weak and hybrid promoters. The P_2 promoter, with a spacing of only 14 nucleotides, is probably inactive because this spacing is unfavourable to expression, the optimum spacing being approximately 17 nucleotides. The P_2 promoter with this spacing is believed to be active, although its relative strength is unknown. The hybrid 1 and strong P_1 promoters have only been found in combination with the inactive form of P_2 (Fluit and Schmitz, 1999). Inactive P_2 promoter may play a role in the low expression of resistance to carbapenems (Jeong *et al*., 2003; Ikonomidis, Ntokou, Maniatis, Tsakris, Pournaras, 2008), that partially justifying the results revealing hidden MBL phenotypes.

The bla_{VIM-1} cassette, identical to that originally described in Italian *Pseudomonas aeruginosa* (Lauretti *et al*., 1999) and in an *Escherichia coli* previously isolated in Greece (Miriagou *et al*., 2003), has also been detected in *Enterobacter cloacae* (Galani, Souli, Chryssouli, Orlandou, Giamarellou, 2005). This gene cassette was inserted into a new type integron In*87* and compared to *Escherichia coli* was probably located on the chromosome. The identity of these gene cassettes suggests extensive spread of resistance gene between various Gram-negative species.

Recently, the bla_{VIM-1} allele has been described in a *Morganella morganii* clinical isolate (Tsakris, Ikonomidis, Spanakis, Poulou, Pournaras, 2007b). The gene was located in a new class 1 integron structure (In*3Mor*) containing a variable structure that is typically described in class 2 integrons. A typical 5′-CS containing an *intI1* gene with a strong P_1 promoter followed directly by an inactive P_2 promoter (without a GGG insertion prior to the –10 hexamer) and an *attI1* site was identified. The variable region of ~3600 bp contained five gene cassettes, including (5′ to 3′) the bla_{VIM-1} cassette, an *aacA7* cassette conferring resistance to aminoglycosides, a *dfrA1* cassette conferring resistance to trimethoprim, a *sat1* cassette conferring resistance to streptothricin and an *aadA1* cassette conferring resistance to streptomycin (Fig. 10). The bla_{VIM-1} gene cassette with its 59-base element was identical to those reported in other Gram-negative bacilli in Greece (Giakkoupi *et al*., 2003b; Miriagou *et al*., 2003; Galani *et al*., 2005). The last cassette was followed by a *qacEΔ1* allele, typical of the 3′-CS of *sul1*-associated class 1 integrons.

The unique structure of In*3Mor* might suggest a different origin of this integron in comparison with other MBL-containing integron structures. It is of interest that the *sat1* gene, which encodes a streptothricin acetyltransferase, is typically detected in the variable region of class 2 integrons (Sunde, 2005; Pan *et al*., 2006).

The *dfrA1-sat1-aadA1* array is a novel arrangement in the class 1 integrons, while it is typically described in the variable region of class 2 integrons consistent with that on

Tn*7* transposons (Sunde, 2005; Pan *et al*., 2006). It could be hypothesized that a simple excision of a *sat1* cassette from Tn*7* was followed by its integration into a class 1 integron that already had *aadA1* (Tsakris *et al*., 2007b).

The bla_{VIM-1} containing integrons are mainly found to be harboured by transferable plasmids in most enteric bacteria species including *Klebsiella pneumoniae* (Giakkoupi *et al*., 2003b), *Klebsiella oxytoca* (Aschbacher *et al*., 2008), *Escherichia coli* (Miriagou *et al*., 2003; Scoulica *et al*., 2004), *Proteus mirabilis* (Galani *et al*., 2007), *Enterobacter aerogenes* (Galani *et al*., 2007), *Citrobacter freundii* (Weile *et al*., 2007). The chromosomal location was also documented on several occasion, including *Proteus mirabilis* (Vourli *et al*., 2006; Tsakris *et al*., 2007a), *Enterobacter cloacae* (Galani *et al*., 2005), *Morganella morganii* (Tsakris *et al*., 2007b). The presence of two class 1 integrons on chromosomal and plasmidic DNA in the same clinical isolate, *Enterobacter cloacae*, has also been reported (Perilli *et al*., 2008). Both integrons presented the typical structure, and had two genes bla_{VIM-1} and *aadA2*.

The first report of the VIM-1 determinant in *Acinetobacter baumannii* in the world has also been described in Greece (Tsakris *et al*., 2006). Five unrelated metallo-β-lactamase positive isolates were found in two Greek hospitals. They carried bla_{VIM-1} gene within typical for Greece class 1 integron with a variable region including from 5′ to 3′ bla_{VIM-1}, *aacA7*, *dhfrI*, and *aadA1* gene cassettes. This class 1 integron contained the *intI1* gene with a strong P_1 promoter, an inactivated (without a GGG insertion) P_2 promoter and *attI1* site. The bla_{VIM-1} gene cassette with its 59-base element was identical to those reported previously in other Gram-negative bacteria from Greece (Giakkoupi *et al*., 2003b; Miriagou *et al*., 2003). *Acinetobacter* spp. may develop resistance to carbapenems through various mechanisms, including production of another carbapenemases, as class D, which was simultaneously detected in these strains (Tsakris *et al*., 2006).

4.2. VIM-2

The most widespread acquired metallo-β-lactamase is VIM-2 enzyme. bla_{VIM-2} gene was identified first in France from a *Pseudomonas aeruginosa* strain (COL-1) isolated from a neutropenic patient in 1996 (Poirel *et al*., 2000b). This isolate was resistant to most β-lactams, including ceftazidime, cefepime, and imipenem, but remained susceptibility to aztreonam. The bla_{VIM-2} allele was embedded into typical class 1 integron, and associated with a ca. 45-kb, not self-transmissible natural plasmid (pNOR2000). The bla_{VIM-2} gene cassette was a single determinant of integron, named In*56* (Fig. 10), as opposed to the class 1 integron that contained bla_{VIM-1} together with at least another gene cassettes (Lauretti *et al*., 1999).

The bla_{VIM-2} gene constitutes an 801-bp-long open reading frame encoding a 266-amino-acid protein, named VIM-2 (Poirel *et al*., 2000b). The G + C content of this frame was 56%, a value that is not typical of *Pseudomonas aeruginosa* genes (ranging from 60.1 to 69.5%), and it could correspond to that of genes found in members of the family *Enterobacteriaceae*.

The initiation codon (ATG) of bla_{VIM-2} was preceded by two promoter regions a weak P_c (regions –35 [TGGACA] and –10 [TAAGCT]) and P_2 (regions –35 [TTGTTA] and –10 [TACAGT]), which lie within the integrase structural gene. The secondary promoter P_2 identified in some class 1 integrons was in its active form, since the insertion of three guanosine molecules 119 bases downstream of the promoter P_c between the –35 and –10 regions of P_2 brought the spacing to 17 bp (Lévesque *et al*., 1994). Compared to the In*56*, In*8* and In*105* had G to T and G to A transitions in the promoter region that lies within the integrase structural gene in these integrons (Yum *et al*., 2002a; Galani, Souli, Koratzanis, Chryssouli, Giamarellou, 2006). These mutations have led to a strong version of the P_1 promoter as compared with In*56* of the first *Pseudomonas aeruginosa* isolate reported to produce VIM-2.

The bla_{VIM-2} gene cassette, which was inserted in the *attI1* recombination site, has a core site (GTTATGC) and an inverse core site (GCATAAC). The 59-base element was 72 bp long. The G + C content of this 59-base element was 58%. The 59-base elements for bla_{VIM-1} and bla_{VIM-2} cassettes clearly differed in size and structure. Only the right and left ends of the 59-base element shared significant homology, while the centre part required three gaps to be introduced in the bla_{VIM-2} 59-base element in order to obtain an optimal alignment (Poirel *et al*., 2000b).

The chromosomal located, integron almost identical to In*56* has been described in an environmental *Pseudomonas pseudoalcaligenes* strain (Quinteira, Ferreira, Peixe, 2005a). The only differences with In*56* were those observed in the promoter region. The Pc promoter found in this strain integron is a strong promoter (–35 [TTGACA] and –10 [TAAACT] (Fluit and Schmitz, 1999), compared to a weak-type promoter of In*56* (Poirel *et al*., 2000b). This strain was resistant to carbapenems (MIC for imipenem and meropenem were >32 µg/ml), but was still susceptible, to aztreonam (MIC, 2 µl/ml) (Quinteira, Ferreira, Peixe, 2005a).

The bla_{VIM-2} gene encode a 266-amino-acid protein, which have a relative molecular mass of 29.7 kDa, and a broad substrate hydrolysis range, including penicillins, cephalosporins, cephamycins, oxacephamycins, and carbapenems, but not monobactams. Kinetic parameters revealed that VIM-2 has a broad hydrolysis profile, including most β-lactams, except monobactams (aztreonam), cefsulodin, cefepime, and cefpirome. VIM-2 activity is higher against imipenem than against meropenem, but the level of resistance to carbapenems remained low (Poirel *et al*., 2000b).

The codon usage differed as well from that of *Pseudomonas aeruginosa* genes (West and Iglewski, 1998). The amino acid sequence of bla_{VIM-2} showed low amino acid identity with most of the Ambler class B carbapenem-hydrolyzing β-lactamases, ranging from 32% to 4% for Bc-II from *Bacillus cereus* to GOB-1 from *Chryseobacterium meningosepticum*, respectively. Amino acid sequence analysis of this protein revealed a putative cleavage site between the alanine and serine residues at positions 20 and 21, respectively (Nielsen, Engelbrecht, Brunak, Von Heijne, 1997).

VIM-2 enzyme is most closely related to VIM-1 (90% amino acid identity), a recently identified metallo-β-lactamase isolated from an Italian *Pseudomonas aeruginosa* clinical isolate (Lauretti *et al*., 1999). VIM-1 and VIM-2 clustered within a subgroup of carbapenem-hydrolyzing β-lactamases. The conserved amino acids among carbapenem-

hydrolyzing β-lactamases that may bind either to Zn^{2+} ions or a water molecule near or within their putative active site were found in VIM-2 (Rasmussen and Bush, 1997; Bush, 1998; Ullah *et al*., 1998; Wang *et al*., 1999a): His-86, His-88, Asp-90, His-149, His-225, Cys-168, and His-210. These amino acids were identical for VIM-2 and VIM-1. Amino acid changes in VIM-2 compared to the sequence of VIM-1 occurred mostly within the NH_2- or COOH-terminal regions.

The publishing data demonstrate that while VIM enzymes share common features in fold and active site architecture, their ability to bind and hydrolyze β-lactams varies considerably. The most noticeable example of this is the difference between VIM-1 and VIM-2, which are structurally very similar. For instance, VIM-2 tends to bind most β-lactams more tightly than VIM-1 and possesses significantly lower K_m values (the affinity of enzyme for a substrate) for benzylpenicillin, ampicillin, piperacyllin, mezlocillin, ticarcillin, cefalothin, cefoxitin, cefotaxime, ceftazidime, cefpirome, moxalactam, and meropenem. The most notable exception is imipenem, where VIM-1 and VIM-2 possess K_m values of 1.5 μM and 10 μM, respectively (Docquier *et al*., 2003a; Walsh *et al*., 2005a). However, VIM-1 is capable of hydrolyzing most β-lactams (piperacillin, azlocillin, ticarcyllin, cefaloridine, cefalothin, cefuroxime, cefotaxime, ceftazidime, cefpirome, and meropenem) more efficiently than VIM-2. Again, the most notable exception is imipenem, where VIM-1 and VIM-2 possess k_{cat} (the enzyme's ability to turn over the substrate) values of 0.2/s and 34/s, respectively. Docquier *et al*. (2003a) speculate that these substantial kinetic differences are due to amino acid substitutions near or at the active site, namely histidine/tyrosine at position 224 and serine/arginine at position 228.

