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# Identification of plasmid OXA and other β-lactamase genes among carbapenem-resistant isolates of *Pseudomonas aeruginosa* from a clinical university hospital in North Eastern Poland

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**SUMMARY**

The aim of the study was to evaluate the prevalence of OXA and other β-lactamase genes, antibiotic susceptibility, and the genetic relatedness among clinical isolates of *P. aeruginosa* resistant to carbapenems. The presence of *bla*$_{OXA}$ genes was demonstrated in 48% of isolates belonging to four PFGE profiles. Most of them contained the *bla*$_{OXA-2}$ gene (88.3%). Other *bla*$_{OXA}$ genes (Ps1310 with *bla*$_{OXA-30}$ and Ps1309 with *bla*$_{OXA-10}$) were found in only two isolates. The tested isolates also contained other β-lactamase genes such as *bla*$_{VIM-2}$, *bla*$_{VIM-4}$, *bla*$_{SHV-5}$, and *bla*$_{TEM-1}$. All isolates were susceptible only to colistin (100%).

*KEY WORDS: P. aeruginosa*, *bla*$_{OXA}$ genes, Antibiotic susceptibility, PFGE.

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*Pseudomonas aeruginosa* is one of the bacterial species most frequently responsible for nosocomial infections. In recent years a significant increase in nosocomial infections has been reported especially in intensive care units (ICUs) and oncology departments (Vojtova *et al.*, 2011; Lucena *et al.*, 2014). Infections caused by this organism are often hard to treat due to the intrinsic resistance to many groups of antibiotics. Particularly dangerous are infections caused by carbapenem-resistant *P. aeruginosa* isolates that are often highly resistant to the majority of β-lactam antibiotics. This type of resistance can be caused by transfer of plasmids with genes encoding β-lactamases (Sacha *et al.,* 2009; Strateva et *al.,* 2009). The high level of acquired resistance as well as the horizontal and clonal spread of resistant *P. aeruginosa* strains has become a serious problem in many hospitals (Zavascki *et al.*, 2006; Strateva *et al.,* 2009; Poirel *et al.*, 2010; Evans *et al.*, 2014).

From September 2012 to December 2013, 428 non-duplicated *P. aeruginosa* strains were isolated from patients hospitalized in the University Hospital in Bialystok. Among them, 45 isolates (10.51%) resistant to carbapenemss were identified, and 25 were selected for further research. Twenty-one of tested isolates were obtained from the ICU (84%). The isolates were cultured from clinical specimens (bronchial secretion, bronchoalveolar lavage, urine, blood, pus, wound swab, and rectal swab). Species identification (GN cards) and susceptibility

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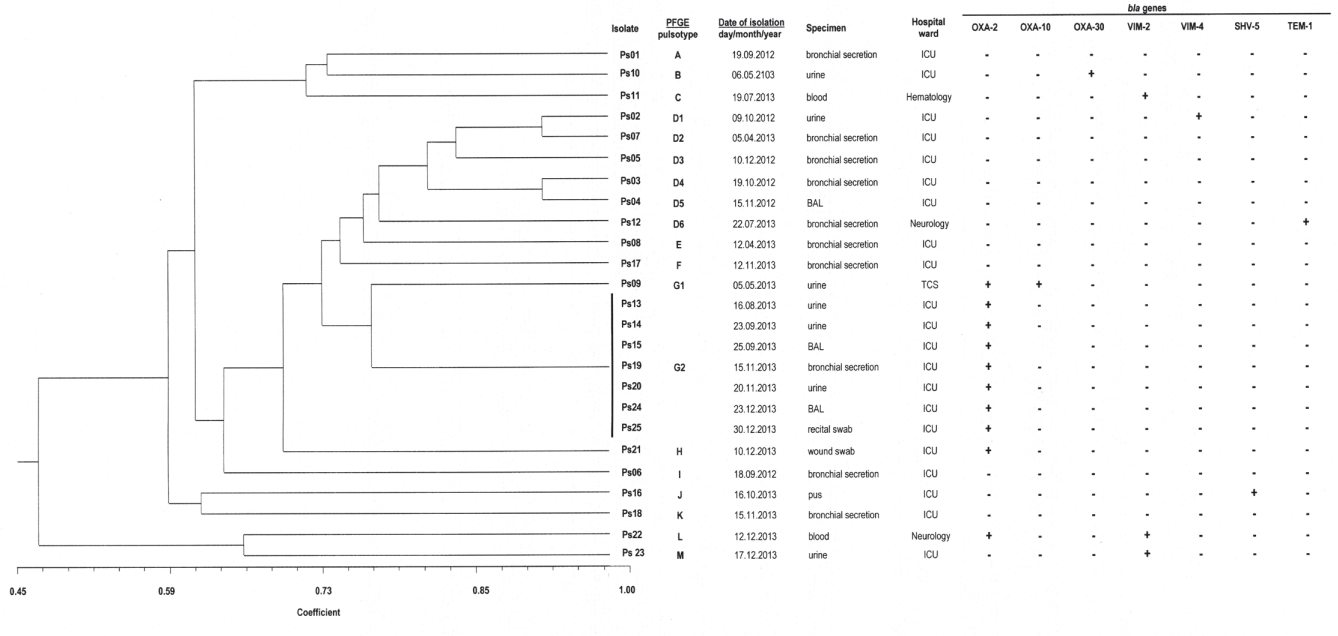
tests (AST-N259 cards) were performed using the VITEK 2 automated system (bioMérieux, USA). The results of susceptibility to antibiotics were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_4.0.pdf).

