# Antibiotic susceptibility and the presence of *bla*$_{IMP}$ and *bla*$_{VIM}$ genes among of *Pseudomonas aeruginosa* strains resistant or susceptible to imipenem

Sacha P,[1*] Wieczorek P,[1] Ojdana D,[1] Jaworowska J,[2] Kaczyńska K,[2] Bajguz A,[3] Tryniszewska E [1]

[1] Department of Microbiological Diagnostics and Infectious Immunology, Medical University of Bialystok, Poland
[2] Department of Microbiological Diagnostics and Infectious Immunology, University Hospital, Bialystok, Poland
[3] Provincial Sanitary-Epidemiological Station, Bialystok, Poland

## ABSTRACT

**Introduction:** *Pseudomonas aeruginosa* rods are increasingly causing serious infections in hospitalized patients. Particularly worrying is the increase of resistance to carbapenems antibiotics.
**Purpose:** To evaluate susceptibility and the occurrence of genes (*bla*$_{IMP}$ and *bla*$_{VIM}$) encoding resistance to carbapenems among *Pseudomonas aeruginosa* strains.
**Materials and methods:** Studies were conducted for 50 strains of *Pseudomonas aeruginosa* (25 susceptible and 25 resistant to imipenem). Susceptibility to antibiotics was tested using the diffusion method and discs with antibiotics and / or strips with gradient concentrations of antibiotics. In the second phase we tested the ability of MBL production by all strains using the CD and DDST technique described in the literature. The next stage of the study was to evaluate the prevalence of carbapenems resistance genes. These studies were performed by PCR technique.
**Results:** The studies found in both groups of *Pseudomonas aeruginosa* rods similar percentage of strains resistant to aminoglycoside antibiotics (from 72% to 88%) and ciprofloxacin (84%). There was no presence of the genes in any of the tested groups of *Pseudomonas aeruginosa*.
**Conclusion:** *Pseudomonas aeruginosa* strains resistant to imipenem no posses *bla*$_{IMP}$ and *bla*$_{VIM}$ genes therefore their resistance was conditioned by the presence of other mechanisms. Antibiotics with high activity against *Pseudomonas aeruginosa* strains resistant to imipenem were polymyxin B (100% susceptible strains) and colistin (96% susceptible strains).
**Key words:** *Pseudomonas aeruginosa*, susceptibility to antibiotics, genes resistance to carbapenems

***Corresponding author:**
Department of Microbiological Diagnostics and Infectious Immunology
Medical University of Bialystok
Waszyngtona 15A, Str.
15-269 Bialystok, Poland
Tel./Fax: +48 85 746 8571
E-mail: sachpt@umb.edu.pl (Paweł Sacha)

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## INTRODUCTION

Carbapenems are an important group of drugs widely used in hospitals. A very broad antibacterial spectrum including all important pathogens and resistance to most β-lactamases are important advantages of these drugs [1,2]. Significant increase of resistant accumulation is observed among non-fermenting rods mainly from the genus *Pseudomonas* and *Acinetobacter*. The phenomenon is strictly associated with the spreading of resistance genes among other bacteria colonizing the hospital environment [3], mostly due to the presence of many mobile form of genetic material (plasmids, integrons) which can be transferred to other bacterial cell of different genus, species and even families. The presence of antibiotic resistance genes is not always associated with their expression. Nevertheless induction factors in the environment of bacterial cells (e.g. antibiotics) or spontaneous mutations may unlock their activity [4, 5].

The natural resistance of bacteria to several groups of antibiotics and irrational behaviour therapy contribute to settlement of the hospital environment by multiresistant strains of *Pseudomonas aeruginosa* [6].

The aim of this study was to analyse an occurrence of $bla_{IMP}$ and $bla_{VIM}$ genes encoding enzymes from the group of metallo-β-lactamases (MBL) in population of hospital *Pseudomonas aeruginosa* strains with resistance to imipenem and determine susceptibility to different groups of antibiotics.