Subsequently, two other *Pseudomonas aeruginosa* had been identified in France that harboured the same bla_{VIM-2} gene cassette (Poirel *et al*., 2001). Both isolates had similar resistance patterns compared to the *Pseudomonas aeruginosa* COL-1 isolate. In these two isolates, the bla_{VIM-2} gene cassettes were embedded in different class 1 integrons, In*58* and In*59* (Poirel *et al*., 2001). Between its 5′-CS and 3′-CS ends, In*58* contained four gene cassettes containing antibiotic resistance genes (Fig. 10). Just downstream of the 5′-CS, an *aacA7* gene encoding an AAC(6′)-I1 aminoglycoside acetyltransferase was located. The bla_{VIM-2} gene was inserted as the second position and was identical to that inserted in In*56* in originally described *Pseudomonas aeruginosa* COL-1. The third cassette contained an *aacC1* gene encoding an AAC(3′)-I and the fourth cassette contained an *aacA4* gene, which encoded AAC(6′)-Ib (Poirel *et al*., 2001). Characterization of In*59* revealed interesting features (Fig. 10). It contained 5′-CS and 3′-CS structures with the same Pc promoter and the bla_{VIM-2} gene cassette identical to those found in In*56* and In*58*. The bla_{VIM-2} gene cassette was flanked by two novel aminoglycoside acetyltransferase cassette-associated genes, named *aacA29a* and *aacA29b* (Poirel *et al*., 2001). These novel *aacA29* aminoglycoside resistance genes showing a G + C content of 55.6% a value suggesting that they may not have originated from *Pseudomonas aeruginosa*, thus further underlining the mobility of gene cassettes. The presence of a 101-bp sequence of a *qacE* cassette upstream of each *aacA29* cassette may have resulted from recombination at the sequence GATATAT of the *qacE* cassette and the core site of the ancestral *aacA29* cassette. The fact that this event took place between two nonhomologous recombining sites suggests a RecA-independent process such as an integrase-mediated process (Hansson,

the case of the integron from Polish *Pseudomonas aeruginosa* encoding resistance to β-lactams and aminoglycosides, and become permanently linked and to move genetically as a pair. Consequently, if one was selected by use of one agent so will be the other (Walsh *et al*., 2003).

The second one interesting bla_{VIM-2}-carrying integron was isolated from environmental *Achromobacter xylosoxidans* strain that was first reported (Shin *et al*., 2005). The nucleotide sequences of the bla_{VIM-2} gene from *Achromobacter xylosoxidans* strains were identical to that of the original VIM-2 producer, *Pseudomonas aeruginosa* COL-1 (Poirel *et al*., 2000b). The structure of the integron was unique, serially arrayed in *intI1, aacA4, aacA4,* bla_{VIM-2}, *aacA4,* and *qacEΔ1.* A bla_{VIM-2} gene in third position in this integron may be less well expressed (Shin *et al*., 2005). Among three *aacA4*, a first gene cassette had a silent point mutation at 734 bp (T→A), and a second had a point mutation at 1636 bp (A→G, Tyr→Cys). The sequences of the third *aacA4* and the bla_{VIM-2} were identical with those previously reported (Lee *et al*., 2002; Pallecchi *et al*., 2001). Each 59-base element had a core (GTTRRRY) and an inverse core site (RYYYAAC) except the last *aacA4* gene cassette deleted core site. In addition, these were the same size as those of prior reports (Riccio *et al*., 2001). This integron contained two promoters, a hybrid type P_1 and an inactive P_2, which may also have an effect on low expression of VIM-2 because of the relatively weak strength of P_1 (Fluit and Schmitz, 1999).

Two unique integrons containing bla_{VIM-2} gene cassette was identified in several *Pseudomonas aeruginosa* strains isolated from Italy and was depicted as In*71* and In*74* (Lagatolla *et al*., 2006). Both In*71* and In*74* had a chromosomal location. Sequence analysis of In*71* revealed the presence of a bla_{VIM-2} allele carried on a gene cassette with a complete *attC* recombination site (59 be), identical to those of In*56* from *Pseudomonas aeruginosa* COL-1 (Poirel *et al*., 2000b) and several other bla_{VIM-2}-containing integrons. The bla_{VIM-2} cassette was followed by an *aacA4* gene cassette. The cassette array was not followed by a 3′-CS typical of *sul1*-associated integrons (Hall and Collis, 1995) but was followed by a *tniC* gene typical of the transposition module of Tn*402* (Radström *et al*., 1994). The latter integron, In*74*, had a structure different from that In*71*, with an *aadB* gene cassette in the first position followed by a bla_{VIM-2} cassette, and its recombination site, identical to that found in In*71*. Since this cassette array has never been described before (Lagatolla *et al*., 2006).

The first case of metallo-β-lactamase has been reported in *Pseudomonas aeruginosa* from India (Toleman, Vinodh, Sekar, Kamat, Walsh, 2007), that is carried on a unique integron but that shows genetic structures similar to those of integrons from the United States and Russia (Lolans, Queenan, Bush, Sahud, Quinn, 2005). The integron had an unusual cassette structure consisting of a tandem array of *aacC7,* bla_{VIM-2}, *dhfrB5*, and *aacC6-II* gene cassettes. The cassette array and integron structure were strikingly similar to those of two other bla_{VIM-2}-harbouring integrons that have recently been sequenced from *Pseudomonas aeruginosa* strains isolated in the United States and Russia (Lolans *et al*., 2005). In particular, all three integrons had the same three cassettes in positions 1 to 3 of their variable regions, i.e., *aacA7,* bla_{VIM-2}, and *dhfrB5* (previously called *dhfrIIe*), which confer resistance to aminoglycosides, β-lactams, and trimethoprim, respectively (Levings, Lightfoot, Elbourne, Djordjevic, Hall, 2006). Additionally, all three integrons

lacked the 3′-CS that is found in the vast majority of class 1 integrons in clinically relevant bacteria and that consists of fused *qacE* and *sul1* gene cassettes, termed *qacEΔ1/sul1.* Instead, the *tniC* gene encoding the resolvase of transposon Tn*5090* (also called *tniR* of Tn*402*) (Radström *et al*., 1994) was found 3′ adjacent to the variable region of each integron. The Indian integron differs in only two respects from the integrons of the Russian and U.S. isolates. First, the fourth gene cassette is *aacC6-II* (Shaw *et al*., 1989), rather than the *aacCA5* gene found in the integrons of the Russian and U.S. isolates (Levings, Partridge, Lightfoot, Hall, Djordjevic, 2005). Second, the integron of the Indian isolate contained an ISPa21-like insertion sequence that has inserted within the 59-base element of the *aacC6-II* gene, an event that would "fix" this gene in the integron, making it refractory to integrase-mediated excision events. The lack of a 3′-CS is characteristic of the class 1 integron harboured by transposon Tn*5090* (also called Tn*402*), the progenitor of the common type of class 1 integron structure that contains the 3′-CS, as seen, for example, in transposon Tn*21* (Liebert, Hall, Summers, 1999). The addition of the *sul1* gene cassette and its subsequent fusion to the Tn*5090*/Tn*402 qacE* gene cassette by integration and deletion events, respectively, gave rise to the common form of the class 1 integron. These three $bla_{\text{VIM-2}}$-harbouring integrons found in *Pseudomonas aeruginosa* strains isolated from widely separated geographical locations probably originated from a widely dispersed Tn*5090* transposon. This transposon has evolved by normal integrase-mediated acquisition and loss of gene cassettes to include the $bla_{\text{VIM-2}}$ gene. The wide dispersal of this genetic structure with this particular gene array may be the reason that the $bla_{\text{VIM-2}}$ MBL is reported more often than any other MBL gene (Walsh *et al*., 2005a; Toleman *et al*., 2007).

Most IMP- and VIM-like gene cassettes are found on class 1 integrons, with variable structures among isolates. The $bla_{\text{VIM-2}}$ gene cassette is frequently embedded into structure of integron with aminoglycoside resistance gene cassettes (Table III). The simultaneous presence of $bla_{\text{VIM-2}}$ and *aacA4* is a common occurrence among integrons carrying metallo-β-lactamase genes. The first report of a class 1 integron containing a carbapenemase gene associated with another β-lactamase gene cassette has been described in *Pseudomonas aeruginosa* (Quinteira, Sousa, Peixe, 2005b). The $bla_{\text{VIM-2}}$ gene cassette was inserted within integron, named In*100*, identical to that reported for In*56* (Poirel *et al*., 2000b), and was found in the first position. An *aacA4* gene cassette was found immediately downstream of the $bla_{\text{VIM-2}}$ cassette. Interestingly, the sequence immediately downstream of *aacA4* gene is identical to that of the bla_{P1b} gene cassette described in In*28*, coding for PSE-1/CARB-2 β-lactamase, carbenicillinase which confers resistance to ampicillin and carbenicillin. The fourth cassette of In*100* contains the *aadA2* gene (Quinteira, Sousa, Peixe, 2005b).

More recently, another β-lactamase OXA-2 gene was detected inside a $bla_{\text{VIM-2}}$-containing class 1 integron structure (Corvec *et al*., 2008), different from what was observed for *Pseudomonas aeruginosa* COL-1 originally described (Poirel *et al*., 2000b). This integron was named In*122*. In the upstream part of this integron, an insertion sequence element, IS*Pa7*, was identified bracketed by two perfect 17 bp inverted repeats. The $bla_{\text{VIM-2}}$ gene cassette was located on the first position with respect to the integrase

gene (Fig. 10). Analysis of the sequences located in the downstream part of the integron revealed an uncommon genetic structure with a *qacG*-like gene (Chu *et al*., 2001). The downstream part of the integron also revealed a gene encoding a putative invertase that shared 98% amino acid identity with TniC found in the 3′-extremity of a *qac* gene cassette in Tn*5090*/Tn*402* (Radström *et al*., 1994). Thus, In*122* may be part of a Tn*402*-like transposon.

Downstream of the bla_{VIM-2} gene cassette, a cassette containing the bla_{OXA-2} gene encoding a narrow-spectrum oxacillinase (penicillinase) was identified. A third gene cassette was an *aacA4* gene, and a fourth gene cassette corresponded to *aadB* gene. The fifth cassette contained a *qacG*-like gene that encoded a 110-amino acid protein. The bla_{VIM-2}-carrying integron was considered likely to be chromosomally located (Corvec *et al*., 2008).

The bla_{VIM-2} gene cassette was always identified throughout the world as part of class 1 integrons varying in size and structure and is now the most prevalent carbapenemase gene (Pallecchi *et al*., 2001; Poirel *et al*., 2001; Walsh *et al*., 2005a). Transposon structure (termed Tn*1332*) likely at the origin of bla_{VIM-2} acquisition that did not directly involve an integron structure has been reported. A plasmid-encoded class II transposon element was identified in a carbapenem-resistant *Pseudomonas putida* isolate (Poirel, Cabanne, Collet, Nordmann, 2006).

Tn*1332* is closely related to Tn*1331* and belonging to the Tn*3* family, that had been identified previously in a *Klebsiella pneumoniae* isolate from Argentina (Tolmasky, 1990). Tn*1331* is closely related to Tn*3*, with an additional 3-kb fragment containing several antibiotic resistance genes, such as, *aacA4, aadA1,* bla_{OXA-9}, and bla_{TEM-1}, but does not possess any *attI1* site (Dery *et al*., 2003). As reported for Tn*1331*, the *aadA1* and bla_{OXA-9} gene cassettes are fused as a single gene cassette that may have arisen as a consequence of a recombination event involving two integrons (Sarno, McGillivary, Sherratt, Actis, Tolmasky, 2002).

Compared to Tn*1331*, Tn*1332* carried the bla_{VIM-2} gene cassette that had been inserted between the *aacA4* and *aadA1* aminoglycoside resistance gene cassettes. In addition, Tn*1332* carried two novel insertion sequences, IS*Ppu17* and IS*Ppu18* (Poirel *et al*., 2006).

IS*Ppu17*, an IS*30* family member, is 1,066 bp long, and its transposase (321 amino acids). The inverted repeats (IRs) of IS*Ppu17* are 22 bp long, and transposition of IS*Ppu17* generated 3-bp duplication at its insertion site. IS*Ppu18*, a member of the IS*4* family, is 1.192 bp long and encodes a 326-amino-acid transposase. The IRs of IS*Ppu18* is 12 bp long, and transposition of IS*Ppu18* generated 4-bp duplication (Poirel *et al*., 2006).

Immediately upstream and downstream of the 38-bp-long IRs of Tn*1332*, 5-bp duplication was identified that was the signature of the transposition process for bla_{VIM-2} acquisition. The common promoter sequences present in the 5′ conserved region of class 1 integrons and responsible for the expression of gene cassettes were absent (Lévesque *et al*., 1995). Thus, the promoter sequences enhancing bla_{VIM-2} expression in Tn*1332* might be the same as those described for Tn*1331* that enhanced bla_{TEM-1} gene expression, being part of the 520-bp direct repeats upstream of the *aacA4* gene (Tolmasky and Crosa, 1993). However, the presence of IS*Ppu17* in Tn*1332* might also be the source of additional promoter sequences involved in expression of the bla_{VIM-2} gene (Poirel *et al*., 2006).

other only by the presence of the *aacC1* cassette between bla_{VIM-2} and *aphA15*. The *aphA15* gene cassette was identical to that often observed in bla_{VIM-1}-containing integrons in Italy (Lauretti *et al*., 1999; Riccio *et al*., 2005; Toleman *et al*., 2005). In the integron from Mazovia, the bla_{VIM-2} cassette was accompanied by *aadB*, identical to that described in In*34* (Partridge and Hall, 2003) and a fused gene, *aadA6/aadA10*. Its 806-bp-long *aadA6* part was almost identical to that from a bla_{VIM-4}-containing integron from Greece (GenBank accession number AY460181), whereas the 19-bp-long 3′ end fully matched the *aadA10* sequence from the integron in plasmid R388-R151 (Partridge, Collis, Hall, 2002).

