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Molecular typing indicates an important role for two international clonal complexes in dissemination of VIM-producing *Pseudomonas aeruginosa* clinical isolates in Hungary

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Abstract

VIM metallo-β-lactamase-producing serotype O11 or O12 *Pseudomonas aeruginosa* isolates infecting or colonising 19 patients from seven hospitals were reported in Hungary between 2003 and 2005. In this study we characterised VIM-producing *Pseudomonas* spp. clinical isolates from two novel locations in Hungary; we identified three new bla_{VIM} carrying integron types and the presence of the bla_{VIM-2} allele in Hungary. By applying various typing techniques, including multilocus sequence typing, we revealed an important role of two international clonal complexes, CC4 and CC11, in the dissemination of bla_{VIM}-positive *P. aeruginosa* in hospitals in Hungary. Isolate P12-Q, a representative strain from France of the major European multiresistant P12 clone, displayed ST111 which, according to eBURST analysis, is the presently calculated founder sequence type of CC4. This is in accordance with the wide geographic distribution of the P12 clone. Our data indicate that, although the CC4 clonal complex includes serotype O1 and O6 isolates as well, it also contains the P12 clone. We characterised a *P. aeruginosa* nosocomial clone with a singleton sequence type (ST313), that may have acquired bla_{VIM-2} and bla_{VIM-4} gene cassettes from a yet unidentified local gene pool in Hungary.

Keywords: Carbapenem; Metallo-β-lactamase; Clonal complex; Serotype; *Pseudomonas aeruginosa*; P12 clone; MLST; CC4; CC11

1. Introduction

Metallo-β-lactamases (MBLs) are emerging both in Europe and worldwide as acquired and worrisome resistance determinants in Gram-negative pathogens [17,29,33]. From the central and eastern European region, the first accounts of VIM-producing *Pseudomonas aeruginosa* were published from Croatia and Poland in 2003 [23,33] and from Hungary in 2004 [18]. During the subsequent year, molecular analysis revealed the involvement of hospitals from three different towns in northwestern Hungary in an outbreak caused by VIM-4-producing *P. aeruginosa* [19].

The bla_{VIM} type metallo-β-lactamase genes are mostly carried by class 1 integrons that can be embedded in transposons. These transposons, in turn, can be located on plasmids, resulting in a highly transmissible genetic apparatus [1,22,26,33]. VIM enzymes confer resistance against all β-lactam antibiotics except for the monobactams. The origin of the bla_{VIM} genes is as yet unknown and their dissemination is mediated by clonal spread as well as by horizontal gene transfer [29,33]. The VIM-type MBLs form two main clusters based on their deduced protein sequence, the VIM-1-like group

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(including VIM-4) and the VIM-2-like group, while VIM-7 represents an outgroup enzyme that is more distantly related to these two clusters. The VIM-4 and VIM-2 enzymes display 90% amino acid identity and their gene cassettes have different 59-base elements suggesting a separate origin for these two gene cassettes [27,33].

Although initially acquired MBL-positive strains have usually been reported sporadically or as causing smaller nosocomial outbreaks, in Italy it was shown that acquired MBLs can rapidly emerge and become endemic [15] and outbreaks caused by MBL-positive isolates were recently described in many geographical regions [4,7,13,16,20,35].

MBL-producing *P. aeruginosa* strains are associated with a higher case-fatality rate and invasive disease [16], and further dissemination of these genes among other Gram-negative clinical pathogens would significantly restrict opportunities for therapeutic use of β-lactam antibiotics [3].

An international comparison of VIM-1-like MBL-positive *P. aeruginosa* strains from Hungary, Sweden, Italy and Greece using the recently developed MLST (multilocus sequence typing) approach was useful in revealing clonal relatedness between the isolates, and it suggested a common ancestry for some of the VIM-1-like MBL-positive *P. aeruginosa* strains currently spreading in Europe [11]. This study indicated that two international clonal complexes, CC4 and CC11 (initially described as BG4 and BG11), are involved in the dissemination of bla_{VIM} in certain European hospitals [8,11].