Plasmid DNA extractions from *P. aeruginosa* isolates were performed with the Plasmid Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions.

Genomic Mini AX Bacteria Kit (A&A Biotechnology, Poland) was used for the isolation of total DNA. Polymerase chain reaction (PCR) for $bla_{OXA}$ genes (Aktas *et al.*, 2008; Lin *et al.*, 2012) and genes encoding other β-lactamases ($bla_{KPC}$, $bla_{VIM}$, $bla_{IMP}$, $bla_{SHV}$, $bla_{TEM}$, $bla_{CTX-M}$) were performed using specific primers and conditions as described previously (Sundsfjord *et al.*, 2004; Ellington *et al.*, 2007; Sacha *et al.*, 2012; Ojdana *et al.*, 2014). Amplicons were sequenced in an external laboratory (Genomed S.A., Warsaw, Poland) and the obtained sequences were compared by using the Basic Local Alignment Search Tool (BLAST) database http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.

Clonal relationships among all tested *P. aeruginosa* isolates were determined by pulsed-field gel electrophoresis (PFGE) of genomic DNA. PFGE typing was performed according to a previously described procedure (Yetkin *et al.*, 2006) with some modifications. Isolates were grown for 16 h at 37°C in tryptic soy broth (TSB) (Oxoid, UK). The cultures were centrifuged and the resulting pellet suspended in 1 mL of TEN buffer (pH 8.0; 0.1M Tris, 0.1 M ethylenediaminetetraacetic [EDTA], 0.15 M NaCl). Next, the suspension was centrifuged again and re-suspended in 100 µL of EC buffer (pH 7.5; 6 mM Tris, 1M NaCl, 0.1M EDTA, 0.5% [w/v] Brij 58, 0.2% [w/v] sodium deoxycholate, 0.5% sodium lauroyl sarcosinate), and cells were embedded into 2% low-gelling-temperature agarose (Type VII; Sigma-Aldrich, USA). After overnight digestion with 50 mg/mL of lysozyme (Sigma-Aldrich) and then 1 mg/mL of