The occurrence of the integron variants correlated well with the geographic distribution of the MBL-producing organisms, and this suggested that their emergence in particular parts of the country had been likely due to a number of independent events. The regional dissemination of MBL producers could be attributed to various phenomena, including their clonal spread, horizontal transmission of resistance determinants, or both (Fiett *et al*., 2006).

The VIM-2 metallo-β-lactamases are mostly detected in non-fermenters bacteria, mainly in *Pseudomonas aeruginosa* (Walsh *et al*., 2005a), but the world epidemiological situation may be changing. This enzyme has been reported in isolates of other species (including members of *Enterobacteriaceae* family), such as *Pseudomonas putida* (Lee *et al*., 2002), *Acinetobacter baumannii* (Yum *et al*., 2002a), *Serratia marcescens* (Yum, Yong, Lee, Kim, Chong, 2002b), *Escherichia coli* (Galani *et al*., 2006), *Klebsiella oxytoca* (Conceição, Brízio, Duarte, Barros, 2005), *Enterobacter cloacae* (Jeong *et al*., 2003), *Citrobacter freundii* (Yan *et al*., 2002), *Providencia rettgeri* (Lee, Kang, Shin, Kim, 2007) and others. Its geographical dissemination is extremely widespread, covering a large array of countries from the European, Asian and American continents. Recently, the bla_{VIM-2} allele was identified in Spain (Gutiérrez *et al*., 2007), USA (Lolans *et al*., 2005), Canada (Pitout *et al*., 2007), India (Toleman *et al*., 2007), Venezuela and Chile (Mendes *et al*., 2004).

4.3. VIM-4

VIM-4 enzyme, encoded by a novel allele bla_{VIM-4}, was reported from a *Pseudomonas aeruginosa* isolate from Larissa, Greece (Pournaras *et al*., 2002). This was the first detection of a VIM-1 variant after its appearance in Italy. The *Pseudomonas aeruginosa* strain was recovered in 2001 from a patient who had received imipenem. This strain was highly resistant to all β-lactam antibiotics but kept moderate antibacterial activity for aztreonam (minimal inhibitory concentration was 16 μg/ml) (Pournaras *et al*., 2002). The isolate carried a class 1 integron that contained as a sole cassette the gene bla_{VIM-4}. Interestingly, a carbapenem-resistant VIM-4-producing *Pseudomonas aeruginosa* isolate was also identified in Sweden, in the same year (2001), that bla_{VIM-4} was described for the first time in Greece, but from a patient that was transferred from Greece (Giske, Rylander, Kronvall, 2003).

The DNA sequence included a part of the 5′ conserved segment followed by a bla_{VIM-1}-type gene that possessed a C instead of an A at nucleotide position 1864

(numbering according to [Lauretti *et al*., 1999]). This transversion resulted in a Ser-to-Arg change at position 175 (numbering according to reference [Galleni *et al*., 2001]) of the VIM-1 MBL. It is notable that position 175 is also occupied by Arg in VIM-2 and VIM-3 enzymes. Residue 175 (position 228 in the recently proposed standard numbering scheme for class B β-lactamases [Galleni *et al*., 2001]) is not among those considered significant for β-lactam hydrolysis by VIM and IMP MBLs (Galleni *et al*., 2001). This novel bla_{VIM-1} variant was designated bla_{VIM-4}.

VIM-4- and VIM-1-encoding genes differ by only one nucleotide and should be considered alleles derived from a common ancestor. On the other hand, bla_{VIM-4} was, most likely, the sole gene in the variable region of the detected class 1 integron while the bla_{VIM-1} integrons found in sporadic isolates in Italy included additional resistance gene cassettes (Lauretti *et al*., 1999; Riccio *et al*., 2001), indicating different phylogenies. Given also that there was not any apparent epidemiological association of the case described here with those in Italy, it can be assumed that the bla_{VIM-4}-encoding integron emerged independently (Pournaras *et al*., 2002).

The same MBL gene, for the first time, was also identified in *Enterobacteriaceae* strains, such as *Klebsiella pneumoniae* and *Enterobacter cloacae,* which were simultaneously recovered from a single patient under carbapenem therapy (Luzzaro *et al*., 2004). The presence of a bla_{VIM} allele identical to bla_{VIM-4} (Pournaras *et al*., 2002) was evident in both cases. In the two isolates investigated (*Klebsiella pneumoniae* and *Enterobacter cloacae*), the bla_{VIM-4} gene was carried on an apparently identical plasmid that could be transferred to *Escherichia coli* by conjugation, and was characterized (pCC416) (Colinon, Miriagou, Carattoli, Luzzaro, Rossolini, 2007). Since the two isolates had been obtained at the same time from the same clinical specimen—while an isolate of *Enterobacter cloacae* clonally related (but not containing the VIM-4 determinant) had been obtained from the same body site 4 weeks earlier—*Klebsiella pneumoniae* most likely represented the original vehicle of the bla_{VIM-4}-coding plasmid. Transfer of the plasmid to *Enterobacter cloacae* had probably occurred *in vivo* (Luzzaro *et al*., 2004).

Plasmid pCC416 carried the MBL gene bla_{VIM-4} and the AmpC-like β-lactamase gene bla_{CMY-4} that were located in two distinct regions. The pCC416 plasmid derived from the IncA/C_2 CMY through acquisition of a segment containing a novel bla_{VIM-4} integron, named In*416*. In*416* was closely related to class 1 integrons carrying bla_{VIM-1} alleles from various Gram-negative species isolated mainly in Europe suggesting a common origin. In*416* contained bla_{VIM-4} as the first gene cassette of a class 1 integron, followed by *aacA7, dfrI, aadA1* and *smr* gene cassettes encoding small multidrug resistance protein. In*416* was associated with a Tn*1696* module as the prototype In*4* integron (Colinon *et al*., 2007).

Greece is endemic region, where after the isolation of previous VIM-type enzymes from different Gram-negative pathogens, a new variant encoded by bla_{VIM-4} gene, has now been detected in several *Pseudomonas aeruginosa* clones (Pournaras *et al*., 2003), which can causing a large outbreak. Interestingly, a novel class 1 integron, In*V4P1*, was described in a highly carbapenem-resistant *Pseudomonas aeruginosa* strain (Maniati *et al*., 2007). The structure of In*V4P1* is the first example where a bla_{VIM-4} allele is found in

that several clonally distinguishable VIM-4-producing *Pseudomonas aeruginosa* strains were present in that country, one of the clones now being considered an endemic strain (Patzer *et al*., 2004). All the isolates possessed an identical class 1 integron, chromosomally encoded, with the aminoglycoside resistance gene cassette *aacA4* at the first position. The second gene cassette position was occupied by the bla_{VIM-4}. The bla_{VIM-4} gene is additionally unique in that it contains a 163 bp direct repeat of the 3'end of the gene. The direct repeat starts 15 bp after the bla_{VIM-4} stop codon and immediately after the natural inverse core site of the 59 base element. The extent of the gene duplication is 169 bp (170 bp) of the 3' end of the bla_{VIM-4} gene. The 59-be at the end of the direct repeat is identical to a normal bla_{VIM-1} 59-be and suggests that a normal bla_{VIM-4} 59-be is identical to a bla_{VIM-1} 59-be (the 59-be of previous bla_{VIM-4} gene cassettes are not represented in the GenBank database) (Patzer *et al*., 2004).

The sequence GTTGAGC found within the bla_{VIM-4} gene coding sequence was located 20 bp downstream of the start of the direct repeat. This pseudo-core sequence matched the core site of a 59-be perfectly, thus effectively forming a shortened 59-be of 34 bp directly after the complete copy of the bla_{VIM-4} gene. The direct repeat also includes its own 59-be and therefore it appears that the bla_{VIM-4} gene cassette has two 59-be (Patzer *et al*., 2004).

The 169 bp (170 bp) duplication that is part of the bla_{VIM-4} gene cassette described here is an insertion that was generated by deletion of part of a bla_{VIM-4} tandem cassette. The repeat is positioned precisely at the end of an inverse core site, and is derived from the original copy of the 59-be that formed part of the first bla_{VIM-4} cassette. The deletion removed most of the 59-be of the first bla_{VIM-4} cassette together with 643 bp of the second copy of the bla_{VIM-4} gene, fusing the first cassette to the end of the second (Patzer *et al*., 2004).

The entire Polish integron sequence revealed an almost identical match with a bla_{VIM-1}-containing class 1 integron isolated from a strain of *Escherichia coli* in Greece (Scoulica *et al*., 2004). The only differences were a single nucleotide change resulting in the substitution Ser 205 Arg of the bla_{VIM-4} gene and two transitions of C to T at positions 60 and 68 of the 59-be of the bla_{VIM-1} gene. Patzer *et al*. (2004) suggests that the integrons found in Polish isolates are older than that found in the Greek isolate.

Two another bla_{VIM-4}-carried class 1 integrons have been described in *Pseudomonas aeruginosa* from Poland (Fiett *et al*., 2006). The bla_{VIM-4} cassettes were identical to each other and to that originally described by Pournaras *et al*. (2002). The *aacA4* gene cassette which followed bla_{VIM-4} from the isolates from East Pomerania was specific and contained a single mutation (amino acid substitution Asp 164 Val) compared to those in In*58* (Poirel *et al*., 2001). A single *Pseudomonas aeruginosa* isolate from Katowice (Upper Silesia) possessed *aacA7, aadA6,* and *orfD* cassettes behind bla_{VIM-4}. It differed from the integron identified in Greece only by the presence of *aacA7* (GenBank accession number AY460181) (Fiett *et al*., 2006).

The same integron has been identified in *Pseudomonas putida* isolates, reported from Belgium (Bogaerts *et al*., 2008), and presents a specific 170 bp 3'-terminal repeat of the bla_{VIM-4} gene. The integron, harboured by a *Pseudomonas aeruginosa* strain, showing high structural similarity to integrons previously isolated in Greece and Poland (Patzer *et al*.,

2004; Scoulica *et al*., 2004), was described in Hungary by Libisch *et al*. (2004). That was the first report of integron-borne metallo-β-lactamase gene in this country. This one integron had a bla_{VIM-4} gene cassette, also containing a duplicated region (170 bp), and was different by a presence in the first position of the bla_{OXA} gene. These data have been confirmed, in addition, a new class 1 integrons containing a large number of gene cassettes have been described (Libisch *et al*., 2006). The first one had a cassette structure consisting of a tandem array of bla_{VIM-4}, *aacA8,* bla_{OXA-2}, *aacA7*, and the second one had the same arrangement of gene cassettes with addition of *aacA7* in the first position (Libisch *et al*., 2006).

More recently, the bla_{VIM-4} allele was recovered, as the first case of MBLs, from *Klebsiella pneumoniae* in Tunisia (Ktari *et al*., 2006), and *Pseudomonas aeruginosa* in Australia (Peleg, Bell, Hofmeyr, Wiese, 2006a).

Many integrons determining the VIM metallo-β-lactamase also encode aminoglycoside-modifying enzymes, but some reports of occurrence the VIM and other β-lactamase genes have been described in the same integron or outside integron.

