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Identification of Two Multidrug-Resistant *Pseudomonas aeruginosa* Clonal Lineages with a Countrywide Distribution in Hungary

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Abstract The aim of this study was to identify class 1 integrons from extended-spectrum and metallo-β-lactamase-negative, multidrug-resistant *Pseudomonas aeruginosa* clinical isolates from Hungary and to characterize the isolates by phenotypic and molecular methods. Fourteen selected *P. aeruginosa* isolates resistant to ceftazidime, gentamicin, and ciprofloxacin were subjected to serotyping, random amplification of polymorphic DNA (RAPD), integron content analysis, and a phenotypic test to detect high-level production of AmpC. Four representative isolates were further analyzed by multilocus sequence typing. Two *P. aeruginosa* multidrug-resistant clonal lineages were identified with a countrywide distribution. The first lineage is characterized by serotype O4, RAPD genotype *A*, sequence type ST175, and the presence of a class 1 integron harbouring *aadB* and *aadA13* gene cassettes in its variable region. The second lineage is characterized by serotype O6, RAPD genotype *B*, sequence type ST395, and a class 1 integron carrying a single *aadB* cassette. The corresponding isolates were recovered from altogether 11 towns in Hungary. ST175 and ST395 are the presently calculated founders of two distinct *P. aeruginosa* clonal complexes that appear to have a wide geographical distribution also outside Hungary. The multidrug-resistant phenotype associated with these two clonal lineages might have contributed to an increase in their frequency and to their subsequent diversification. Both *P. aeruginosa* lineages displayed $\geq$8-fold synergy with boronic acid/ceftazidime combinations, suggesting an AmpC-mediated resistance to ceftazidime. Our observations underscore the role of class 1 integrons in the spread of aminoglycoside resistance by clonal dissemination among *P. aeruginosa* clinical isolates in Hungary.

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Introduction

Pseudomonas aeruginosa is one of the most important clinical pathogens in the nosocomial setting and a common causative agent of bacteremia, pneumonia, and urinary tract infections. Multidrug-resistance among nosocomial isolates of *P. aeruginosa* is a matter of major concern [4]. Several different factors can contribute to multidrug-resistant (MDR) phenotype in *P. aeruginosa*, including the overproduction of the chromosomal AmpC cephalosporinase, upregulated efflux systems, and the acquisition of various resistance determinants [8, 14]. The occurrence of acquired metallo-β-lactamase (MBL) and extended-spectrum β-lactamase (ESBL) determinants has been examined among *P. aeruginosa* clinical isolates in Hungary in earlier works [11–13, 20]. According to these observations, MBL producers occur at a rate below 1% and ESBL producers occur only sporadically among *P. aeruginosa* clinical isolates in Hungary. The aim of this study was to detect and characterize class 1 integrons from MBL and ESBL-negative, MDR *P. aeruginosa* clinical isolates from Hungary and to analyze these isolates by phenotypic and molecular techniques.

Materials and Methods

Bacterial Strains

The MDR *P. aeruginosa* clinical isolates were provided by Hungarian microbiological laboratories between March 2005 and January 2007. Isolates producing MBLs or ESBLs were excluded from this study. Fourteen isolates were selected for characterization displaying resistance to ceftazidime (minimal inhibitory concentration (MIC) $\geq$32 μg/mL), ciprofloxacin (MIC $\geq$4 μg/mL), and gentamicin (MIC $\geq$256 μg/mL) and a balanced geographical distribution throughout Hungary (Table 1). *P. aeruginosa* strains PA1975 and PA2040 [21] were used as positive controls in phenotypic detection of AmpC. The ATCC 27853 *P. aeruginosa* strain was used as the quality control strain.

Antibacterial Susceptibility Tests and Detection of AmpC-Mediated Resistance

Antimicrobial susceptibility tests were performed as recommended by the Clinical and Laboratory Standards Institute (CLSI). MICs were determined by agar dilution and interpreted according to the current CLSI breakpoints [2]. The agar dilution method was performed using phenylboronic acid as the AmpC inhibitor at the concentration of 200 mg/L alone and in combination with ceftazidime to detect high-level AmpC-mediated resistance, as recommended [1, 9]. With this method a $\geq$8-fold synergy (MIC potentiation ratio) with the boronic acid/ceftazidime combination was reported for AmpC-producing *Pseudomonas* spp. [9].