| Isolate | PFGE pulsotype | Date of isolation day/month/year | Specimen | Hospital ward | *bla* genes | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | OXA-2 | OXA-10 | OXA-30 | VIM-2 | VIM-4 | SHV-5 | TEM-1 |
| Ps01 | A | 19.09.2012 | bronchial secretion | ICU | - | - | - | - | - | - | - |
| Ps10 | B | 06.05.2103 | urine | ICU | - | - | + | - | - | - | - |
| Ps11 | C | 19.07.2013 | blood | Hematology | - | - | - | + | - | - | - |
| Ps02 | D1 | 09.10.2012 | urine | ICU | - | - | - | - | + | - | - |
| Ps07 | D2 | 05.04.2013 | bronchial secretion | ICU | - | - | - | - | - | - | - |
| Ps05 | D3 | 10.12.2012 | bronchial secretion | ICU | - | - | - | - | - | - | - |
| Ps03 | D4 | 19.10.2012 | bronchial secretion | ICU | - | - | - | - | - | - | - |
| Ps04 | D5 | 15.11.2012 | BAL | ICU | - | - | - | - | - | - | - |
| Ps12 | D6 | 22.07.2013 | bronchial secretion | Neurology | - | - | - | - | - | - | + |
| Ps08 | E | 12.04.2013 | bronchial secretion | ICU | - | - | - | - | - | - | - |
| Ps17 | F | 12.11.2013 | bronchial secretion | ICU | - | - | - | - | - | - | - |
| Ps09 | G1 | 05.05.2013 | urine | TCS | + | + | - | - | - | - | - |
| Ps13 | | 16.08.2013 | urine | ICU | + | - | - | - | - | - | - |
| Ps14 | | 23.09.2013 | urine | ICU | + | - | - | - | - | - | - |
| Ps15 | | 25.09.2013 | BAL | ICU | + | | - | - | - | - | - |
| Ps19 | G2 | 15.11.2013 | bronchial secretion | ICU | + | - | - | - | - | - | - |
| Ps20 | | 20.11.2013 | urine | ICU | + | - | - | - | - | - | - |
| Ps24 | | 23.12.2013 | BAL | ICU | + | - | - | - | - | - | - |
| Ps25 | | 30.12.2013 | recital swab | ICU | + | - | - | - | - | - | - |
| Ps21 | H | 10.12.2013 | wound swab | ICU | + | - | - | - | - | - | - |
| Ps06 | I | 18.09.2012 | bronchial secretion | ICU | - | - | - | - | - | - | - |
| Ps16 | J | 16.10.2013 | pus | ICU | - | - | - | - | - | + | - |
| Ps18 | K | 15.11.2013 | bronchial secretion | ICU | - | - | - | - | - | - | - |
| Ps22 | L | 12.12.2013 | blood | Neurology | + | - | - | + | - | - | - |
| Ps 23 | M | 17.12.2013 | urine | ICU | - | - | - | + | - | - | - |

**BAL**, bronchoalveolar lavage; **ICU,** intensive care units; **TCS**, thoracic surgery

FIGURE 1 - *Dendrogram of carbapenem resistant P. aeruginosa isolates.*

protease (Sigma-Aldrich), genomic DNA in the agarose plugs was restricted by 20 U of *Xba*I (EurX, Poland) for 6 h at 37°C. DNA fragments were resolved in 1.2% agarose gel (Pulsed Field Certified Agarose, BIO-RAD, USA) with 0.5 × Tris-borate-EDTA (TBE) buffer at 6 V/cm at 14°C using a CHEF Mapper XA Chiller System (BIO-RAD). Pulse times were 4–35 s for 20 h. The gel was stained with ethidium bromide (Sigma-Aldrich) (5 μg/mL), and photographed under UV light using a ChemiDoc XR System (BIO-RAD). NTSYS pc 2.02 (Exeter Software, USA) and the unweighted pair group method with arithmetic mean algorithm (UPGMA) was used to prepare a dendrogram of genetic relatedness between tested isolates. Band position tolerance was set on 1.5%.

The presence of $bla_{\text{OXA}}$ genes was demonstrated

TABLE 1 - *Comparison of antibiotic susceptibility of P. aeruginosa isolates with and without $bla_{OXA}$ genes.*