The coexistence of most prevalent VIM-type genes (bla_{VIM-1}, bla_{VIM-2} and bla_{VIM-4}) and extended-spectrum β-lactamase (ESBL) gene in a single clinical isolate contribute to the broad β-lactam-resistant phenotype. Co-transfer of two enzyme genes, VIM and ESBL, in the same strain has been documented for *Enterobacteriaceae*, with both bla_{VIM-1} and bla_{CTX-M} in *Escherichia coli* (Scoulica *et al*., 2004; Aschbacher *et al*., 2008), bla_{SHV-5} in *Klebsiella pneumoniae* (Kassis-Chikhani *et al*., 2006; Cagnacci *et al*., 2008), bla_{SHV-12} in *Enterobacter cloacae* (Perilli *et al*., 2008), with both bla_{VIM-2} and bla_{GES-7} or bla_{IBC-1} in *Escherichia coli* (Galani *et al*., 2004; Galani *et al*., 2006), bla_{PER-1} in *Providencia* spp. (Lee *et al*., 2007), and with both bla_{VIM-4} and bla_{SHV-2a} or bla_{SHV-12} in *Enterobacter cloacae* (Luzzaro *et al*., 2004; Ikonomidis *et al*., 2007b), bla_{CTX-M} in *Klebsiella pneumoniae* (Ktari *et al*., 2006). These non-MBL genes were found outside integrons, but in most cases were located within the same plasmid. Association between VIM and ESBLs still appears to be a rare event in *Pseudomonas* spp. and was reported only in *Pseudomonas aeruginosa* for VIM-2 with either PER-1 (Docquier, Luzzaro, Amicosante, Toniolo, Rossolini, 2001; Yakupogullari, Poirel, Bernabeu, Kizirgil, Nordmann, 2008), and VIM-11 with GES-1 (Pasteran *et al*., 2005) and also in *Pseudomonas putida* VIM-2 and PER-1 (Bogaerts *et al*., 2008). Among *Pseudomonas aeruginosa* genes encoding narrow spectrum β-lactamase, such as bla_{P1b} and bla_{OXA-2} were inserted into the same array of integron (Quinteira *et al*., 2005b; Corvec *et al*., 2008).

Among VIM-type metallo-β-lactamases VIM-1, -2 and -4 are absolutely frequently worldwide present in clinical pathogens than described below enzymes.

4.4. VIM-3

Strains producing the VIM-3 metallo-β-lactamase were first identified in Taiwan in 2001, which was derived from a multidrug-resistant *Pseudomonas aeruginosa* (Yan *et al*., 2001a). All examined isolates were resistant to cefotaxime and ceftazidime, but susceptibilities to aztreonam, piperacillin, and carbapenems appeared to be diverse. VIM-3 is almost

4.5. VIM-5

The first published bla_{VIM-5} variant (Bahar *et al*., 2004) derived from *Pseudomonas aeruginosa* was originally reported in an imipenem-resistant *Klebsiella pneumoniae* isolate from Turkey (Midilli *et al*., 2003). It is a closely related to VIM-1 (Fig. 10), and compared with VIM-1, VIM-5 differs by five amino acid substitutions: Ala130Lys, His224Leu, Glu225Ala, Ser228Arg, Lys291Thr (numbering is according to the BBL scheme) (Galleni *et al*., 2001). *Pseudomonas aeruginosa* isolate that produced VIM-5 enzyme was resistant to imipenem, meropenem, aztreonam, ceftazidime, piperacillin and piperacillin with tazobactam. This was the first report of a metallo-β-lactamase-producing *Pseudomonas aeruginosa* isolate from Turkey. The presence of VIM-5 enzyme, which was detected in a *Klebsiella pneumoniae* isolated from a different Turkish area, suggests regional spread of this resistance determinant (Bahar *et al*., 2004).

In another region of Turkey gene encoding VIM-5 MBL was also identified in *Enterobacter cloacae* strain. The gene, bla_{VIM-5}, was located on an approximately 23-kbp nonconjugative plasmid (pEDV5) in a class 1 integron. The bla_{VIM-5} gene was embedded into integron as a single antibiotic resistance gene cassette, followed by *orfD* and *qacEÄ1* gene (Gacar *et al*., 2005).

The molecular mass of VIM-5 was calculated as 28 kDa. All β-lactams with significant differences in k_{cat} and K_m values were hydrolyzed by VIM-5, with the exception of aztreonam (Gacar *et al*., 2005). Notably, ceftazidime and cefepime were less effectively hydrolyzed by VIM-5 than VIM-1 (Franceschini *et al*., 2000) and VIM-2 (Poirel *et al*., 2000b), while cefotaxime was hydrolyzed at a comparable efficacy to that of VIM-2. The behaviour of VIM-5 against carbapenems was similar to that of VIM-1 and VIM-2, with greater efficiency on imipenem than meropenem.

4.6. VIM-6

Metallo-β-lactamase, VIM-6, was identified from two *Pseudomonas putida* isolates from Singapore (Koh *et al*., 2004b). These isolates were highly resistant to β-lactams, with MICs of >32 μg/ml for imipenem and meropenem, >256 μg/ml for ceftazidime, and 128 μg/ml for aztreonam. The complete bla_{VIM-6} genes were sequenced, and their sequences were found to differ from that of VIM-2 at nucleotide positions 179 (A to G) and 443 (A to G) and from that of VIM-3 at nucleotide positions 178 (A to C) and 179 (A to G). Analysis of plasmid DNA revealed plasmidic localization of bla_{VIM-6} gene. By following the BBL numbering scheme (Galleni *et al*., 2001), this novel enzyme, now named VIM-6, differs from VIM-2 by two amino acid changes at position 59 (Gln to Arg) and 165 (Asn to Ser) and from VIM-3 by only one amino acid at position 59 (Lys to Arg) (Koh *et al*., 2004b). VIM-2, VIM-3, and VIM-6 appear to form a cluster (with at least 99.2% amino acid identity within the cluster) whose amino acid sequences differ from those of the other VIM enzymes by about 10% (Koh *et al*., 2004b).

4.7. VIM-7

The first case of a mobile metallo-β-lactamase gene, bla_{VIM-7}, was found in the bacterial population of the United States. The gene has been detected and well characterized from a carbapenem-resistant *Pseudomonas aeruginosa* strain from Houston (Toleman, Rolston, Jones, Walsh, 2004). Two years later, bla_{VIM-7} remerged in another clonally unrelated *Pseudomonas aeruginosa* isolate in the same centre (Aboufaycal *et al*., 2007), which had identical sequence of integron as the strain described previously (Toleman *et al*., 2004).

The bla_{VIM-7} is preceded by a ribosome binding site (AGGAG) 6 bp upstream of the start codon. The presence of a conserved core site 13 bp upstream of the ribosome binding site, together with an inverse core site 7 bp after the stop codon and a 59-bp element, demonstrates that bla_{VIM-7} is harboured on a gene cassette. Additionally, an *attI1* site identified immediately upstream of the gene cassette containing the promoter of the *intI* gene suggests that bla_{VIM-7}, like other metallo-β-lactamases, is harboured on an integron. The gene encoding the enzyme, bla_{VIM-7}, like the bla_{VIM-1} and bla_{VIM-3} genes, is harboured on a gene cassette which gives it the potential to move readily from one genome to another. The bla_{VIM-7} genetic locus does differ in that no additional antimicrobial resistance genes were found directly downstream of bla_{VIM-7}. The sequence of DNA (600 bp) downstream of bla_{VIM-7} in the recombinant plasmid pMATVIM-7 (Toleman *et al*., 2004) displays no homology with any antimicrobial resistance genes or the 3′ conserved sequence found in most class 1 integrons. It is possible that the 3′-CS conserved section among many class 1 integrons, consisting *qacEΔ1*gene, fused to *su11* is present further downstream of the bla_{VIM-7} gene that is not represented in the clone pMATVIM-7. However, the 3′-CS is not found in all class 1 integrons (e.g., Tn*402*) and is not required for mobility.

The bla_{VIM-7} allele was located on a 24-kb plasmid and has been shown to be highly mobile and can be expressed in *Enterobacteriaceae* and *Pseudomonas* spp. (Toleman *et al*., 2004).

VIM-7 enzyme shares only 77% identity with VIM-1 and 74% with VIM-2 and therefore constitutes a third subgroup among the VIM-type β-lactamases (Fig. 11), whereas VIM-1 to VIM-6 have 89 to 99% identity (Lauretti *et al*., 1999; Poirel *et al*., 2000b; Yan *et al*., 2002). These amino acid variations among VIM-7 and the other VIM-type enzymes cluster in the leader sequence but are also found throughout the mature protein. The amino acid changes within the mature protein are often changes involving functionally different residues, namely Gln 48 Lys, Ser 61 Lys, Asp 64 Gly, Ser 192 Arg, Tyr 195 Phe, Asn 216 Asp, Glu 225 Lys, and His 219 Arg. The most likely site for cleavage occurs between amino acid positions 26 and 27 (YSA/QP), which would leave a mature peptide of 25 392 Da. However, though divergent from other VIM-like proteins, VIM-7 shares even less homology with other metallo-β-lactamases and therefore may be considered part of the VIM family (Fig. 11). These data and the genetic context of the bla_{VIM-7} gene indicate that VIM-7 is only distantly related to the other VIM-like metallo-β-lactamases. It is therefore unlikely that VIM-7 has arrived on the North

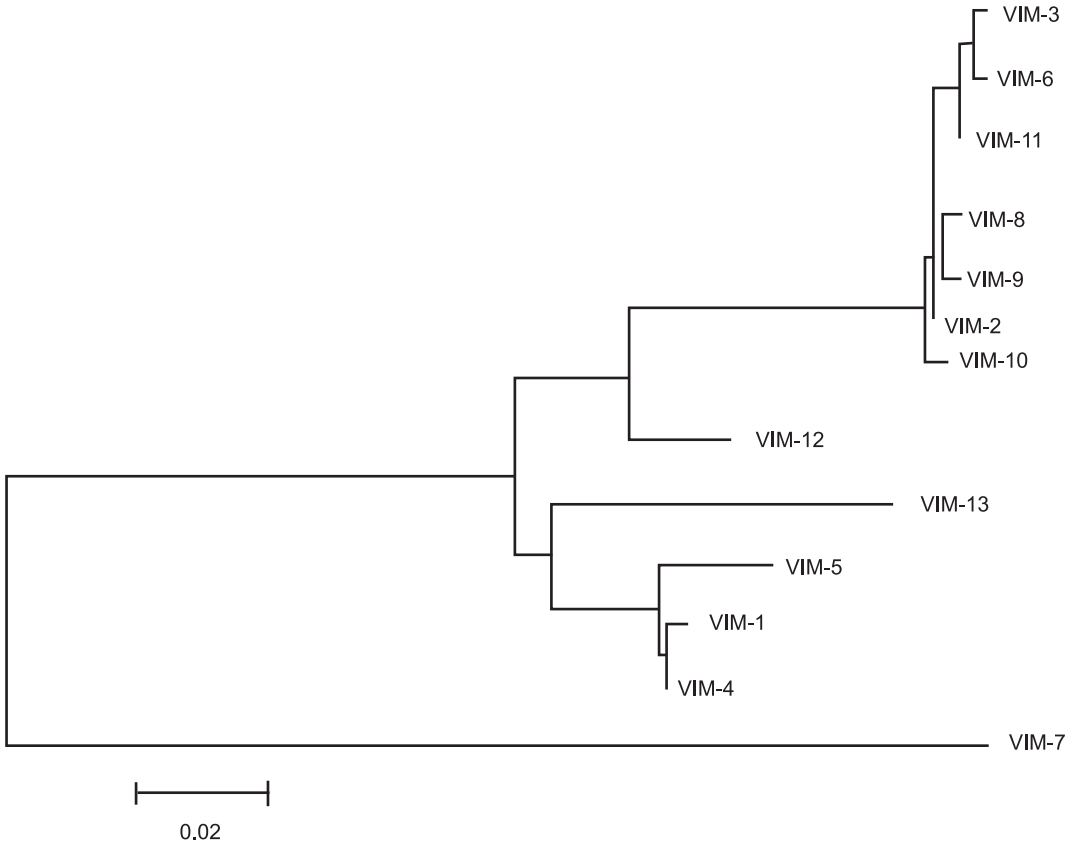

Fig. 11:

American continent via immediate dissemination from Europe or East Asia, where the other VIM enzymes have been reported. It is probable that VIM-7 has arisen independently within the United States, possibly by the therapeutic use of broad-spectrum β-lactams (cephalosporins or carbapenems) (Toleman *et al*., 2004).