We have been monitoring metallo-β-lactamase(MBL)-producing clinical isolates at the National Centre for Epidemiology in Hungary since 2003 [18]. In addition to the description of a cluster of MBL-producing *P. aeruginosa* infections in 2005 in northwestern Hungary [19], we also identified VIM-producing *Pseudomonas* spp. strains from other hospitals where these resistance determinants had not been detected before. Our aim was to characterise these isolates by molecular and phenotypic tools and to investigate their epidemiological relationship.

2. Materials and methods

2.1. Bacterial strains

VIM-producing *Pseudomonas* spp. strains first characterised in this study are listed in Table 1 together with earlier identified isolates PA396, PA555 and MB197 [19]. The ATCC 27853 *P. aeruginosa* strain was used as quality control strain. *P. aeruginosa* strain P12-Q, also listed in Table 1, was isolated from French patient "Q" during a study related to the major European multiresistant serotype O12 *P. aeruginosa* clone P12 [21].

2.2. Antibacterial susceptibility tests

Test organisms were inoculated onto plates of Mueller-Hinton agar (Oxoid, Basingstoke, UK) as recommended by the Clinical and Laboratory Standards Institute [5]. Minimal inhibitory concentrations (MICs) were determined by the

Table 1
Comparison of representative VIM-producing *Pseudomonas* spp. isolates from Hungary and isolate P12-Q according to various characteristics

Isolate	Town	Hospital[a]	Ward	Specimen	Time of isolation[b]	Species	Serotype	Integron[c]	VIM allele	PFGE type	RAPD type	Allelic profile[d]	ST	CC[e]
PA396	Budapest	KHK	ICU	Urine	08/2002	*P. aeruginosa*	O12	E	VIM-4	a1	A1	39-5-2-4-4-3	229[f]	4
PA555	Pécs	BMK	ICU	Urine	10/2003	*P. aeruginosa*	O12	D	VIM-4	a2	A2	39-5-2-4-4-3	229[f]	4
06–117	Budapest	OITI	ICU	Nasal swab	08/2002	*P. aeruginosa*	O12	A	VIM-4	a3	A3	17-5-5-4-4-3	111	4
P12-Q	Rodez	HR	Nursing home	Cutaneous pus	07/1993	*P. aeruginosa*	O12	ND	–	a4	A4	17-5-5-4-4-3	111	4
MB197	Győr	PAK	ICU	Blood	06/2005	*P. aeruginosa*	O11	F	VIM-4	b1	B1	38-11-3-13-1-2-4	235	11
06–150	Gyula	PKK	Traumatology	Wound secretion	09/2006	*P. aeruginosa*	O11	D	VIM-4	b2	B2	38-11-3-13-1-2-4	235	11
05–97	Budapest	SZIK	Burn unit	Wound secretion	04/2005	*P. aeruginosa*	O1	B	VIM-4	c	C1	47-8-7-6-8-11-40	313	S
05–207	Budapest	SZIK	Burn unit	Wound secretion	06/2005	*P. aeruginosa*	O1	B	VIM-4	c	C1			
05–98	Budapest	SZIK	Burn unit	Wound secretion	04/2005	*P. aeruginosa*	O1	C	VIM-2	c	C2	47-8-7-6-8-11-40	313	S
07–08	Veszprém	CSFK	Traumatology	Urine	01/2007	*P. putida*	–	D	VIM-4	–	–			

[a] Hospitals are indicated by the abbreviation of their Hungarian name.
[b] Time of isolation is given as mm/yyyy.
[c] Integrons A–D correspond to those in Fig. 1 with accession numbers EF693948, EF693946, EF693947 and AY702100, respectively. Integrons E and F are described in detail in refs. [18,19] with accession numbers AY509609 and DQ357197, respectively. ND stands for not determined.
[d] Allelic profiles indicate allele numbers for the *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE* genes, respectively [8].
[e] S stands for singleton.
[f] Allelic numbers and STs for these two isolates were determined previously [11].

agar dilution method [5] for β-lactam antibiotics and by the Etest (AB Biodisk, Solna, Sweden) for other antibiotics. Antimicrobial susceptibility test discs were purchased from Oxoid (Basingstoke, UK). To detect MBL production, the MBL Etest and the Combined Disk method [34] were used.

