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Distribution of carbapenem resistance mechanisms in *Pseudomonas aeruginosa* isolates among hospitalised children in Poland: Characterisation of two novel insertion sequences disrupting the *oprD* gene

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ABSTRACT

This study aimed to analyse the distribution of carbapenem resistance mechanisms among *Pseudomonas aeruginosa* clinical isolates. Fifty-five *P. aeruginosa* isolates, resistant both to imipenem and meropenem, from children hospitalised in 2009–2010 were studied. All strains were genotyped by pulsed-field gel electrophoresis (PFGE). Mutations in the *oprD* gene and the occurrence of insertion sequences (ISs) were determined by DNA sequencing. Mex efflux systems were determined by analysis using the efflux pump inhibitor Phe-Arg β-naphthylamide. Metallo-β-lactamase (MBL) production was determined with Etest MBL strips and PCR for bla_{VIM} and bla_{IMP}. PFGE show high genetic diversity among the isolates. Mutations inactivating the *oprD* gene were detected in 44 strains (80%). Frameshift mutations detected in 20 isolates were the most common cause of inactivation of the *oprD* gene. Point mutations leading to premature stop codons were found in 12 isolates, and various substitutions were found in 6 isolates. Disruption of the coding sequence of *oprD* by ISs was found in six isolates. Two novel ISs (IS*Pa51* and IS*Pa52*) were detected. Increased activity of different Mex systems was observed in 27 isolates (49%). Ten isolates simultaneously overexpressed two ($n = 3$) or three ($n = 7$) types of Mex efflux system. Seven (13%) *P. aeruginosa* strains were found to have minimum inhibitory concentrations (MICs) of >64 mg/L both for imipenem and meropenem (two VIM-4, four VIM-2 and one IMP-1). These results show a significant diversity of *P. aeruginosa* strategies for resistance development. Noteworthy, a variety of ISs were found to disrupt the *oprD* gene.

1. Introduction

Carbapenems (e.g. imipenem and meropenem) are the most frequently used antibiotics in the therapy of serious infections caused by multiresistant *Pseudomonas aeruginosa*. However, the growing threat of carbapenem resistance in *P. aeruginosa* may significantly decrease the efficacy of these last-resort antibiotics [1,2]. Low-level resistance to carbapenems [minimum inhibitory concentrations (MICs) of 8–32 mg/L] in *P. aeruginosa* is often associated with reduced drug uptake resulting from OprD porin loss [3] combined with stable derepression of the chromosomal AmpC β-lactamase gene [4]. Furthermore, imipenem resistance may be conferred by extended-spectrum AmpC β-lactamases, which showed high rates of imipenem and cefepime hydrolysis [5]. Upregulation of one of the several efflux systems such as MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY–OprM [6,7] was also reported to decrease carbapenem efficacy in *P. aeruginosa*. High-level carbapenem resistance (MIC > 32 mg/L) is associated with the production of carbapenemases, mostly metallo-β-lactamases (MBLs) [8]. Acquired MBLs include numerous variants of the most prevalent VIM and IMP enzymes as well as the less common SPM, GIM, NDM, AIM and SIM enzymes [9].

The aim of this study was to analyse the distribution of major resistance mechanisms among carbapenem-resistant *P. aeruginosa* clinical isolates from children.

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2. Materials and methods

2.1. Clinical strains and susceptibility testing

In this study, 55 *P. aeruginosa* isolates resistant both to imipenem and meropenem obtained from children hospitalised in a reference tertiary hospital (Children's Memorial Health Institute, Warsaw, Poland) in the period 2009–2010 were analysed. The isolates were identified by standard laboratory methods.

MICs were determined using the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [10]. *P. aeruginosa* ATCC 27853 was used as a control strain for antimicrobial susceptibility testing.

2.2. Genotyping of P. aeruginosa clinical strains

P. aeruginosa strains were genotyped by pulsed-field gel electrophoresis (PFGE) of *Spe*I-digested genomic DNA using a CHEF-DR II System (Bio-Rad, Hercules, CA). Electrophoresis was run at 12 °C with a pulse time of 5–45 s at 6 V/cm in TBE buffer [45 mM Tris base, 45 mM boric acid, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0; Bio-Rad] for 20 h. PFGE patterns were analysed using BioNumerics v.6.6 (Applied Maths, Sint-Martens-Latem, Belgium) using the band-based dice similarity coefficient and the unweighted pairs geometric-matched analysis (UPGMA) dendrogram with a position tolerance of 1% for optimisation and band comparison.

2.3. Analysis of the oprD gene by PCR and DNA sequencing

The sequence of the entire *oprD* gene was analysed in all of the isolates tested. The nucleotide sequence of the *oprD* gene was investigated by PCR with previously designed primers [6]. Primers OprD-F (CGCCGACAAGAAGAACTAGC) and OprD-R (GTCGATTACAGGATCGACAG) were used for *oprD* amplification and sequencing. An additional primer (OprD-F2, GCCGACCACCGTCAAATCG) was used for sequencing. The *oprD* variants of carbapenem-resistant *P. aeruginosa* strains tested were compared with the *oprD* gene of the reference strain *P. aeruginosa* PAO1.