4.8. VIM-8

The novel variant of bla_{VIM-2} gene was described in 2004 in South America, Colombia, where the considerable increase in the level of the imipenem resistance among *Pseudomonas aeruginosa* has been observed. At least from 1998 onwards, this rise in resistance was substantially due to the type A outbreak-related *Pseudomonas aeruginosa* strain, which produced a novel bla_{VIM-8} gene (Crespo *et al*., 2004). This gene had three polymorphisms compared with the sequence of bla_{VIM-2}. Two of these nucleotide changes were silent (position 492 A to G and 693 G to A), but the third A to G at nucleotide 45 determined a Thr139Ala substitution at amino acid 139. Thr139 is also modified (to Ile)

The crystal structure of dizinc (II) VIM-2 (Garcia-Saez, Docquier, Rossolini, Dideberg, 2008) revealed that the Asn 165 side chain forms an H bond with the peptide carbonyl of Phe117, flanked in the protein sequence by two Zn ligands (His116 and His118). The Asn 165 Ser mutation is expected to remove the interaction between loops L7 and L8, possibly enhancing loop flexibility and resulting in a relatively wider active-site groove, a situation that would better accommodate cephalosporins with bulkier substituents at C-3. In line with these observations, a number of pieces of evidence account for the impact of second-shell ligands in enzymatic activity (Iyobe S *et al*., 2000; Tomatis, Rasia, Segovia, Vila, 2005), suggesting that mutations outside the active site also contribute to tuning the MBL catalytic performance.

More recently, the bla_{VIM-11} has also been identified in a new hospital in Taiwan (Lu *et al*., 2008). Interestingly, this gene was recovered from *Acinetobacter baumannii*, and was embedded in class 1 integron as a gene cassette. The emergence of carbapenem-resistant *Acinetobacter baumannii* in a new regional hospital 3 years after its establishment was associated with increasing carbapenem usage and patient transfer between hospitals, which brought carbapenem-resistant *Acinetobacter* spp. from other hospitals.

4.10. VIM-12

The VIM group MBLs is clustered into three evolutionary lineages, driven by VIM-1, VIM-2, and VIM-7 (Fig. 11). Interestingly, a novel type of bla_{VIM} genes is bla_{VIM-12} allele, classified as intermediate between bla_{VIM-1} and bla_{VIM-2} (Pournaras *et al*., 2005). The bla_{VIM-12} is a hybrid of two genes ($bla_{VIM-1/VIM-2}$), and was originally identified in 2005 in *Klebsiella pneumoniae* clinical isolate from Greece.

The nucleotide sequences of DNA revealed a class 1 integron named In*h12*. The variable region of In*h12* was approximately 2.1 kbp and included bla_{VIM-12} gene cassette flanked by two copies of an *aacA7* gene cassette similar to those encountered frequently among VIM-encoding class 1 integrons (Fig. 10). A typical 5′ conserved segment containing an *intI1* gene with a strong P_1 promoter followed directly by an activated P_2 promoter (including a GGG insertion) and an *attI1* site was identified. *qacEΔ1/sul1* sequences were also present at the 3′-CS of In*h12*. The bla_{VIM-12} gene (798 bp) shared 97.7%, 94.5%, and 80.2% nucleotide homology with the bla_{VIM-1}, bla_{VIM-2}, and bla_{VIM-7} genes, respectively. This novel MBL differed from bla_{VIM-1} by 18 nucleotides. Notably, these changes were all located at the 3′ end and matched exactly the nucleotides found at the corresponding positions in the bla_{VIM-2} gene. The bla_{VIM-12} could therefore be viewed as a bla_{VIM-1}/bla_{VIM-2} hybrid being identical to bla_{VIM-1} from the 5′ end up to nucleotide 663 and to bla_{VIM-2} from nucleotide 614 up to its 3′ end (Pournaras *et al*., 2005). Furthermore, the 59-base element of the bla_{VIM-12} gene cassette (72 bp in length) was identical to the element commonly found in bla_{VIM-2} cassettes (Poirel *et al*., 2000b; Pallecchi *et al*., 2001; Poirel *et al*., 2001) and differed significantly from the 59 bp of the bla_{VIM-1} gene cassettes (Lauretti *et al*., 1999; Riccio *et al*., 2001; Miriagou *et al*., 2003). In*h12* integron containing bla_{VIM-12} gene was originally located on a transferable plasmid (p2873) (Pournaras *et al*., 2005).

The pattern of the nucleotide changes of the bla_{VIM-12} gene, compared with bla_{VIM-1} and bla_{VIM-2}, leads to the hypothesis that this novel variant might have been formed by a recombination event between bla_{VIM-1}- and bla_{VIM-2}-containing sequences. The possibility that bla_{VIM-12} belongs to a distinct VIM lineage cannot be excluded (Pournaras *et al*., 2005). It is probable that bla_{VIM-12} has arisen within hospital environments, where VIM-1- and VIM-2-producing microorganisms are endemic (Tsakris *et al*., 2000; Giakkoupi *et al*., 2003b; Pournaras *et al*., 2003).

The sequence of the 266-amino acid VIM-12 polypeptide (28 kDa) is 97.0% and 93.6% identical with those of VIM-1 and VIM-2, respectively. VIM-12 differs from VIM-1 by 8 amino acid residues at positions 246, 251, 257, 258, 284, 287, 294, and 299 (Galleni *et al*., 2001). All eight residues are located at the protein surface and are distant from the active site (Docquier *et al*., 2003a). VIM-12 bears at its N-terminal region the SGEPS amino acid signature which is characteristic of the VIM-1 β-lactamases (the corresponding signature for VIM-2 is SVDSS (Kontou *et al*., 2007).

VIM-12 represents a novel and unique member of the family of known metallo-β-lactamases, exhibiting atypical substrate specificity (Kontou *et al*., 2007). The hydrolytic activity of VIM-12 is optimal at pH 6.7 and 40°C. Only penicillin G was efficiently hydrolyzed by this enzyme. VIM-12 also exhibited a moderate rate of hydrolysis of imipenem. Imipenem acts as competitive inhibitor, as expected, with respect to the fact that imipenem represents a true substrate of VIM-12 and not a dead-end competitive inhibitor. Hydrolysis of meropenem, ceftazidime, cefoxitin, and cefotaxime was not observed, in contrast to other VIM-type β-lactamases. Meropenem cannot be hydrolyzed but can bind the enzyme acting as a noncompetitive inhibitor. Meropenem contains bulky rings that contribute to a less flexible overall structure. This structural difference could explain why this antibiotic is not suitable for correct recognition and processing by VIM-12, although it has been reported as an appropriate substrate for other VIM-type β-lactamases. Interestingly, it seems that VIM-12 contains allosteric site for binding of meropenem (and possibly for ceftazidime and other antibiotics) and these sites favour inactivation of VIM-12 in a non-competitive mode, something that has never been reported before for VIM-type β-lactamases. Typically, aztreonam was not hydrolyzed by VIM-12, a fact consistent with the behavior of all VIM-type metallo-β-lactamases (Kontou *et al*., 2007).

A recent report on the identification of the same bla_{VIM-12} gene in an *Escherichia coli* clinical isolate (Ikonomidis *et al*., 2007a) indicates that either VIM-12 already started to spread among Gram-negative bacteria or it has been elusive so far among clinical isolates. An outbreak caused by a single clone of *Klebsiella pneumoniae* carrying the novel plasmid-mediated bla_{VIM-12} carbapenem-hydrolyzing β-lactamase gene has been recently described in other Greek hospital (Tokatlidou *et al*., 2008).

4.11. VIM-9, VIM-10, VIM-13, VIM-14, VIM-15, VIM-16, VIM-18

Several sequences of new bla_{VIM} genes have been submitted to the EMBL database but have not been formally published (Tables 2 and 3) (Walsh *et al*., 2005a). Two genes,

Table 3: Origin, bacterial hosts of the VIM-type metallo-β-lactamases and genetic context of structures carrying bla_{VIM} genes

Enzyme	Organism	Accession number	Country	Isolation year(s)	Genetic context of structures carrying *bla_{VIM}* genes*	Integron	Localisation	Reference
VIM-1	*Pseudomonas aeruginosa*	Y18050	Italy	1997	***[bla_{VIM-1}]*** → *[aacA4]* → *[aphA15]* → *[aadA1]* → *[qacEΔ1/sul1]*	In*70.2*	Ch	(Lauretti *et al.*, 1999) (Riccio *et al.*, 2005)
	Pseudomonas aeruginosa	AJ581664	Italy	1999	***[bla_{VIM-1}]*** → *[aacA4]* → *[aphA15]* → *[aadA1]* → *[qacEΔ1/sul1]*	In*70.2*	Ch	(Rossolini *et al.*, 2000) (Riccio *et al.*, 2005)
	Pseudomonas aeruginosa	AJ581665	Italy	1999	***[bla_{VIM-1}]*** → *[aacA4]* → *[aphA15]* → *[aadA1]* → *[qacEΔ1/sul1]*	In*70.3*	Ch	(Rossolini *et al.*, 2000) (Riccio *et al.*, 2005)
	Achromobacter xylosoxidans	AJ278514 AJ278515	Italy	1998	***[bla_{VIM-1}]*** → *[aacA4]* → *[aphA15]* → *[aadA1]* → *[qacEΔ1/sul1]*	In*70*	Tn402-like /pAX22	(Riccio *et al.*, 2001)
	Pseudomonas putida	AJ439689	Italy	1999-2000	***[bla_{VIM-1}]*** → *[aacA4]* → *[aadA1]* → *[qacEΔ1/sul1]*	In*110*	pVA304	(Lombardi *et al.*, 2002)
	Escherichia coli	-	Greece	2001	***[bla_{VIM-1}]*** → *[aacA7]* → *[dhfrI]* → *[aadA]* → *[qacEΔ1/sul1]*	+	p541	(Miriagou *et al.*, 2003)
	Klebsiella pneumoniae	-	Greece	2002	***[bla_{VIM-1}]*** → *[aac6]* → *[dhfrI]* → *[aadA]* → *[qacEΔ1/sul1]*	+	p	(Giakkoupi *et al.*, 2003b)
	Escherichia coli	AY152821	Greece	2001	*[aac(6')-Ib]* → ***[bla_{VIM-1}]*** → *[repeat]* → *[qacEΔ1/sul1]*	+	p	(Scoulica *et al.*, 2004)
	Enterobacter cloacae	AY648125	Greece	2003	***[bla_{VIM-1}]*** → *[aac(6')-IIc]* → *[qacEΔ1/sul1]*	In*87*	Ch	(Galani *et al.*, 2005)
	Pseudomonas aeruginosa	-	Italy	1996-1997, 1999, 2000-2002	***[bla_{VIM-1}]*** → *[aacA4]* → *[aphA15]* → *[aadA1]* → *[qacEΔ1/sul1]*	In*70-like*	Ch	(Lagatolla *et al.*, 2006)
	Morganella morganii	DQ522239	Greece	2005	***[bla_{VIM-1}]*** → *[aacA7]* → *[dfrA1]* → *[sat1]* → *[aadA]* → *[qacEΔ1/sul1]*	In*3Mor*	Ch	(Tsakris *et al.*, 2007b)

Table 3: Contd.

Enzyme	Organism	Accession number	Country	Isolation year(s)	Genetic context of structures carrying bla_{VIM} genes*	Integron	Localisation	Reference
	Escherichia coli	AY970968	Spain	2003	***[bla$_{VIM-1}$]*** → *[aacA4]* → *[dfrII]* → *[aadA1]* → *[catB2]* → *[qacEΔ1/sul1]*	In*113*	pMVH202	(Tórtola *et al.*, 2005)
VIM-1	*Klebsiella pneumoniae*	AY987853	Spain	2003	***[bla$_{VIM-1}$]*** → *[aacA4]* → *[dfrII]* → *[aadA1]* → *[catB2]* → *[qacEΔ1/sul1]*	In*113*	pMVH202	(Tórtola *et al.*, 2005)
	Pseudomonas aeruginosa	-	France	2004-2005	***[bla$_{VIM-1}$]*** → *[aacA4]* → *[aphA15]* → *[aadA1]* → *[qacEΔ1/sul1]*	In*70.2-like*	Ch	(Corvec *et al.*, 2006)
	Acinetobacter spp. baumannii	DQ112355	Greece	2004-2005	***[bla$_{VIM-1}$]*** → *[aacA7]* → *[dhfr1]* → *[aadA1]* → *[qacEΔ1/sul1]*	+	(?)	(Tsakris *et al.*, 2006)
	Klebsiella pneumoniae	AJ870988	France	2004	***[bla$_{VIM-1}$]*** → *[aacA7]* → *[dhfr1]* → *[aadA1]* → *[qacEΔ1/sul1]*	+	pPBH01	(Kassis-Chikhani *et al.*, 2006)
	Pseudomonas aeruginosa	AJ784256	Italy	1999-2002	***[bla$_{VIM-1}$]*** → *[smr]* → *[orf1]* → *[aacA4]* → *[Pse-1]* → *[qacEΔ1/sul1]*	+	Ch	(Toleman *et al.*, 2005)
	Pseudomonas aeruginosa	AJ784804	Italy	1999-2002	***[bla$_{VIM-1}$]*** → *[aacA4]* → *[aphA15]* → *[aadA1]* → *[qacEΔ1/sul1]*	In*70-like*	Ch	(Toleman *et al.*, 2005)
	Pseudomonas aeruginosa	AJ784805	Italy	1999-2002	***[bla$_{VIM-1}$]*** → *[aacA4]* → *[aphA15]* → *[aadA1]* → *[qacEΔ1/sul1]*	In*70-like*	Ch	(Toleman *et al.*, 2005)
	Pseudomonas aeruginosa	-	Italy	1999-2002	***[bla$_{VIM-1}$]*** → *[aacA4]* → *[[??]]* → *[aacA4]* → *[qacEΔ1/sul1]*	+	Ch	(Toleman *et al.*, 2005)
	Enterobacter cloacae	-	Spain	2005-2006	***[bla$_{VIM-1}$]*** → *[aacA4]* → *[aadA1]* → *[qacEΔ1/sul1]*	+	p	(Tato *et al.*, 2007)
	Klebsiella oxytoca	-	Spain	2005-2006	***[bla$_{VIM-1}$]*** → *[aacA4]* → *[aadA1]* → *[qacEΔ1/sul1]*	+	p	(Tato *et al.*, 2007)

Table 3: Contd.