2.3. Molecular techniques

Detection of bla_{VIM} genes and class 1 integrons by PCR and their sequencing were performed as described [19]. The oligonucleotides used for sequencing and PCR mapping of integrons from the VIM-positive isolates were those described in refs. [18,19], and the following: VIM4-R2, 5′-CTACTCGG CGACTGAGCG-3′, VIM2-R2, 5′-AGTTCAGCCG-CCAGA ACG-3′; SulI-R1, 5′-GCCGAACACCGTCACCAT-3′; ORF5-R1, 5′-ACGAAGGTC-TCCGCGAAT-3′; aacA7-R, 5′-TTG CGCTGTTGGTAAGTTG-3′. The partial nucleotide sequence of the variable region of class 1 integrons A–D (Fig. 1) were deposited in GenBank under accession numbers EF693948, EF693946, EF693947 and AY702100.

2.4. Pulsed-field gel electrophoresis (PFGE) and serotyping

PFGE was performed according to the method described by Poh et al. with modifications [25]. Genomic DNA inserts were digested at 37 °C for 2.5 h with 20 U of SpeI enzyme. Electrophoresis was performed in a CHEF-DRII apparatus (Bio-Rad, Richmond, CA). DNA fingerprints were compared by the Fingerprinting II Informatix™ software (Bio-Rad) using a 1% band position tolerance and a cut-off value of 80% similarity by the Dice coefficient [11,30,32] to identify PFGE genotypes. Within a certain PFGE type, isolates not sharing an identical pattern were assigned to distinct subtypes. The 80% cut-off value corresponds to the criteria developed by Tenover et al. [11,12,32]. The statistical significance of the clusters of the dendrogram was tested by cophenetic correlation analysis, performed in Fingerprinting II Informatix™. The cut-off value for acceptance of the dendrogram was set at a minimum cophenetic correlation coefficient (CCC) value of 70% [11].

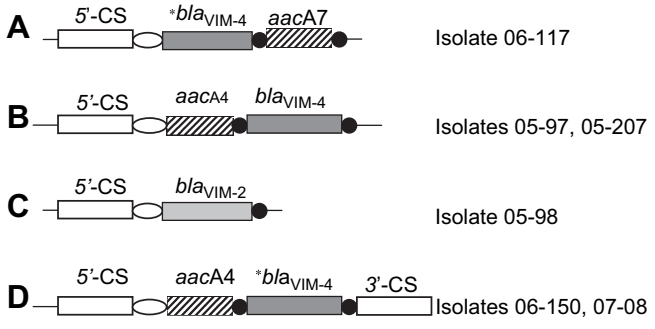

Fig. 1. The partial or full variable region of integrons of MBL-producing *P. aeruginosa* isolates first characterised in this study. Empty ellipses represent the attI1 site, black circles the 59-base elements. Asterisks indicate bla_{VIM} cassettes with the 170 bp duplication. 5′-CS and 3′-CS stand for the 5′ and 3′ conserved sequences, respectively.

The anti-*P. aeruginosa* in vitro agglutinating sera (Bio-Rad, Marnes-la-Coquette, France) were used for detection of serotypes.