Characterisation of Class 1 Integrons and Serotyping

Polymerase chain reaction (PCR) amplification and sequencing of integrons were performed as described earlier [22]. The anti-*P. aeruginosa in vitro* agglutinating sera (Bio-Rad, Marnes-la-Coquette, France) were used for serotyping. The nucleotide sequences of variable regions of class 1 integrons A and B were deposited in GenBank under accession Nos. EU863269 and EU863270.

Random Amplification of Polymorphic DNA (RAPD)

Random Amplification of Polymorphic DNA (RAPD) typing was performed as described previously [15] and analyzed by Fingerprinting II Informatix^{TM} software (Bio-Rad) using a cutoff value of 80% similarity by the Dice coefficient to identify RAPD genotypes [6, 13]. The statistical significance of the clusters was tested by cophenetic correlation (CC) analysis [17], performed in Fingerprinting II Informatix.

Multilocus Sequence Typing (MLST)

Multilocus Sequence Typing (MLST) was performed according to the protocol published by Curran et al. [3]. Nucleotide sequences were searched against the MLST database (www.pubmlst.org/paeruginosa) for assignment of sequence types (STs). The eBURST software was used for phylogenetic analysis as described [5]. 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) [3].

Mating-Out Assays

Mating-out assays were carried out on MH agar plates using isolates 05-140 and 05-340 as donors and the Rif^{R} *Pseudomonas putida* strain UWC1 as recipient [7]. Transconjugants were selected on plates containing 16 μg/mL gentamicin and 300 μg/mL rifampicin. The initial donor/recipient ratio was 0.2. Mating plates were incubated at 37°C for 14 h.

Results

Detection and Sequencing of Class 1 Integrons

Various features for the analyzed 14 MDR *P. aeruginosa* isolates are shown in Table 1. PCR experiments identified class 1 integrons in all isolates. In seven isolates, a variable region of about 1.5 kb was detected, whereas in the remaining seven isolates, a variable region of about 0.7 kb was detected (Table 1). The full variable regions of these integrons were sequenced for all isolates.

The 1.5-kb integron harbored two cassettes: an *aadB* gene encoding an aminoglycoside 2′-*O*-adenylyltransferase that inactivates gentamicin and tobramycin, followed by an *aadA13* cassette encoding and aminoglycoside-3′-adenylyltransferase that confers resistance to streptomycin and spectinomycin (Integron A, Table 1) [18, 19]. The 0.7-kb integron carried a single *aadB* gene cassette (Integron B). One isolate, 05-310, harbored a second class 1 integron of about 1.65 kb. Partial sequencing demonstrated that a bla_{OXA-4} gene [16] is located next to its 3'conserved sequence (3'CS).

Serotyping and RAPD Analysis

All isolates harboring integron A could be assigned to serotype O4 and those carrying integron B could be assigned to serotype O6. The RAPD experiment established two clusters within the isolates in good correlation with serotyping (RAPD genotypes *A* and *B*, respectively;

Table 1 Various characteristics of 14 selected multidrug-resistant *P. aeruginosa* clinical isolates from Hungary