Enzyme	Organism	Accession number	Country	Isolation year(s)	Genetic context of structures carrying bla_{VIM} genes*	Integron	Localisation	Reference
VIM-2	*Pseudomonas aeruginosa*	AF191564	France	1996	***[bla$_{VIM-2}$]*** → *[qacEΔ1/sul1]*	In*56*	p	(Poirel *et al.*, 2000b)
	Pseudomonas aeruginosa	AF263520	France	1997	*[aacA7]* → ***[bla$_{VIM-2}$]*** → *[aacC1]* → *[aacA4]* → *[qacEΔ1/sul1]*	In*58*	Ch	(Poirel *et al.*, 2001)
	Pseudomonas aeruginosa	AF263519	France	1997	*[aacA29a]* → ***[bla$_{VIM-2}$]*** → *[aacA29b]* → *[qacEΔ1/sul1]*	In*59*	Ch	(Poirel *et al.*, 2001)
	Achromobacter xylosoxidans	AY686225	Korea	2001-2003	*[aacA4]* → *[aacA4]* → ***[bla$_{VIM-2}$]*** → *[aacA4]* → *[qacEΔ1/sul1]*	+	Ch	(Shin *et al.*, 2005)
	Pseudomonas aeruginosa	AJ515707	Poland	2002	***[bla$_{VIM-2}$]*** → *[aacA4]* → *[qacEΔ1/sul1]*	In*58-like*	Ch	(Walsh *et al.*, 2003)
	Pseudomonas aeruginosa	AY560837	Portugal	2000	***[bla$_{VIM-2}$]*** → *[aacA4]* → *[bla$_{P1b}$]* → *[aadA2]* → *[qacEΔ1/sul1]*	In*100*	Ch	(Quinteira *et al.*, 2005b)
	Pseudomonas aeruginosa	AM296017	India	2003	*[aacA7]* → *[bla$_{VIM-2}$]* → *[dhfrB5]* → *[aacC6-II]* → *[ISPa21]* → *[tnpA]* → *[tniC]*	+	Tn5090 (Tn402)/ pVIM-2_MAT	(Toleman *et al.*, 2007)
	Pseudomonas aeruginosa	AY507153	France	1996-2004	***[bla$_{VIM-2}$]*** → *[bla$_{OXA-2}$]* → *[aacA4]* → *[aadB]* → *[qacG]* → *[tniC]*	In*122*	Tn5090 (Tn402)/Ch	(Corvec *et al.*, 2008)
	Pseudomonas putida	DQ174113	France	2004	*[ISPpu17]* → *[aacA4]* → ***[bla$_{VIM-2}$]*** → *[aadA1]* → *[bla$_{OXA-9}$]* → *[tnpR]* → *[bla$_{TEM-1}$]* → *[ISPpu18]*	-	Tn1332/p	(Poirel *et al.*, 2006)
	Escherichia coli	AY781413	Greece	2004	***[bla$_{VIM-2}$]*** → *[qacEΔ1/sul1]*	In*8*	Ch	(Galani *et al.*, 2006)
	Providencia rettgeri	-	Korea	2004	***[bla$_{VIM-2}$]*** → *[??]*	(?)	(?)	(Lee *et al.*, 2007)
	Pseudomonas putida	EF614235	Spain	2003	*[aac(6')-32]* → ***[bla$_{VIM-2}$]*** → *[tnpA]* → *[qacEΔ1/sul1]*	+	pV2GY3	(Gutiérrez *et al.*, 2007)

Table 3: Contd.

Enzyme	Organism	Accession number	Country	Isolation year(s)	Genetic context of structures carrying bla_{VIM} genes*	Integron	Localisation	Reference
	Pseudomonas aeruginosa	AM180753	Italy	1999	***[bla_{VIM-2}]*** → *[aacA7]* → *[tniC]*	In*71*	Tn402/ Ch	(Lagatolla *et al*., 2006)
VIM-2	*Pseudomonas aeruginosa*	DQ353808	Italy	2002	*[aadB]* → ***[bla_{VIM-2}]*** → *[qacEΔ1/sul1]*	In*74*	Ch(?)	(Lagatolla *et al*., 2006)
	Acinetobacter spp.	-	Korea	2000-2001	***[bla_{VIM-2}]*** → *[??]*	(?)	(?)	(Lee *et al*., 2003)
	Acinetobacter spp.	-	Korea	2003-2004	***[bla_{VIM-2}-like]*** → *[??]*	(?)	(?)	(Yong *et al*., 2006)
	Citrobacter freundii	-	Taiwan	2000	***[bla_{VIM-2}]*** → *[??]*	(?)	p	(Yan *et al*., 2002)
	Pseudomonas aeruginosa	-	Turkey	(?)	***[bla_{VIM-2}]*** → *[aacA4]* → *[qacEΔ1/sul1]*	In*60*	Ch	(Yakupogullari *et al*., 2008)
	Pseudomonas aeruginosa	-	Canada	2002-2005	***[bla_{VIM-2}]*** → *[qacEΔ1/sul1]*	+	(?)	(Pitout *et al*., 2007)
	Pseudomonas aeruginosa	-	Canada	2002-2005	***[bla_{VIM-2}]*** → *[aacC1]* → *[qacEΔ1/sul1]*	+	(?)	(Pitout *et al*., 2007)
	Pseudomonas aeruginosa	-	Canada	2002-2005	***[bla_{VIM-2}]*** → *[aacA4]* → *[aacC1]* → *[qacEΔ1/sul1]*	+	(?)	(Pitout *et al*., 2007)
	K. oxytoca	-	Portugal	(?)	***[bla_{VIM-2}]*** → *[aacA4]* → *[aadA1]* → *[bla_{OXA-2}]* → *[qacEΔ1/sul1]*	+	p	(Conceição *et al*., 2005)
	Escherichia coli	-	Greece	2001	***[bla_{VIM-2}]*** → *[bla_{IBC-1}]* → *[qacEΔ1/sul1]*	(?)	(?)	(Galani *et al*., 2004)
	Pseudomonas putida	-	Argentina	2005	***[bla_{VIM-2}]*** → *[??]*	(?)	(?)	(Almuzara *et al*., 2007)
	Pseudomonas aeruginosa	-	Greece	2001	***[bla_{VIM-2}]*** → *[qacEΔ1/sul1]* *[aacA29]* → ***[bla_{VIM-2}]*** → *[qacEΔ1/sul1]*	In*56-like* In*59-like*	(?) (?)	(Giakkoupi *et al*., 2003)
	Pseudomonas aeruginosa	-	Brazil	2000-2001	***[bla_{VIM-2}]*** → *[??]*	(?)	(?)	(Sader *et al*., 2005)

Table 3: Contd.

Enzyme	Organism	Accession number	Country	Isolation year(s)	Genetic context of structures carrying bla_{VIM} genes*	Integron	Localisation	Reference
	P. pseudoalcaligenes	AY685199	Portugal	(?)	***[bla_{VIM-2}]*** → *[qacEΔ1/sul1]*	In*56*	Ch	(Quinteira *et al.*, 2005a)
VIM-3	*Pseudomonas aeruginosa*	AF300454	Taiwan	1997-2000	***[bla_{VIM-3}]*** → *[??]*	(?)	Ch	(Yan *et al.*, 2001a)
	Pseudomonas aeruginosa	DQ393784	Taiwan	2003	***[bla_{VIM-3}]*** → *[orf2]* → *[aacA4]* → *[aacA4]* → *[aadB]* → *[aacA4]* → *[qacEΔ1/sul1]*	+	Ch	(Yan *et al.*, 2006)
	Pseudomonas aeruginosa	-	Taiwan	2000, 2002	***[bla_{VIM-3}]*** → *[??]*	+	(?)	(Huang *et al.*, 2007)
	Pseudomonas aeruginosa	EF138817	Taiwan	2003	***[bla_{VIM-3}]*** → *[orf2]* → *[aacA4]* → *[aacA4]* → *[aadB]* → *[aacA4]* → *[qacEΔ1/sul1]*	In*450*	Tn6001/ Ch	(Tseng *et al.*, 2007)
VIM-4	*Pseudomonas aeruginosa*	AY135661	Greece	2001	***[bla_{VIM-4}]*** → *[qacEΔ1/sul1]*	+	(?)	(Pournaras *et al.*, 2002)
	Pseudomonas aeruginosa	-	Sweden	2001	***[bla_{VIM-4}]*** → *[??]*	(?)	(?)	(Giske *et al.*, 2003)
	Pseudomonas aeruginosa	AJ585042	Poland	1997-2001	*[aacA4]* → ***[bla_{VIM-4}]*** → *[qacEΔ1/sul1]*	+	Ch	(Patzer *et al.*, 2004)
	Pseudomonas aeruginosa	AY509609	Hungary	2002	*[bla_{OXA-10}]* → *[aacA4]* → ***[bla_{VIM-4}]*** → *[qacEΔ1/sul1]*	+	Ch(?)	(Libisch *et al.*, 2004)
	Klebsiella pneumoniae	AM181293	Tunisia	2005	***[bla_{VIM-4}]*** → *[aacA7]* → *[dhfrAI]* → *[aadA1]* → *[qacEΔ1/sul1]*	+	Ch	(Ktari *et al.*, 2006)
	Pseudomonas aeruginosa	AY509609	Hungary	2003-2005	*[bla_{OXA}]* → *[aacA4]* → ***[bla_{VIM-4}]*** → *[qacEΔ1/sul1]*	+	(?)	(Libisch *et al.*, 2006)
	Pseudomonas aeruginosa	AY702100	Hungary	2003-2005	*[aacA4]* → ***[bla_{VIM-4}]*** → *[qacEΔ1/sul1]*	+	(?)	(Libisch *et al.*, 2006)

Table 3: Contd.

Enzyme	Organism	Accession number	Country	Isolation year(s)	Genetic context of structures carrying *bla*$_{VIM}$ genes*	Integron	Localisation	Reference
VIM-4	*Pseudomonas putida*	-	Belgium	2004-2007	***[aacA4]*** → ***[bla$_{VIM-4}$]*** → ***[qacEΔ1/sul1]***	+	(?)	(Bogaerts *et al*., 2008)
VIM-5	*Klebsiella pneumoniae*	AY144612	Turkey	2002	***[bla$_{VIM-5}$]*** → ***[??]***	(?)	(?)	(Midilli *et al*., 2003)
	Pseudomonas aeruginosa	AY456196	Turkey	2003	***[bla$_{VIM-5}$]*** → ***[??]***	(?)	(?)	(Bahar *et al*., 2004)
	Enterobacter cloacae	DQ023222	Turkey	before 2002	***[bla$_{VIM-5}$]*** → ***[orfD]*** → ***[qacEΔ1/sul1]***	+	pEDV5	(Gacar *et al*., 2005)
VIM-6	*Pseudomonas putida*	AY165025	Singapore	2000	***[bla$_{VIM-6}$]*** → ***[??]***	(?)	p	(Koh *et al*., 2004b)
VIM-7	*Pseudomonas aeruginosa*	AJ536835	USA	2001	***[bla$_{VIM-7}$]*** → ***[??]***	+	p	(Toleman *et al*., 2004)
	Pseudomonas aeruginosa	-	USA	2003	***[bla$_{VIM-7}$]*** → ***[??]***	+	(?)	(Aboufaycal *et al*., 2007)
VIM-8	*Pseudomonas aeruginosa*	AY524987	Colombia	1999-2002	***[bla$_{VIM-8}$]*** → ***[??]***	(?)	(?)	(Crespo *et al*., 2004)
VIM-9	*Pseudomonas aeruginosa*	AY524988	United Kingdom	2004	***[bla$_{VIM-9}$]*** → ***[??]***	(?)	(?)	(**)
VIM-10	*Pseudomonas aeruginosa*	AY524989	United Kingdom	2004	***[bla$_{VIM-10}$]*** → ***[??]***	(?)	(?)	(**)
VIM-11	*Pseudomonas aeruginosa*	AY605049	Argentina	2002	***[bla$_{VIM-11}$]*** → ***[??]***	+	(?)	(Pasteran *et al*., 2005)
	Pseudomonas aeruginosa	AY635904	Italy	2004	***[bla$_{VIM-11}$]*** → ***[??]***	(?)	(?)	(**)

Table 3: Contd.