2.5. Random amplification of polymorphic DNA (RAPD)

RAPD typing was performed as described previously using 40 ng template DNA and primer 208 (5′-ACG GCC GAC C-3′) [11]. Genomic template DNA was purified by the High Pure PCR template preparation kit (Roche, Mannheim, Germany). PCR products were separated on 1.5% (w/v) agarose gels and analysed by Fingerprinting II Informatix™ software (Bio-Rad) using a 1% band position tolerance and a cut-off value of 80% similarity by the Dice coefficient to identify RAPD genotypes. Within a certain RAPD type isolates not sharing an identical pattern were assigned to distinct subtypes. By use of an 80% cut-off value RAPD could assign isolates to different clusters that corresponded to the clonal complexes identified by MLST [11].

2.6. Multilocus sequence typing (MLST)

MLST was performed according to the protocol published by Curran et al. [8] with modifications [11]. Nucleotide sequences were determined on both strands and searched against the MLST database (http://pubmlst.org/paeruginosa/) for assignment of allelic numbers and sequence types (STs). The eBURST software was used for phylogenetic analysis as described by the eBURST manual (http://eburst.mlst.net/eBURST_readme.pdf) and by ref. [10]. Clonal complexes were defined as a group of isolates with either identical STs or STs that varied at one or two loci (single- or double-locus variants) [8]. The clonal complexes (CC4 and CC11) discussed in this work correspond to the previously described BG4 and BG11 complexes [8,9,11], respectively.

3. Results

3.1. Detection of acquired MBLs by phenotypic methods

A collection of *Pseudomonas* spp. clinical isolates non-susceptible to ceftazidime and imipenem, provided by Hungarian regional clinical microbiology laboratories between September 2002 and February 2007, were screened for MBL production. Table 1 shows various characteristics of six MBL-positive clinical isolates that were identified in two Hungarian towns and two other hospitals where MBL producers had not been reported before. Table 1 also lists the previously described VIM-4 positive isolates PA396, PA555, MB197 [19] and the MBL-negative isolate P12-Q [19,21]. The imipenem-EDTA, ceftazidime-EDTA and cefepime-EDTA disk tests and the MBL Etest were positive for all isolates listed in Table 1 excluding P12-Q. The only exception was the imipenem-EDTA disk test for *P. putida* isolate 07–08 that had an increase of 6 mm in the diameter of the inhibitory zone in the presence of EDTA (Table 2). This value is slightly below the 7 mm cut-off value [34].

Table 2
Antibiotic MICs for selected VIM-producing *Pseudomonas* spp. isolates from Hungary and for P12-Q

Isolate	IPM (mg/l)	MEM (mg/l)	CAZ (mg/l)	FEP (mg/l)	TZP (mg/l)	AZT (mg/l)	AMK (mg/l)	GEN (mg/l)	CIP (mg/l)	PO (mg/l)
PA396	64	>32	256	256	>256	32	32	8	>32	2
PA555	>256	>32	256	256	>256	8	>256	>256	>32	2
06–117	>256	>32	>256	256	>256	8	64	16	>32	4
P12-Q	4	2	32	32	>256	8	16	128	32	4
MB197	>256	>32	128	>256	>256	8	>256	64	>32	4
06–150	>256	>32	>32	128	>256	8	>256	128	>32	2
05–97	>256	>32	16	32	32	2	>256	32	2	2
05–207	>256	>32	64	128	64	4	>256	64	2	2
05–98	>256	>32	32	32	32	2	>256	8	2	2
07–08	32	>32	32	16	32	16	4	4	>32	8

Abbreviations: IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; FEP, cefepime; TZP, piperacillin-tazobactam; AZT, aztreonam; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; PO, polymyxin.

3.2. Antibiotic susceptibility

Minimal inhibitory concentrations of the commonly used anti-*Pseudomonas* drugs are shown in Table 2. All isolates proved multidrug-resistant; however, it is noteworthy that all but one *P. aeruginosa* isolate in Table 2 remained susceptible to aztreonam by current CLSI breakpoints. The three isolates examined from the SZIK hospital in Budapest (05–97, 05–98 and 05–207) were also susceptible to both piperacillin-tazobactam and ciprofloxacin, which is distinct from other characterised VIM-producing *P. aeruginosa* isolates from Hungary [19].