| *Isolate* | | *Breakpoints (mg/l) of isolates with blaOXA genes* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *(N=11)* | | *AN* | *FEP* | *CAZ* | *CIP* | *CS* | *GM* | *IPM* | *MEM* | *TM* |
| Ps1309 *1 | | ≥64 | 16 | 16 | ≥4 | ≤0.5 | ≥16 | 4 | ≥16 | ≥16 |
| Ps1310 *2 | | ≥64 | ≥64 | ≤1 | 0.5 | ≤0.5 | 8 | ≥16 | 4 | ≥16 |
| Ps1313 *3 | | ≥64 | 8 | 16 | ≥4 | ≤0.5 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1314 *3 | | ≥64 | 8 | 16 | ≥4 | ≤0.5 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1315 *3 | | ≥64 | 16 | ≥64 | ≥4 | ≤0.5 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1319 *3 | | ≥64 | 8 | 16 | ≥4 | 2 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1320 *3 | | ≥64 | 8 | 16 | ≥4 | 2 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1321 *4 | | ≥64 | 16 | ≥64 | ≥4 | 2 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1322 *4 | | ≥64 | ≥64 | ≥64 | ≥4 | 2 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1324 *3 | | ≥64 | 8 | 16 | ≥4 | 2 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1325 *3 | | 16 | 8 | 16 | ≥4 | 2 | ≥16 | ≥16 | 8 | ≥16 |
| | S | 9.1% | 54.5% | 9.1% | 9.1% | 100% | - | - | - | - |
| % | I | - | - | - | - | - | - | 9.1% | 18.2% | - |
| | R | 90.9% | 45.5% | 90.9% | 90.9% | - | 100% | 90.9% | 81.8% | 100% |
| *Isolate* | | *Breakpoints (mg/l) of isolates without blaOXA genes* | | | | | | | | |
| *(N=14)* | | *AN* | *FEP* | *CAZ* | *CIP* | *CS* | *GM* | *IPM* | *MEM* | *TM* |
| Ps1201 | | 8 | 8 | 4 | ≥4 | ≤0.5 | 8 | ≥16 | ≥16 | ≤1 |
| Ps1202 *5 | | ≥64 | ≥64 | ≥64 | 2 | ≤0.5 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1203 | | 16 | 32 | 16 | 1 | 1 | 8 | ≥16 | ≥16 | ≤1 |
| Ps1204 | | 16 | 16 | 16 | ≥4 | ≤0.5 | 8 | ≥16 | ≥16 | ≤1 |
| Ps1205 | | ≤2 | 8 | 8 | 1 | ≤0.5 | ≤1 | 8 | ≥16 | ≤1 |
| Ps1206 | | ≤2 | ≥64 | ≥64 | ≥4 | 2 | 4 | ≥16 | ≥16 | ≤1 |
| Ps1307 | | ≤2 | ≤1 | 2 | ≥4 | 2 | ≤1 | ≥16 | 4 | ≤1 |
| Ps1308 | | ≤2 | 8 | 8 | 0.5 | 2 | ≤1 | ≥16 | ≥16 | ≤1 |
| Ps1311 *6 | | ≥64 | 16 | ≥64 | 2 | 2 | ≥16 | ≥16 | ≥16 | ≥16 |
| Ps1312 *7 | | ≤2 | 8 | 4 | 0.5 | 1 | ≤1 | ≥16 | ≥16 | ≤1 |
| Ps1316 *8 | | ≥64 | 8 | 16 | ≥4 | ≤0.5 | ≥16 | ≥16 | 4 | ≥16 |
| Ps1317 | | ≤2 | 8 | 8 | ≤0.25 | 2 | 2 | 4 | ≥16 | ≤1 |
| Ps1318 | | 4 | 2 | 4 | ≤0.25 | 2 | 2 | ≥16 | 4 | ≤1 |
| Ps1323 *6 | | ≥64 | ≥64 | ≥64 | ≥4 | ≤0.5 | ≥16 | ≥16 | ≥16 | 8 |
| | S | 57.1% | 57.1% | 50% | 28.6% | 100% | 50% | - | - | 71.4% |
| % | I | 14.3% | - | - | 14.3% | - | - | 14.3% | 21.4% | |
| | R | 28.6% | 42.9% | 50% | 57.1% | - | 50% | 85.7% | 78.6% | 28.6% |