Enzyme	Organism	Accession number	Country	Isolation year(s)	Genetic context of structures carrying *bla*$_{VIM}$ genes*	Integron	Localisation	Reference
VIM-11	*Pseudomonas aeruginosa*	-	Taiwan	(?)	***[bla***$_{VIM-11}$***]*** → *[??]*	+	(?)	(Lu *et al*., 2008)
VIM-12	*Klebsiella pneumoniae*	DQ143913	Greece	2005	*[aacA7]* → ***[bla***$_{VIM-12}$***]*** → *[aacA7]* → *[qacEΔ1/sul1]*	In*h12*	p2873	(Pournaras *et al*., 2005)
	Escherichia coli	-	Greece	2006	*[aacA7]* → ***[bla***$_{VIM-12}$***]*** → *[aacA7]* → *[qacEΔ1/sul1]*	In*h12*	(?)	(Ikonomidis *et al*., 2007a)
	Klebsiella pneumoniae	-	Greece	2006-2007	*[aacA7]* → ***[bla***$_{VIM-12}$***]*** → *[aacA7]* → *[qacEΔ1/sul1]*	In*h12*	p	(Tokatlidou *et al*., 2008)
VIM-13	*Pseudomonas aeruginosa*	DQ365886	Spain	2006	***[bla***$_{VIM-13}$***]*** → *[??]*	(?)	(?)	(**)
VIM-14	*Pseudomonas aeruginosa*	AY635904.1	Italy	2004	***[bla***$_{VIM-14}$***]*** → *[??]*	(?)	(?)	(**)
VIM-18	*Pseudomonas aeruginosa*	AM778091	India	2007	***[bla***$_{VIM-18}$***]*** → *[??]*	(?)	(?)	(**)

NOTE

* According to reference and/or GenBank [Available at: *http://www.ncbi.nlm.nih.gov/Genbank/index.html.* Accessed June 6, 2008];

(**) Data only in GenBank ;

[??] - no data; **(?)** – no precise data; **+** – presence of integron; **Ch** – chromosome; **p** – plasmid; **Tn** – transposone.

bla$_{VIM-1}$**,** ***bla***$_{VIM-2}$**,** ***bla***$_{VIM-3}$**,** ***bla***$_{VIM-4}$**,** ***bla***$_{VIM-5}$**,** ***bla***$_{VIM-6}$**,** ***bla***$_{VIM-7}$**,** ***bla***$_{VIM-8}$**,** ***bla***$_{VIM-9}$**,** ***bla***$_{VIM-10}$**,** ***bla***$_{VIM-11}$**,** ***bla***$_{VIM-12}$**,** ***bla***$_{VIM-13}$**,** ***bla***$_{VIM-14}$**,** ***bla***$_{VIM-18}$ – VIM-type metallo-β-lactamase genes; ***aacA4*****,** ***aacA7*****,** ***aac6*****,** ***aac(6')-Ib*****,** ***aac(6')-Ilc*****,** ***aacA*****,** ***aacC1*****,** ***aacA29a*****,** ***aacA29b*****,** ***aacA29*****;** ***aacC6-II*****,** ***aac(6')-32*****,** ***aacC-A5*****,** ***aacA8*** – aminoglycoside acetyltransferase genes; ***aphA15*****,** ***aph15*****,** ***aph*** – aminoglycoside phosphotransferase gene; ***aadA1*****,** ***aadA*****,** ***aadA2*****,** ***aadB*****,** ***aadA6*** – aminoglycoside adenyltransferase genes; ***dhfrI*****,** ***dfrA1*****,** ***dhfrA1*****,** ***dhfr*****,** ***dfrII*****,** ***dhfrB5*****,** ***dfrI*** – dihydrofolate reductase genes; ***sat1*** – streptothricin acetyltransferase 1 gene; ***catB2*** – chloramphenicol acetyltransferase gene; ***smr*** – small multidrug resistance protein gene; ***orf1*****,** ***orfII*****,** ***orfIII*****,** ***orfD*** – open reading frames; ***Pse-1*** – PSE-1 β-lactamase; ***bla***$_{P1b}$ – PSE-1/CARB-2 β-lactamase gene; **IS*Pa21*****,** **IS*Ppu17*****,** **IS*Ppu18*** – insertion sequences; ***tnpA*****,** ***tnpR*** – transposase genes; ***tniC*** – invertase gene; ***bla***$_{OXA}$**,** ***bla***$_{OXA-2}$**,** ***bla***$_{OXA-9}$**,** ***bla***$_{OXA-10}$**,** ***bla***$_{OXA-58}$**,** ***bla***$_{OXA-69}$ – OXA-type β-lactamase genes; ***qacG*****,** ***qacF'*** – quaternary ammonium compound-resistance protein genes; ***bla***$_{TEM-1}$ – TEM-1 β-lactamase gene; ***bla***$_{IBC-1}$ – IBC-1 β-lactamase gene;

N-terminus to the start of the loop, SPM-1 exhibits 31% identity to IMP-1, whereas, from (but not including) the loop to the C-terminus, SPM-1 exhibits 47% identity to IMP-1 (Arakawa *et al*., 1995; Toleman *et al*., 2002). Although it is more speculation, it is interesting to raise the possibility that SPM-1 could be a hybrid protein of two ancestral class B type enzymes, one being more IMP-1-like than the other. The alignment of SPM-1 with the other class B enzymes clearly shows that SPM-1 contains the classic HXHXD motif (residues 116 to 120) (Galleni *et al*., 2001) as well as key histidine (positions 165 and 221) and cysteine (position 184) residues. This motif and other residues coordinate two zinc ions per molecule of enzyme, which are required for the bridging of water molecules involved in the hydrolytic pathway (Toleman *et al*., 2002).

The genetic context of bla_{SPM-1} is unique in that it is immediately associated with a novel genetic element designated common region (CR; now IS*CR*) (Toleman *et al*., 2006b), and not with integrons and transposons (Poirel *et al*., 2004a). These elements constitute a tool for dissemination of bla_{SPM-1} gene. Interestingly, these common elements differ significantly in *Pseudomonas aeruginosa* strains collected from different areas of Brazil even though the bla_{SPM} genes are identical (Walsh *et al*., 2005a).

The spread of clonally related carbapenem-resistant *Pseudomonas aeruginosa* containing bla_{SPM-1} was found in Recife, Brazil (Poirel *et al*., 2004a). Upstream of bla_{SPM-1} gene IS*CR4* was identified. The genetic structures surrounding the bla_{SPM-1} revealed an open reading frame *orf495* (transposase gene of IS*CR4*) encoding 495 amino acids (Fig. 12). The product of *orf495* had 81% amino acid identity with *orf2*, identified in the multidrug resistance region of an SXT-type conjugative transposon from *Vibrio cholerae* (Hochhut *et al*., 2001) and considered a recombinase of the so-called *CR2* element (Partridge and Hall, 2003), 58% identity with *orfA*, identified in an *Escherichia coli* strain (Cloeckaert *et al*., 2000) and part of the so-called *CR3* (Partridge and Hall, 2003). Analysis

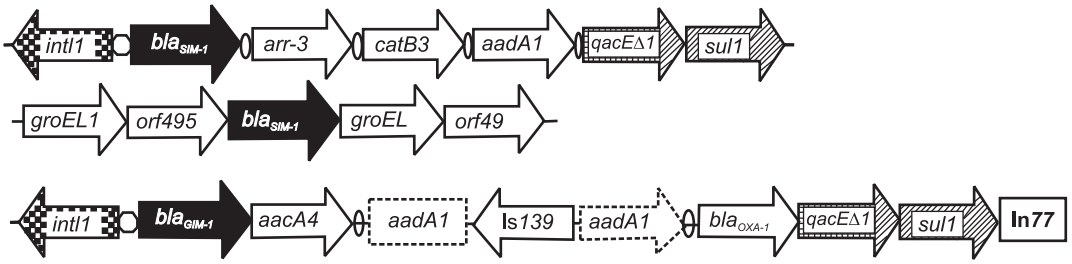

Fig. 12: Open reading frames are indicated by arrows; the *attC* recombination site (59-base element sequence) of gene cassettes are indicated by ovals; the gray circles represent the recombination site *attI1*. The *intI* integrase gene, that encodes an integrase, is part of the 5'-CS; the 3'-CS located downstream of the integrated gene cassettes include the sulphonamide resistance gene *sul1* and the disinfectant resistance determinant *qacEΔ1*, the *orf5* is not shown.

IntI1, IntI1 DNA integrase gene; bla_{SIM-1}, metallo-beta-lactamase SIM-1 gene; *arr-3*, rifampin ADP-ribosylating transferase gene; *catB3*, chloramphenicol acetyltransferase gene; *aadA1*, 3'-(9)-O-adenylyltransferase gene, *qacEΔ1*, QacEdelta1 multidrug exporter (disinfectant resistance protein and ethidium bromide resistance protein) gene; *sul1*, Sul1 dihydropteroate synthase gene; *groEL1*, GroEL protein 1, gene; *groEL2*, GroEL protein 2 gene; *orf495*, transponase gene of IS*CR4* element; bla_{GIM-1}, metallo-beta-lactamase GIM-1 gene; *aacA4*, 6'-N- aminoglycoside acetyltransferase type II gene, IS*1394*, insertion element; bla_{OXA-2}, OXA-2 beta-lactamase gene.

of the nucleotide sequences located between *orf495* and bla_{SPM-1} revealed a similar 33-bp right-hand boundary, sharing 31, 24, and 24 bp with those of *CR3*, *CR2*, and *CR1*, respectively. Thus, *orf495* may be part of a novel transposable structure called IS*CR4* (Poirel *et al*., 2004a; Toleman *et al*., 2006b). Upstream of *orf495*, the sequence encoding the C-terminal extremity of a putative GroEL chaperonin protein (called GroEL1) was present, with its product sharing 89 and 87% amino acid identity with GroEL proteins from *Desulfitobacterium hafniense* and *Stenotrophomonas maltophilia*, respectively, with two hairpin structures likely playing a role in transcription ending. Downstream of bla_{SPM-1}, sequence encoding an N-terminally truncated part of a similar protein (called GroEL2) was present. The C-terminal extremity of the GroEL2-encoding sequence present downstream of bla_{SPM-1} was very similar (94% nucleotide identity) to that of GroEL1. In addition, the noncoding sequences located downstream of both *groEL* genes were identical. The 5′ end of *orf495* was identified in the 3′-end extremity of the *groEL2* gene in examined sequence (Poirel *et al*., 2004a), as well as in that reported by Toleman *et al.* (105). Thus, at least part of *orf495* is also present on both sides of bla_{SPM-1}. These data indicated a possible duplication of part of the target genetic structures subsequent to a mobilization process that could result, as suggested (Partridge and Hall, 2003), from a one-ended mobilization event enhancing the spread of bla_{SPM-1}. Analysis of the 220 bp separating *orf495* from bla_{SPM-1} revealed putative promoter sequences made of a -35 motif (TTGAAT) provided by the *CR4* boundary and a putative original –10 motif (TACAAT) of bla_{SPM-1}, constituting a hybrid promoter (Poirel *et al*., 2004a).

Similar genetic organization surrounding the bla_{SPM-1} gene was also detected in another institution, in Rio de Janeiro (Carvalho *et al*., 2006). DNA sequencing confirmed the bla_{SPM-1} gene and revealed upstream of bla_{SPM-1}, a transposable the IS*CR4* element, comprising an open reading frame (*orf495*) whose product shares significant identity with putative recombinases of other CR elements (Rasmussen and Bush, 1997). This sequence was identical to the previously published sequence of SPM-1-positive *Pseudomonas aeruginosa* isolate obtained from Recife, by Poirel *et al*. (2004a).