3.3. Detection by PCR and sequencing of MBL genes

PCR experiments demonstrated that all isolates positive by MBL phenotypic tests were bla_{VIM}-positive and that these genes were located on class 1 integrons (Fig. 1). Sequencing of bla_{VIM} cassettes and adjoining parts of the integrons revealed that all but one isolate carried a $bla_{\text{VIM-4}}$ allele.

Isolate 06–117 from the OITI hospital in Budapest carried a $bla_{\text{VIM-4}}$ gene with a 170 bp duplication next to the 5′-CS sequence, followed by an *aacA7* cassette (Fig. 1A). Two of the three *P. aeruginosa* clinical isolates from SZIK hospital in Budapest harboured an *aacA4* gene in the first position and $bla_{\text{VIM-4}}$ in the second position, while isolate 05–98 recovered in the same department was a VIM-2-producing strain (Fig. 1, integrons B and C). PCR mapping experiments revealed that in integron B, the $bla_{\text{VIM-4}}$ cassette was not followed by 3′-CS. For *P. aeruginosa* isolate 06–150 from Gyula and for *P. putida* isolate 07–08 from Veszprém, a previously described integron [19] was identified (Fig. 1, integron D). The $bla_{\text{VIM-4}}$ cassette of this integron, similarly to that from isolate 06–117, also had a 170 bp duplication, but in this case $bla_{\text{VIM-4}}$ was next to the 3′-CS.

3.4. Serotyping and PFGE

The newly identified *P. aeruginosa* MBL-producing isolates could be assigned to serotypes O1, O11 and O12 (Table 1). PFGE experiments revealed that the four serotype O12 isolates in Table 1 shared ≥83% genetic similarity by the Dice coefficient (PFGE subtypes a1–a4). Isolate MB197 shared 80% similarity with isolate 06–150 (PFGE subtypes b1 and b2), while all three serotype O1 clinical isolates from hospital SZIK had an identical PFGE pattern (Table 1, PFGE type c), and this pattern was clearly unrelated to those of the other isolates using a cut-off value of 80% similarity. Thus, a small cluster of MBL-positive infections involving 3 patients was identified at this burn unit. The CCC value of the PFGE dendrogram was 97%.

3.5. RAPD

The RAPD experiment established 3 clusters within the isolates, in good correlation with macrorestriction analysis. The first cluster (RAPD type A, subtypes A1–A4) contained all serotype O12 isolates sharing ≥84% similarity by the Dice coefficient (Table 1). The serotype O1 and O11 isolates clustered to two distinct RAPD genotypes using the 80% cut-off value: isolates 06–150 and MB197 displayed 86% similarity with each other and thus belonged to RAPD type B, while isolates 05–97, 05–98 and 05–207 were assigned to RAPD type C (≥95% similarity). The CCC value of the RAPD dendrogram was 93%.

3.6. MLST

Allelic profiles were determined for representative VIM-producing *P. aeruginosa* isolates from Hungary and for the VIM-non-producing isolate P12-Q (Table 1). Isolates PA396 and PA555 had been MLST-typed in an earlier study [11]. All isolates had allelic profiles that were already present in the MLST database.

Overall, MLST results were consistent with clonal relationships determined by PFGE and RAPD. Our data revealed that all serotype O12 VIM-producing isolates from Hungary belonged to the previously described CC4 (Fig. 2B, ref. [11]), although the isolate characterised in this study (06–117) had a different sequence type (ST111) from that of isolates PA396 and PA555 (ST229). VIM-non-producing isolate P12-Q from France also displayed ST111; thus, this representative isolate of the P12 clone also belonged to CC4.