## MATERIALS AND METHODS

**Antibiotic susceptibility.** Studies concerning susceptibility to antibiotics and the ability to produce carbapenemases were performed on 50 strains (25 strains resistant to imipenem and 25 susceptible) of *Pseudomonas aeruginosa* isolated from clinical specimens.

The strains were identified to the species level by using the GNI card and the VITEK 2 system (f. bioMérieux, USA) according with the manufacturer's instructions. Reference strains of ATCC 27853 *Pseudomonas aeruginosa*, ATCC 25922 *Escherichia coli* and ATCC 35 218 *Escherichia coli* were used for the control of performed studies.

Antibiotic susceptibility testing was performed on Mueller-Hinton agar (f. Oxoid, UK), using strips with gradient concentrations of antibiotics (E-test strips; f. bioMérieux, France and M.I.C Evaluator strips; f. Oxoid, UK) and discs (f. BD BBL, USA. Cultures of *Pseudomonas aeruginosa* on blood agar (f. Emapol, Poland) were used for preparation bacterial suspension with a density of 0.5 MacFarland scale (~ $10^8$cfu/ml) in 0.9% NaCl. Next, the bacteria were inoculated on Mueller-Hinton agar with using a sterile swab and after placing the strips with gradient concentrations or discs with antibiotics incubated for 18 hours under aerobic conditions at $37^0$ C. The MICs values (lowest concentration inhibiting bacterial growth) were read after incubation or were measured zones of inhibition around the disc with antibiotic. Tests were performed and results interpreted according to CLSI guidelines [7].

The ability of metallo-β-lactamase production was evaluated by using two phenotypic methods – CD (Combination Disc) and DDST (Double Disk Synergy Test).

In CD method for determination of MBL production two discs containing imipenem at a concentration 10μg (IMP-10; f. BD BBL, USA) was used. The suspensions of strains were inoculated on Mueller-Hinton agar and next two discs containing imipenem were placed on agar. To one of them a solution 0.5 M EDTA in a volume of 10 μl per disc was added. The study was performed in accordance with the procedure described by *Yong et al.* [8]

In DDST method discs containing imipenem (IMP-10; f. BD BBL, USA), ceftazidime (CAZ-30; f. BD BBL, USA) and sterile discs (without antibiotic) with a diameter of 6 mm (f. Emapol, Poland) were used 0.5 M EDTA solution was applied (10μl) to the disc without antibiotic. The study was performed in accordance with the procedure described by *Lee et al.* [9].

**PCR analysis.** Bacterial DNA was isolated using commercial kits Genomic mini (f. A&A, Poland).The PCR reaction was prepared in volume of 25 μl mixture containing 2 μl of isolated DNA, 1 μl (1μM) of each primer, 12.5 μl Master Mix (f.DNA, Poland) with 0.2 mM dNTP, 1xbufor reaction, 2 mM $MgCl_2$, and 0.04 U/μl polimerase DNA. The mixture was made up with 7.5μl deionised water to a final volume. The PCR reaction was performed in a thermocycler Cyclone 96 PEQLAB (f. Biotechnology, GmbH, Germany) using primers and reaction protocol described by *Ellington* et al. [10]. Obtained amplicons were subjected to horizontal electrophoresis for 90 min in 1.5% agarose (LE Basic GQT, f. Prone, Spain) in the chamber Sub-Cell GT (f. Bio-Rad, USA) and analyzed for the presence of $bla_{IMP}$ (188 bp) and $bla_{VIM}$ (390 bp) genes in ChemiDoc XRS system (f. Bio-Rad, USA).