Finally, a strain *Pseudomonas aeruginosa* that produces the MBL SPM-1 carries two IS*CR* elements, IS*CR4* and a new element, IS*CR12* that encodes a putative transposase with 89% identity to the IS*CR3* transposase and 75% identity to the IS*CR4* transposase (Toleman *et al*., 2006b).

Kinetic analysis demonstrated that SPM-1 has a broad hydrolytic profile across a wide range of β-lactam antibiotics (Murphy, Simm, Toleman, Jones, Walsh, 2003). Considerable variation was observed within the penicillin, cephalosporin, and carbapenem subfamilies. However, on the whole, SPM-1 appears to preferentially hydrolyze cephalosporins. The highest k_{cat}/K_m ratios (in micromolar per second) overall were observed for this subgroup.

SPM-1 was found to have high K_m values for the carbapenems, with meropenem having a K_m of 281 μM, whereas the K_m values for IMP-1 (Laraki et al., 1999a), VIM-1 (Franceschini *et al*., 2000), and VIM-2 (Poirel *et al*., 2000b) were 10, 48, and 5 μM, respectively. The K_m value for imipenem was 37 μM, similar to that reported for IMP-1 (39 μM) (Laraki et al., 1999a), while VIM-1 and VIM-2 have been reported to have significantly lower K_m values for imipenem: 1.5 and 10 μM, respectively (Franceschini *et*

al., 2000, Poirel *et al*., 2000b). SPM-1 was found to have k_{cat} values of 63 s^{-1} for meropenem and 33 s^{-1} for imipenem, which are significantly higher than the k_{cat} values of VIM-1 (for imipenem, 2 s^{-1}; for meropenem, 13 s^{-1}) (Franceschini *et al*., 2000) and VIM-2 (for imipenem, 10 s^{-1}; for meropenem, 1 s^{-1}) (Poirel *et al*., 2000b). The k_{cat}/K_m values of SPM-1 for both imipenem and meropenem are comparable to those of IMP-1, VIM-1, and VIM-2. SPM-1 showed no hydrolysis of the monobactams (aztreonam) or the serine β-lactamase inhibitor clavulanic acid (Murphy *et al*., 2003), like IMP-1 and VIM-1.

After the first report of SPM-1 in a *Pseudomonas aeruginosa* isolate (Toleman *et al*., 2002), this enzyme has disseminated throughout Brazil (Gales, Menezes, Silbert, Sader, 2003). The dissemination of an epidemic *Pseudomonas aeruginosa* producing SPM-1 has been demonstrated in distinct regions of Brazil (Gales *et al*., 2003). High carbapenem resistance rates have been reported among *Pseudomonas aeruginosa* isolates, resulting from the presence of an epidemic clone designated SP clone (Gales, Jones, Turnidge, Rennie, Ramphal, 2001; Sader *et al*., 2001). The first SPM-1-producing *Pseudomonas aeruginosa* strain also belongs to clone SP (Gales *et al*., 2003). A survey performed by Gales *et al*. (2003) showed that among the isolates belonging to SP clone almost all isolates were positive by PCR for bla_{SPM-1}. The SP clone usually exhibit high levels of resistance to carbapenems, broad-spectrum cephalosporins, fluoroquinolones and aminoglycosides (Gales *et al*., 2003). Risk factors for SPM involved the use quinolones as a predictor for the acquisition of multidrug-resistant *Pseudomonas aeruginosa* carrying the bla_{SPM} gene. It is possible that quinolones could select for other resistance mechanisms present in MBL-producing isolates and thus indirectly select for these strains (Nouér, Nucci, de-Oliveira, Pellegrino, Moreira, 2005). On the other hand, it would be expected that in the case of colonization or infection by bla_{SPM}, antibiotic pressure could play a lesser role, since horizontal transmission would be the main mechanism of acquisition (Paterson, 2002; Zavascki, Gaspareto, Martins, Gonçalves, Barth, 2005). However, it is possible that exposure to a particular antibiotic increases the burden of colonization by a clonal strain, increasing the possibility of horizontal transmission (Nouér et al*., 2005).*

The bla_{SPM-1} has already been detected in a large number of strains in centres of several Brazilian cities (Gales *et al*., 2003; Poirel *et al*., 2004a; Zavascki *et al*., 2005; Carvalho *et al*., 2006; Martins *et al*., 2007), and the first nosocomial outbreak of *Pseudomonas aeruginosa* producing SPM-1 MBL has been described in southern Brazil (Zavascki *et al*., 2005). In some Brazilian centres the SPM can be found at least a 50% and more amongst MBL-producing *Pseudomonas aeruginosa* (Sader *et al*., 2005). SPM-1-producing isolates may have been silently disseminating in Brazil for a long time (Pellegrino, Casali, Nouér, Riley, Moreira, 2008). *Pseudomonas aeruginosa* strain carrying the bla_{SPM-1} gene, but does not produce carbapenemase was reported (Pellegrino *et al*., 2008). The bla_{SPM-1} gene was identical in sequence to the gene of other fully carbapenem-resistant isolates and the gene presented by Toleman *et al.* (2002), including the IS*CR4* sequence, the promoter region, and the coding sequence.

For the Brazilian region characteristically is that the high prevalence of SPM-1 metallo-β-lactamase and 16S rRNA methylase (RmtD) coproduction among imipenem-resistant *Pseudomonas aeruginosa* isolates (Doi, Ghilardi, Adams, de Oliveira Garcia, Paterson, 2007b). Coproduction of SPM-1 and RmtD is a common phenomenon observed

features of the recombination site structures (core site GTTRRRY, inverse core site RYYYAAC, and the internal domains 2L and 2R). Finally, the *aadA1* gene cassette is followed by another β-lactamase gene cassette accommodating bla_{OXA-2} (Fig. 12) (Castanheira *et al*., 2004).

The nucleotide sequence of bla_{GIM-1} showed a G+C content of 42.1% and encoded a mature protein of 25 501 Da. The amino acid sequence of GIM-1 had low identity with other clinically significant MBL genes that displayed most identity with IMP variants IMP-6 (43.5%), IMP-1 (43.1%), and IMP-4 (43.1%), followed by VIM variants ranging from a high of 31.2% with VIM-7 to 28.8% with VIM-1, VIM-4, and VIM-5 and only 28.0% similarity with SPM-1. Phylogenetic relatedness of GIM-1 to other metallo-β-lactamase is presented on Figure 9.

GIM-1 possess the major consensus features of the MBLs class B1 family (Bush, 1998; Galleni *et al*., 2001; Garau *et al*., 2004), such as the principal zinc binding motif HXHXD (residues 116 to 120) and the other residues involved in the coordination of the Zn^{2+} ions (Hys196, Cys221, and Hys293, according to the BBL numbering system) (Galleni *et al*., 2001). However, the zinc-binding motif of GIM-1 was unique in that there was a serine at position 117 and a glutamic acid residue at position 119, amino acid residues that were not found at these positions in other MBL enzymes (Castanheira *et al*., 2004).

GIM-1 demonstrates a hydrolytic profile similar to that of IMP-1, but is arguably a weaker enzyme. The low k_{cat}/K_m ratios determined for GIM-1 with most β-lactam antimicrobial agents reflect high substrate affinities (K_m) and low substrate turnover rates (k_{cat}). GIM-1 has no clear preference for any substrate and did not hydrolyze azlocillin, aztreonam, and the serine-β-lactamase inhibitors. For the carbapenems, GIM-1 showed 10 times greater turnover of imipenem than meropenem (k_{cat} values of 27.1 s^{-1} and 2.7 s^{-1}, respectively). However, the affinity of the enzyme for imipenem is 10 times higher than for meropenem (K_m of 287.5 and 25.4 μM, respectively), which makes the k_{cat}/K_m ratios for the two compounds very similar (0.094 for imipenem and 0.106 μM^{-1} s^{-1} for meropenem). This finding contrasts those for other clinically important class B β-lactamases, which show larger k_{cat}/K_m ratios for imipenem than for meropenem (Castanheira *et al*., 2004).

5.3. SIM Metallo-β-Lactamase

A novel acquired MBL gene, bla_{SIM-1}, was detected in seven clinical isolates of *Acinetobacter baumannii* from Korea, where VIM-2- and IMP-1-type MBL-producing *Pseudomonas aeruginosa* and *Acinetobacter* spp. have been prevalent (Lee *et al*., 2005). The SIM-1 (**S**eoul **IM**ipenemase) protein is a new member of subclass B1, and exhibits 64 to 69% identity with the IMP-type MBLs (IMP-9 and IMP-12, respectively), which are its closest relatives.

The bla_{SIM-1} gene was associated as gene cassette with a class 1 integron, which included three additional gene cassettes (*arr-3*, *catB3*, and *aadA1*) (Fig. 12). The bla_{SIM-1} gene cassette was located in the first position of integron. The *attC* (59-base element) recombination site of the bla_{SIM-1} cassette was 88 bp long and not related to those of

known bla_{IMP} cassettes but was very similar (90% identity) to that of a cassette (*ypar13*) of the *Pseudomonas alcaligenes* In*55044* super-integron (Vaisvila *et al*., 2001), suggesting that the source of the bla_{SIM-1} cassette could be a similar element. It is interesting that the other cassettes carried *arr-3*, a rifampin ADP ribosyltransferase gene, and *catB3*, a chloramphenicol acetyltransferase gene, which are rarely found in MBL-encoding integrons (Houang *et al*., 2003). These two genes were located in the second and third position of integron, and the last gene cassette had *aadA1* gene. DNA analysis suggests that the bla_{SIM-1} gene was located on the chromosome (Lee *et al*., 2005).

SIM-1 enzyme is capable of hydrolyzing a broad array of β-lactams, including penicillins, narrow- to expanded-spectrum cephalosporins, and carbapenems. All SIM-1 producing *Acinetobacter baumannii* isolates exhibited relatively low imipenem and meropenem MICs (8 to 16 μg/ml) and had a multidrug resistance phenotype. SIM-1 is only not active against aztreonam and piperacillin (Lee *et al*., 2005).

Clinical features of patients suggest that the acquisition of SIM-1-producing *Acinetobacter baumannii* was not strictly related to the use of carbapenems. Only one patient had received meropenem therapy before the isolation bla_{SIM-1}-carrying *Acinetobacter baumannii*. The first four SIM-1-producing isolates were detected sporadically, from different settings, during a period of 14 months, while the last three isolates were detected over a period of 11 days from patients in the same ward, suggesting the occurrence of a small outbreak. Of the two clonal lineages of SIM-1-producing isolates, one was represented by the sporadic isolates, and the other was represented by those involved in the outbreak (Lee *et al*., 2005).

The presence of apparently the same integron in isolates belonging to different clonal lineages indicates that horizontal transfer of this gene has occurred and suggests that further spread of the resistance may occur in the future (Lee *et al*., 2005).

6. CONCLUSIONS

During the last decade, acquired metallo-β-lactamases (MBLs) have started to emerge among *Pseudomonas aeruginosa* isolates and other Gram-negative nosocomial pathogens as *Acinetobacter* spp. and *Enterobacteriaceae* family (Livermore and Woodford, 2000; Bush, 2001; Nordmann and Poirel, 2002; Walsh *et al*., 2005a; Queenan and Bush, 2007). The production of these enzymes, which exhibit an exceedingly broad substrate specificity and which are not susceptible to conventional β-lactamase inhibitors (Franceschini *et al*., 2000; Poirel *et al*., 2000b; Murphy *et al*., 2003; Lagatolla *et al*., 2004), enables the microbial host to be resistant to virtually all β-lactams (including carbapenems) and drastically reduces the repertoire of agents useful for antimicrobial chemotherapy. Only the aztreonam remains fully active *in vitro*.

Acquired MBL genes are often clustered with other resistance determinants, especially with aminoglycoside-modifying enzymes, within the variable region of integrons or sometimes IS*CR* elements (Nordmann and Poirel, 2002, Walsh *et al*., 2005a; Bebrone, 2007). The recombination system based on integrons and mobile gene cassettes plays a major role in the dissemination of these genes among various pathogens.

Resistance to carbapenem due to the production of metallo-β-lactamases in Gram-negative pathogens is an international public health problem. Dissemination of MBLs among Gram-negative bacteria in large geographic health care regions is dynamic processes that require continuous molecular surveillance.

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