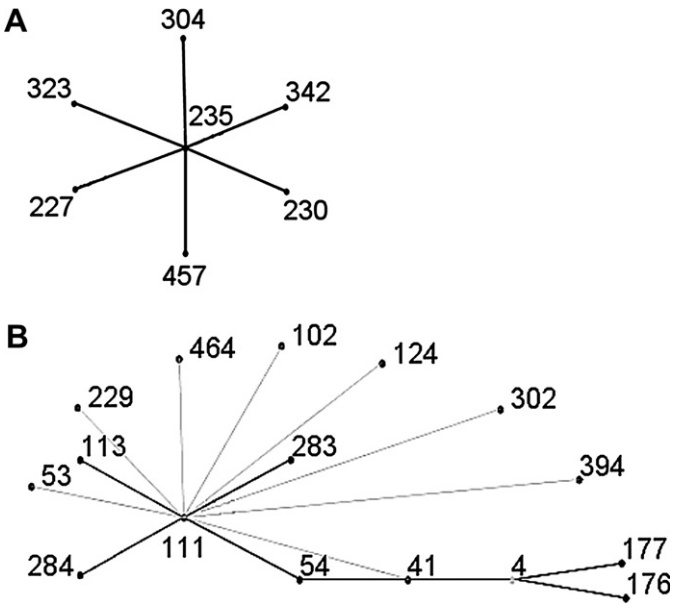

Fig. 2. eBURST diagram of STs belonging to CC11 (A) and CC4 (B) generated by software available at http://eburst.mlst.net/ [10]. Numbers indicate STs, with ST235 and ST111 in the centres as the presently calculated founder STs, respectively. Black lines connect single-locus variant STs (SLVs). In B, double-locus variants (DLVs) of ST111 are indicated with connecting grey lines to the founder by use of the related eBURST tool.

The two O11 isolates from Győr and Gyula (MB197 and 06–150) displayed ST235 and thus belonged to the previously identified CC11 complex (Fig. 2A, ref. [11]). Isolates 05–97 and 05–98 from SZIK hospital in Budapest shared ST313, in agreement with their close clonal relatedness by PFGE and RAPD. This ST does not belong to any presently known CC, and thus it is a singleton ST.

4. Discussion

Acquired MBLs are now considered as one of the major emerging challenges to antimicrobial chemotherapy, with an increasing number of reports from all five continents [4,13,16,20,33,35]. While a number of studies have examined the genetic context of the acquired MBL genes [17,23,26,33], there is only limited information available about the clonal relationships of VIM-producing strains from different countries.

In this study, we characterised six bla_{VIM} carrying *Pseudomonas* spp. clinical isolates from two novel towns and hospitals in Hungary. While all VIM-positive *P. aeruginosa* isolates were previously recovered from western Hungary [19], a VIM-4 producing strain has now been identified in Gyula, at the eastern border of the country. The three new integron types described in this study, together with the four other variants characterised previously [19], demonstrate a high diversity of MBL-carrying integrons in Hungary.

The structure of integron A is unique in that it harbours a bla_{VIM} cassette with a 170 bp duplication in the first position of the integron. These unusual bla_{VIM} cassettes have thus far been exclusively detected in the last position of the integron, left upstream of the 3′-CS element, in isolates from Poland, Greece, Hungary and Belgium [19,23,31]. As the gene cassettes are preferentially inserted into the attI site of the integron [6], this finding suggests that a bla_{VIM-4} cassette with an already existing 170 bp duplication was likely inserted into the first position of integron A.

Integron D, which was initially detected in Pécs, southern Hungary, from a serotype O12 strain, has now been recovered from a different serotype (an O11 *P. aeruginosa* isolate), and also from a different species, a *P. putida* isolate (Table 1). Interestingly, we also recently isolated an *Aeromonas hydrophila* clinical strain in Budapest carrying the same integron (B. Libisch and C.G. Giske, unpublished). These observations indicate a role for horizontal transfer in its dissemination and/or the repeated acquisition of this integron by various clinical strains.