Strain	Town	Hospital[a]	Ward	Sample[b]	Time of isolation (month/year)	Serotype	RAPD type	Size of integron (kb)	Gene cassettes	Integron code
05-58	Gyula	PKK	Nephrology	Urine	03/2005	O4	*A1*	1.5	*aadB, aadA13*	A
05-140	Budapest	XIX.KSZ	Urology	Urine	04/2005	O4	*A2*	1.5	*aadB, aadA13*	A
05-314	Dombóvár	SZLE	Urology	Wound swab	08/2005	O4	*A3*	1.5	*aadB, aadA13*	A
05-521	Veszprém	VK	Inernal Medicine	Urine	11/2005	O4	*A4*	1.5	*aadB, aadA13*	A
06-151	Balassagyarmat	KAK	ICU	Trachea	09/2006	O4	*A5*	1.5	*aadB, aadA13*	A
06-169	Zalaegerszeg	ZK	Surgery	Punktátum	11/2006	O4	*A6*	1.5	*aadB, aadA13*	A
07-10	Szolnok	HGK	ICU	Wound swab	01/2007	O4	*A7*	1.5	*aadB, aadA13*	A
05-92	Budapest	OORI	Rehabilitation	Tracheal asp.	04/2005	O6	*B1*	0.7	*aadB*	B
05-310	Budapest	OORI	Rehabilitacion	Urine	08/2005	O6	*B2*	0.7,1.65[c]	*aadB*	B
05-340	Miskolc	MMK	ICU	Tracheal asp.	07/2006	O6	*B3*	0.7	*aadB*	B
05-345	Budapest	OSI	Inernal Medicine	Urine	08/2005	O6	*B4*	0.7	*aadB*	B
06-154	Debrecen	DK	Pulmonary	Sputum	09/2006	O6	*B5*	0.7	*aadB*	B
06-161	Székesfehérvár	FMK	Gastroenterology	Wound swab	10/2006	O6	*B6*	0.7	*aadB*	B
06-167	Pápa	PK	ICU	Trachea	11/2006	O6	*B7*	0.7	*aadB*	B

[a] Hospitals are indicated with the abbreviation of their Hungarian name

[b] asp. stands for aspirate

[c] This 1.65-kb intergon carries a $bla_{\text{OXA-4}}$ gene next to the 3'CS

Table 1). Within the RAPD, genotypes isolates not sharing an identical pattern were assigned to RAPD subtypes displaying ≥80% identity by the Dice coefficient (Table 1). The CC coefficient value of the dendrogram was 97%.

MLST Analysis

Two isolates from each RAPD genotype were selected for MLST typing, representing two different subtypes and towns of origin of RAPD genotypes *A* and *B*, respectively. Isolates 05-314 and 06-154, showing the lowest level of identity (that is 80%) by the Dice coefficient to other isolates within RAPD genotypes *A* and *B*, respectively, were included in the MLST analysis. Isolates 05-140 and 05-314 displayed sequence type ST175, whereas isolates 05-340 and 06-154 could be assigned to ST395. Both STs were already present in the *P. aeruginosa* MLST database (http://pubmlst.org/paeruginosa/).

Detection of AmpC-Mediated Resistance

None of the isolates were inhibited in their growth by 200 mg/L boronic acid alone. All isolates showed a ≥8-fold MIC potentiation ratio (MPR) with ceftazidime in the presence of boronic acid (Table 2). In 71% (10/14) of the tested isolates, susceptibility to ceftazidime (MIC ≤ 4 μg/mL) could be restored by boronic acid. The control AmpC overexpressing isolates PA2040 and PA1975 showed MPRs higher than the proposed cutoff value of 8 [9]. Isolate 05-310 produces an integron-borne OXA-4 β-lactamase, which has no ceftazidime hydrolyzing activity [16]. Thus, the presence of OXA-4 did not have an impact on the outcome of the AmpC synergy test performed.

Mating-Out Assays

No transconjugant colonies were obtained with representative donor isolates 05-140 and 05-340 in our mating-out assays under the experimental conditions applied. These results suggest that clonal spread rather than horizontal gene transfer might be the fundamental mechanism behind the countrywide dissemination of integrons A and B in Hungary among MDR *P. aeruginosa* isolates.

Discussion

Isolates concurrently resistant to ceftazidime, ciprofloxacin, and gentamicin constituted about 4% of all *P. aeruginosa* clinical isolates in 2006 in Hungary according to the Hungarian Bacteriological Surveillance Database (http://www.oek.hu). We selected 14 ESBL and MBL-negative isolates displaying this phenotype for analysis. Two large clusters were identified among these isolates based on their serotype (O4 or O6), RAPD genotype (*A* or *B*), and integron content (A or B), respectively (Table 1).