2.4. Sequencing of insertion sequences inside the oprD gene

Initial sequencing of the *oprD* gene was conducted using the abovementioned primers (OprD-F, OprD-F2 and OprD-R). For six strains (nos. 208/2009, 241/2009, 21/2010, 39/2010, 131/2010 and 153/2010), the PCR products with OprD-F and OprD-R were more or less two times longer than in other analysed strains. In addition, strain-specific internal primers for further sequencing were designed to cover the *oprD* inserts: is208/09-F, 5′-CGCTTGCCGTCCTTGTTCTTG; is208/09-R, 5′-GTGGGATGGTGTAGTTGCTGTTCA; is21/10;131/10-F, 5′-GCGTTTGCCGTCCTTGTTCTTG; is21/10;131/10-R, 5′-GTGGGATGGTGTAGTTGCTGTTCA; is39/10-F, 5′-CGTCACGCCAAGAAAGGTAAC; is39/10-R, 5′-AGTGGGATGGTGTAGTTGCTGTT; is153/10-F, 5′-CAGCTTGCGACCTTTGTGG; and is153/10-R, 5′-ATAGAGGGAGGCGCTGAGGTTGTC. Insertion sequences (ISs) were assigned by BLAST alignment and new ISs were named by ISFinder database staff (http://www-is.biotoul.fr).

2.5. Phenotypic analysis of Mex efflux pumps

The following reporter antibiotics were used as phenotypic markers of Mex efflux pump upregulation: carbenicillin (MexAB–OprM); erythromycin (MexCD–OprJ); gentamicin (MexXY–OprM); and norfloxacin (MexEF–OprN) [11]. In addition, imipenem was used as an indirect indicator of MexEF–OprN upregulation since it induces expression of this pump [12]. Furthermore, meropenem was used in this study to check whether it could be useful as an additional MexAB–OprM efflux upregulation reporter. The MICs of tested antibiotics were determined twice both in the presence and absence of the broad-spectrum efflux pump inhibitor Phe-Arg β-naphthylamide (PAβN) (50 mg/L) using the agar dilution method according to CLSI recommendations [10]. At least a three doubling dilution decrease in the resistance level was considered a positive result. *P. aeruginosa* PT629, PAO 7H and Mug GR1 were used as reference strains for MexAB–OprM, MexEF–OprN and MexXY–OprM, respectively. Upregulation of the MexCD–OprJ efflux pump in the tested isolates was assigned against *P. aeruginosa* PAO1 strain.

2.6. Detection of VIM and IMP metallo-β-lactamases

Etest MBL strips (AB BIODISK, Solna, Sweden) were used for phenotypic screening of MBL activity in the tested strains. Specific PCR primers were used to detect the presence of bla_{VIM} and bla_{IMP} [11]. For amplification and sequencing of bla_{VIM}, the primers VimF (TTATGGAGCAGCAACGATGT) and VimR (CGAATGCGCAGCACCAGG) were used. Primers ImpF (ATGAGCAAGTTATCTTAGTATTC) and ImpR (GCTGCAACGACTTGTTAG) were used for amplification and sequencing of bla_{IMP}.

3. Results

The 55 *P. aeruginosa* isolates tested displayed the following MIC_{90} values (MICs for 90% of the organisms): imipenem, 128 mg/L; meropenem, 64 mg/L; cefotaxime, >128 mg/L; cefoperazone, >128 mg/L; ceftazidime, 64 mg/L; cefepime, 32 mg/L; piperacillin/tazobactam, 128 mg/L; aztreonam, 64 mg/L; amikacin, 128 mg/L; gentamicin, >128 mg/L; tobramycin, >128 mg/L; and ciprofloxacin, 32 mg/L. PFGE distinguished all of the tested isolates, revealing high diversity of the carbapenem-resistant *P. aeruginosa* isolates in Children's Memorial Health Institute (Fig. 1).

The *oprD* gene sequencing results are shown in Table 1. All mutations in the tested isolates are presented in comparison with *oprD* sequence of the reference strain PAO1. Mutations inactivating the *oprD* gene were detected in 44 (80%) of the tested isolates. Frameshift mutations detected in 20 isolates were the most common cause of inactivation of the *oprD* gene. Point mutations leading to premature stop codons were found in 12 isolates, and other substitutions were found in 6 isolates. Disruption of the coding sequence of the *oprD* gene by a large insertion was found in the remaining six isolates. No PCR product for *oprD* was obtained for 11 isolates (see Table 2), which may indicate gene deletion.

Sequencing of the abovementioned large insertions showed specific ISs inside the *oprD* gene in each of the six isolates. Four of the six isolates carried already known ISs: IS*Pre2*, IS*1394*, IS*Pa26* and IS*Pa1635-like* were found in isolates 208/2009, 241/2009, 131/2010 and 153/2010, respectively (GenBank accession nos. KF682462–5). The *oprD* gene in strains 39/2010 and 21/2010 was disrupted by novel ISs, which were designated IS*Pa51* and IS*Pa52*, respectively (Fig. 2). The nucleotide sequences of the both novel ISs were deposited in the GenBank database (accession nos. KF682466 and KF682467).

With the exception of the novel IS*Pa51*, the ISs had their transposase genes arranged anti-parallel. In addition, IS*Pa26* found in strain 131/2010 was exceptionally placed upstream of the *oprD* coding sequence.

In the phenotypic studies of the Mex-type efflux systems, the MICs of reporter antibiotics were determined in the absence and presence of PAβN, a broad-spectrum efflux inhibitor (Table 2). Increased activity of different Mex systems was detected in 27