## RESULTS

Antibiotic susceptibility of *Pseudomonas aeruginosa* strains are presented in two figures. The group of strains resistant to imipenem (Fig.1)

**Table 1.** MICs of antibiotics among *Pseudomonas aeruginosa* strains susceptible to imipenem.

| **Strains** | **MIC (mg/L)** | | | | | | **PCR** | **PCR** | **CD** | **DDST** |
|---|---|---|---|---|---|---|---|---|---|---|
| ***P.aeruginosa*** | **Ciprofloxacin** | **Netilmicin** | **Amikacin** | **Gentamicin** | **Meropenem** | **Imipenem** | ***bla*$_{VIM}$** | ***bla*$_{IMP}$** | | |
| *Psa-w- 021* | **0.25** | **12** | >256 | >256 | **1.5** | **3** | - | - | - | - |
| *Psa-w- 040* | **0.25** | **4** | 128 | **2** | **0.125** | **2** | - | - | - | - |
| *Psa-w- 509* | >32 | 96 | >256 | 16 | >32 | **4** | - | - | - | - |
| *Psa-w- 515* | >32 | >256 | 64 | 16 | 16 | **2** | - | - | - | - |
| *Psa-w- 518* | >32 | >256 | >128 | 32 | >32 | **4** | - | - | - | - |
| *Psa-w- 604* | >32 | >256 | **16** | **4** | 16 | **2** | - | - | - | - |
| *Psa-w- 606* | >32 | >256 | 128 | 32 | 32 | **4** | - | - | - | - |
| *Psa-w- 609* | >32 | >256 | 64 | 16 | 16 | **2** | - | - | - | - |
| *Psa-w- 610* | >32 | >256 | >256 | 64 | 32 | **4** | - | - | + | - |
| *Psa-w- 614* | >32 | >256 | >256 | 32 | 16 | **4** | - | - | - | - |
| *Psa-w- 618* | >32 | >256 | 96 | 16 | 8 | **4** | - | - | - | - |
| *Psa-w- 626* | >32 | >256 | >256 | 16 | 16 | **2** | - | - | - | - |
| *Psa-w- 631* | >32 | >256 | 64 | 8 | 16 | **2** | - | - | - | - |
| *Psa-w- 651* | >32 | >256 | >256 | 24 | >32 | **2** | - | - | - | - |
| *Psa-w- 660* | >32 | >256 | 128 | 16 | 16 | **1** | - | - | - | - |
| *Psa-w- 713* | **0.25** | **12** | **16** | **4** | **1.5** | **4** | - | - | - | - |
| *Psa-w- 725* | >32 | >256 | 128 | 32 | 16 | **2** | - | - | - | - |
| *Psa-w- 726* | >32 | >256 | **16** | 32 | 32 | **4** | - | - | - | - |
| *Psa-w- 728* | >32 | >256 | >256 | 16 | 8 | **2** | - | - | + | - |
| *Psa-w- 734* | >32 | >256 | 192 | 8 | 8 | **4** | - | - | - | - |
| *Psa-w- 737* | >32 | >256 | >256 | 16 | 16 | **4** | - | - | - | - |
| *Psa-w- 750* | 16 | >256 | 32 | 16 | 32 | **2** | - | - | - | - |
| *Psa-w- 753* | >32 | >256 | 128 | 16 | 16 | **4** | - | - | - | - |
| *Psa-w- 755* | **0.25** | >256 | **8** | **4** | **2** | **3** | - | - | - | - |
| *Psa-w- 760* | >32 | >256 | >256 | 16 | 16 | 4 | - | - | - | - |

**R.** resistant strains (mg / L); **S.** susceptible strains (mg / L);

**Criteria for antibiotic resistance (MIC mg / L):** ciprofloxacin (R ≥ 4; S ≤ 1). netilmicin (R ≥ 32; S ≤ 12). amikacin (R ≥ 32; S ≤ 16). gentamicin (R ≥ 8; S ≤ 4). meropenem (R ≥ 16; S ≤ 4). imipenem (R ≥ 16; S ≤ 4);