A recent work indicated that MLST was a key epidemiological tool for studies of regional and global epidemiology of MDR *P. aeruginosa*, and underscored the importance of two clonal complexes, CC4 and CC11, in the dissemination of bla_{VIM} harbouring *P. aeruginosa* [11]. Our present study reveals that two MLST-typed representative serotype O11 isolates from Hungary also belong to CC11, a clonal complex that already contains bla_{VIM}-positive isolates from Italy, Greece, Sweden and Poland [9,11]. MB197 is a representative isolate in an outbreak of VIM-4-producing *P. aeruginosa* involving hospitals from three different towns in northwestern Hungary [19]. Thus, previous findings for CC11 are further extended by this study, which supports the notion of a common ancestry for some of the bla_{VIM}-positive serotype O11 *P. aeruginosa* isolates emerging in Europe.

Empel and colleagues recently proposed that CC11 might play a role in dissemination of bla_{PER-1} genes as well, and highlighted the fact that this international clonal complex was detected in three distant Russian cities as well as in Turkey [9]. According to eBRUST analysis of currently available STs in the *P. aeruginosa* MLST database, ST235 is the ancestral or founder strain type of CC11, which has given rise to descendent sequence type strains of this clonal complex (Fig. 2A) [10]. This is in accordance with the already demonstrated wide geographic distribution of ST235 in Russia, Turkey, Poland [9] the USA [14] and Hungary.

Isolates 05–97 and 05–98 from SZIK hospital had an identical PFGE pattern and MLST profile (the singleton ST313), but carried two different bla_{VIM} gene cassettes, bla_{VIM-4} and bla_{VIM-2}, respectively. These molecular characteristics, together with their antibiotic resistance profile, raise the possibility that this serotype O1 *P. aeruginosa* clone may have acquired the bla_{VIM} cassettes from a local gene pool in Hungary. This hypothesis is further supported by the fact that the bla_{VIM-2} cassette is in the first position of the integron where the newly acquired gene cassettes are preferentially integrated [6].

In many European countries, it has been repeatedly demonstrated over the past 20 years that serotype O12 dominates among multiresistant *P. aeruginosa* isolates and that most of these O12 isolates belong to clone P12 [2,19,21,24,28]. Our MLST experiments with the O12 isolates revealed that isolate

06–117 displays ST111 and thus belongs to CC4. The previously MLST-typed VIM-positive serotype O12 isolates from Hungary and Sweden [11] and most other serotype O12 isolates [8] also belong to CC4. Interestingly, P12-Q, a representative isolate of the major European P12 clone, also displayed ST111. According to eBURST analysis of currently available STs, ST111 is the founder ST of CC4 (Fig. 2B). This is in accordance with the wide geographic distribution of the P12 clone in Europe [2,19,21,24] and the occurrence of ST111 isolates in the USA as well [14]. The multidrug-resistant phenotype associated with the P12 clone may have contributed to an increase in the frequency of ST111 isolates, with subsequent diversification of this sequence type [10]. The patterns of descent within this clonal complex will be uncovered in more detail when a larger number of MLST and serotyped isolates become available for analysis.

80% (25/31) of the molecularly characterised VIM-producing *P. aeruginosa* isolates from Hungary belonged to CC4 and CC11 or displayed close clonal relationship by PFGE with the representative MLST-typed CC4 and CC11 isolates ([11,19] and this work). There is a need for extensive experimental studies examining the determinants that contribute to the special characteristics of these two *P. aeruginosa* clonal complexes.

In conclusion, this is the first report of VIM-2 MBL-producing *P. aeruginosa* from Hungary and also of the occurrence of isolates belonging to the international clonal complex CC11. In this study, we have demonstrated a high diversity of MBL carrying integrons in Hungary, and we propose that a combination of different factors such as clonal spread and local acquisition of MBL genes may contribute to the appearance of VIM-positive *Pseudomonas* spp. strains in our country. We also provide supporting evidence for the special role of two clonal complexes, CC4 and CC11, in dissemination of MBL-producing *P. aeruginosa*.

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