Table 2 Results of the phenotypic tests to detect AmpC mediated resistance to ceftazidme

Isolate	MIC (μg/mL)		MPR[b]
	Ceftazidime	Ceftazidime/BA[a]	
05-58	64	8	8
05-140	128	4	32
05-314	32	4	8
05-521	32	4	8
06-151	64	4	16
06-169	128	4	32
07-10	64	4	16
05-92	>256	8	>32
05-310	256	4	64
05-340	>256	8	>32
05-345	32	4	8
06-154	128	4	32
06-161	128	8	16
06-167	64	4	16
PA1975	64	4	16
PA2040	>256	4	>64
ATCC27853	1	1	1

[a] Boronic acid, used at the concentration of 200 mg/L in combination with ceftazidime by the agar dilution method

[b] MPR = MIC potentiation ratio, the fold synergy observed with the boronic acid/ceftazidime combination

Our data demonstrated a strong association between the MDR phenotype and the presence of class 1 integrons. A low level of diversity was found among the integrons identified in the analyzed isolates. *aadB* was previously shown to be the most prevalent aminoglycoside-resistance mechanism among gentamicin-resistant *P. aeruginosa* isolates [18], and in our study it was present in all isolates. The aminoglycoside 2′-*O*-adenylyltransferase encoded by *aadB* inactivates gentamicin and tobramycin but not netilmicin and amikacin [18]. This was in accordance with the observation that 12 of the 14 tested isolates remained susceptible to amikacin (data not shown). We detected no integron-borne ceftazidime- and ciprofloxacin-resistance determinants in this study. Further experiments revealed that all isolates displayed a ≥8-fold MIC potentiation ratio with boronic acid/ceftazidime combinations (Table 2), suggesting an AmpC-mediated resistance to ceftazidime.

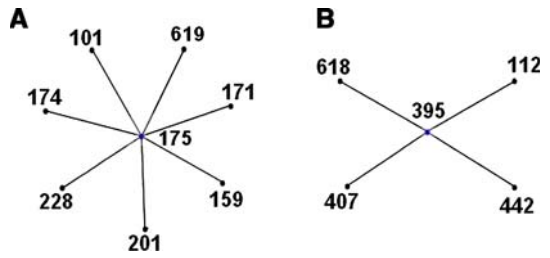

Fig. 1 eBURST diagram of sequence types belonging to two distinct *P. aeruginosa* clonal complexes with founder sequence types ST175 (**a**) and ST395 (**b**) generated by software available at http://eburst.mlst.net/ [5]. Numbers indicate STs, with ST175 and ST395 in the centers as the presently calculated founder STs, respectively. Numbers around the founders, connected to them by black lines, represent single-locus variant STs (SLVs) of the founders. These SLVs share six identical alleles with the founders out of the seven housekeeping genes analyzed [5]

Both MLST-typed representative isolates of the serotype O4 cluster could be assigned to ST175. According to eBURST analysis of the currently available sequence types in the MLST database, ST175 is the founder sequence type of an earlier not described *P. aeruginosa* clonal complex (Fig. 1a). STs belonging to this clonal complex were also identified in the United Kingdom and Canada (Fig. 1a; http://pubmlst.org/paeruginosa/). Representative isolates of the serotype O6 cluster could be assigned to ST395 by MLST. ST395 is the founder sequence type of another *P. aeruginosa* clonal complex that contains isolates from the United Kingdom and the United States as well (Fig. 1b; http://pubmlst.org/paeruginosa/) [10]. Thus, these two *P. aeruginosa* clonal complexes appear to have a wide geographical distribution also outside Hungary. The MDR phenotype associated with isolates belonging to ST175 and ST395 might have contributed to an increase in their frequency and to their subsequent diversification [5, 13].

In conclusion, our work identified two MDR *P. aeruginosa* clonal lineages with a countrywide distribution in Hungary and uncovered an important role of the AmpC and *aadB* determinants in the emergence of MDR clinical isolates. The corresponding isolates were recovered from altogether 11 towns in Hungary. We also provide supporting evidence for the role of integrons in the spread of aminoglycoside resistance by clonal dissemination, presumably through the transfer of colonized patients between different epidemiological settings. Further studies are warranted to investigate the different factors that are involved in and influence this process.

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