**PCR *bla*$_{VIM}$ and PCR *bla*$_{IMP}$:** Result of the presence genes after PCR;

**CD, DDST:** Phenotypic tests for detection of metallo-ß-lactamase production.

**Table 2.** MICs of antibiotics among *Pseudomonas aeruginosa* strains resistant to imipenem.

| **Strains** ***P.aeruginosa*** | **MIC (mg/L)** | | | | | | **PCR** ***bla*$_{VIM}$** | **PCR** ***bla*$_{IMP}$** | **CD** | **DDST** |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Ciprofloxacin** | **Netilmicin** | **Amikacin** | **Gentamicin** | **Meropenem** | **Imipenem** | | | | |
| *Psa-o- 203* | 4 | **12** | **8** | 128 | >32 | >32 | - | - | - | - |
| *Psa-o- 204* | 4 | **12** | **8** | **4** | 16 | >32 | - | - | - | - |
| *Psa-o- 205* | 8 | **8** | **8** | 8 | >32 | >32 | - | - | - | + |
| *Psa-o- 206* | 4 | **8** | **12** | 8 | >32 | >32 | - | - | - | - |
| *Psa-o- 208* | 4 | **12** | **8** | **4** | 16 | >32 | - | - | - | - |
| *Psa-o- 506* | **0.5** | **6** | **8** | **4** | >32 | >32 | - | - | - | - |
| *Psa-o- 520* | >32 | >256 | 192 | 32 | >32 | >32 | - | - | + | + |
| *Psa-o- 603* | 24 | >256 | 192 | 32 | >32 | 32 | - | - | - | - |
| *Psa-o- 607* | 2 | **8** | **16** | **4** | 16 | >32 | - | - | - | - |
| *Psa-o- 620* | >32 | >256 | 64 | 24 | >32 | >32 | - | - | + | - |
| *Psa-o- 635* | >32 | >256 | >256 | 64 | >32 | 32 | - | - | - | - |
| *Psa-o- 649* | >32 | >256 | >256 | 16 | >32 | 16 | - | - | - | - |
| *Psa-o- 650* | >32 | >256 | >256 | 64 | >32 | >32 | - | - | - | - |
| *Psa-o- 661* | >32 | >256 | 128 | >256 | 32 | 16 | - | - | | - |
| *Psa-o- 665* | >32 | >256 | >256 | 32 | >32 | 16 | - | - | + | - |
| *Psa-o- 674* | >32 | >256 | 128 | 16 | 16 | 16 | - | - | - | - |
| *Psa-o- 680* | >32 | >256 | 96 | 24 | >32 | >32 | - | - | - | - |
| *Psa-o- 685* | >32 | >256 | >256 | 64 | >32 | 16 | - | - | - | - |
| *Psa-o- 706* | >32 | >256 | >256 | 16 | 8 | 32 | - | - | - | - |
| *Psa-o- 709* | >32 | >256 | >256 | 32 | 8 | >32 | - | - | - | - |
| *Psa-o- 716* | >32 | >256 | >256 | 16 | >32 | >32 | - | - | - | - |
| *Psa-o- 735* | >32 | >256 | 256 | 16 | 8 | 16 | - | - | - | - |
| *Psa-o- 736* | 4 | >256 | **16** | **4** | 8 | >32 | - | - | - | - |
| *Psa-o- 908* | **0.5** | >256 | 128 | >256 | >32 | >32 | - | - | + | - |
| *Psa-o- 909* | **0.5** | >256 | >256 | >256 | >32 | >32 | - | - | + | + |

**R.** resistant strains (mg / L); **S.** susceptible strains (mg / L);

**Criteria for antibiotic resistance (MIC mg / L):** ciprofloxacin (R ≥ 4; S ≤ 1). netilmicin (R ≥ 32; S ≤ 12). amikacin (R ≥ 32; S ≤ 16). gentamicin (R ≥ 8; S ≤ 4). meropenem (R ≥ 16; S ≤ 4). imipenem (R ≥ 16; S ≤ 4);