AN, amikacin; FEP, cefepime; CAZ, ceftazidime; CIP, ciprofloxacin; CS, colistin; GM, gentamicin; IPM, imipenem; MEM, meropenem; TM, tobramycin; S, susceptible; I, intermediate; R, resistant; *1, isolates with $bla_{OXA-2}$ and $bla_{OXA-10}$; *2, isolates with $bla_{OXA-30}$; *3, isolates with $bla_{OXA-2}$; *4, isolates with $bla_{OXA-2}$ and $bla_{VIM-2}$; *5, isolates with $bla_{VIM-4}$; *6, isolates with $bla_{VIM-2}$; *7, isolates with $bla_{TEM-1}$; *8, isolates with $bla_{SHV-5}$.

in 48% (12/25) of the tested isolates (Figure 1). Most of the $bla_{OXA}$-positive isolates contained the $bla_{OXA-2}$ gene (88.3%). Only two isolates contained other $bla_{OXA}$ genes (Ps1310 isolate with $bla_{OXA-30}$ and Ps1309 with $bla_{OXA-10}$).

Molecular typing of 25 non-repetitive *P. aeruginosa* isolates identified 13 PFGE profiles. Presented results indicated that seven (63.6%) of the isolates with $bla_{OXA}$ genes belonged to one large profile (G2), and were isolated from August to December 2013 from ICU patients. Only two strains, Ps1309 (G1) and Ps1310 (B), isolated at a different time (only in May 2013), contained other genes.

Plasmid-encoded VIM β-lactamases were identified in five *P. aeruginosa* isolates (Ps1311, Ps1321, Ps1322, Ps1323 with $bla_{VIM-2}$, and Ps1202 with $bla_{VIM-4}$). Moreover, two (Ps1321 and Ps1322) of the five isolates contained $bla_{OXA-2}$ genes. Other β-lactamase genes were observed in only two isolates (Ps1312 with TEM-1 and Ps1316 with SHV-5).

The previously observed higher frequency of class D β-lactamases versus class A indicates the geographical distribution of these genes (Lee *et al.*, 2005). The most common enzyme was OXA-10 (40.8%), followed by OXA-1 (22.5%) and OXA-2 (20.4%). The $bla_{OXA-10}$ gene is the most frequently encountered gene in *P. aeruginosa* (Yan *et al.*, 2006; Heintz *et al.*, 2010; Vatcheva-Dobrevska *et al.*, 2013). Also in our study, the majority of detected β-lactamases belonged to group D in comparison to groups B and A (44%, 20%, and 8%, respectively). In contrast to the above-cited studies, OXA-2 was the most prevalent in carbapenem-resistant *P. aeruginosa* at the Clinical University Hospital in Bialystok (Poland).

Antimicrobial susceptibility testing of the eleven $bla_{OXA}$-positive isolates showed a multidrug-resistant phenotype for all the isolates. More than 70% of the isolates were resistant to the tested antibiotics (Table 1). Susceptibility was limited only to colistin and cefepime (100% and 54.5%, respectively). In the case of $bla_{OXA}$-negative isolates, more than 50% were susceptible to all tested antibiotics (with the exception of ciprofloxacin and carbapenems).

In the current study, 20% of a total of 25 *P. aeruginosa* isolates were positive for the production of VIM enzymes. Our study showed that $bla_{VIM-2}$ was the most frequently detectable gene among the carbapenemase genes investigated. Other studies indicate that VIM-2 is the worldwide dominant metallo-β-lactamase (MBL) gene associated with nosocomial outbreaks due to MBL-producing *P. aeruginosa* (Viedma *et al.*, 2012; Andremont *et al.*, 2013; Castaniera *et al.*, 2014).

In conclusion, our study suggests that carbapenem-resistant *P. aeruginosa* isolates belonging to different PFGE profiles may spread in the hospital setting for a long time. The prevalence of resistant genes among clinical *P. aeruginosa* isolates revealed that oxacillinase production is a common mechanism of resistance in our hospital. These results suggest that resistance to carbapenems and other β-lactam antibiotics (80%) of most of our isolates is determined by mechanisms of resistance other than β-lactamase production (with the exception of the five isolates that produced VIM-type enzymes). Overuse of antibiotics is causing the contamination of the hospital environment by multidrug-resistant strains of *P. aeruginosa*. Hospitals should take the necessary measures to limit the spread of multidrug-resistant *P. aeruginosa* strains. This will contribute to more effective treatment and reduce the risk of the spread of resistant genes among other bacterial species.

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