**PCR *bla*$_{VIM}$ and PCR *bla*$_{IMP}$:** Result of the presence genes after PCR;

**CD, DST:** Phenotypic tests for detection of metallo-ß-lactamase production.

showed high level resistance to aminoglycosides (amikacin 68% and 80% gentamicin), ciprofloxacin and ticarcillin with clavulanic acid (68%). On the other hand, the groups of strains susceptible to imipenem (Fig.2) were more susceptible to aminoglycoside antibiotics (84% - 88%) and ticarcillin with clavulanic acid (48%) than the group of strains resistant to imipenem. The highest activity in both groups of strains was demonstrated for polymyxin B (100%) and colistin (96% susceptible strains). The tables 1 and 2 present the results of phenotypic tests for MBL production (methods of CD and DDST), the MIC values of antibiotics for various strains, determined with the use of strips containing gradient concentrations of the antibiotic (E-test or MIC Evaluator tests) and the results of occurrence of genes *bla*$_{IMP}$ and *bla*$_{VIM.}$ The study of phenotypic ability to production of metallo-β-lactamase by CD method showed that feature in 2 susceptible strains (Psa-w-610 and a Psa-w-728) and 5 strains resistant to imipenem (tab. 1 and 2). Using the DDST method this feature only in three strains resistant to imipenem (Psa-o-205, 520 and 909) was detected. Moreover only for two strains (the Psa-o-520 and 909) obtained results were positive in both methods (CD and DDST) (Table 2). Frequently the highest MIC values were observed for netilmicin (> 256 mg/L) and ciprofloxacin (> 32 mg/L). The most different the values of MICs were observed for amikacin (8 mg/L to > 256 mg/L) or gentamicin (2 mg/L to> 256 mg/L). Studies carried out by PCR technique did not confirm presence of *bla*$_{IMP}$ and *bla*$_{VIM}$ genes responsible for production of metallo-β-lactamases in all studied strains (resistant and susceptible to imipenem). There was no presence of the genes in group of strains in which obtained positive result in DDST and CD techniques.

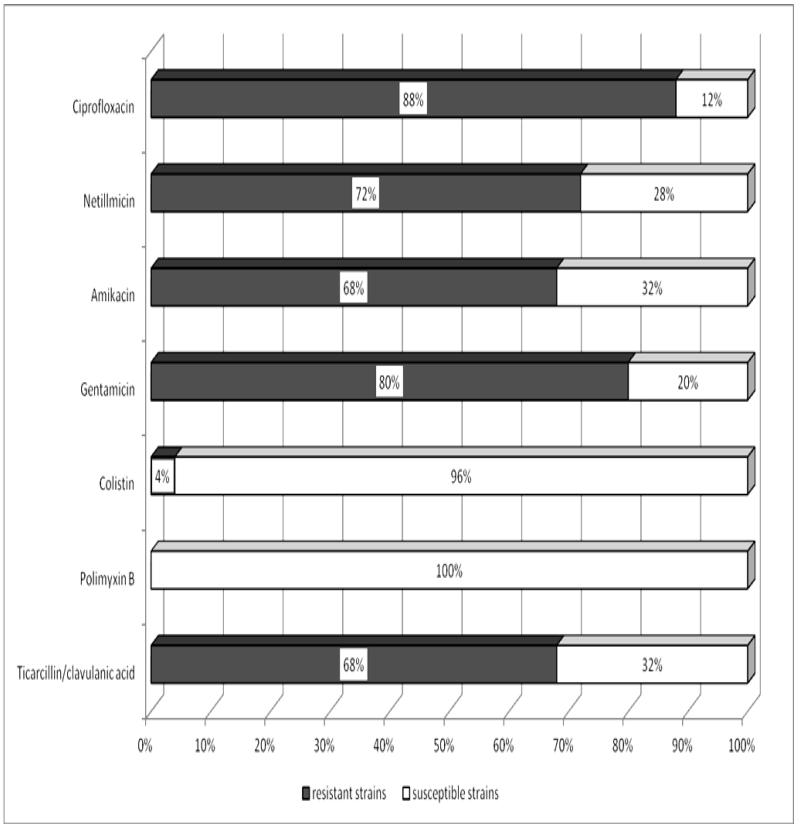


**Fig. 1.** Susceptibility to antibiotics of *Pseudomonas aeruginosa* strains resistant to imipenem.

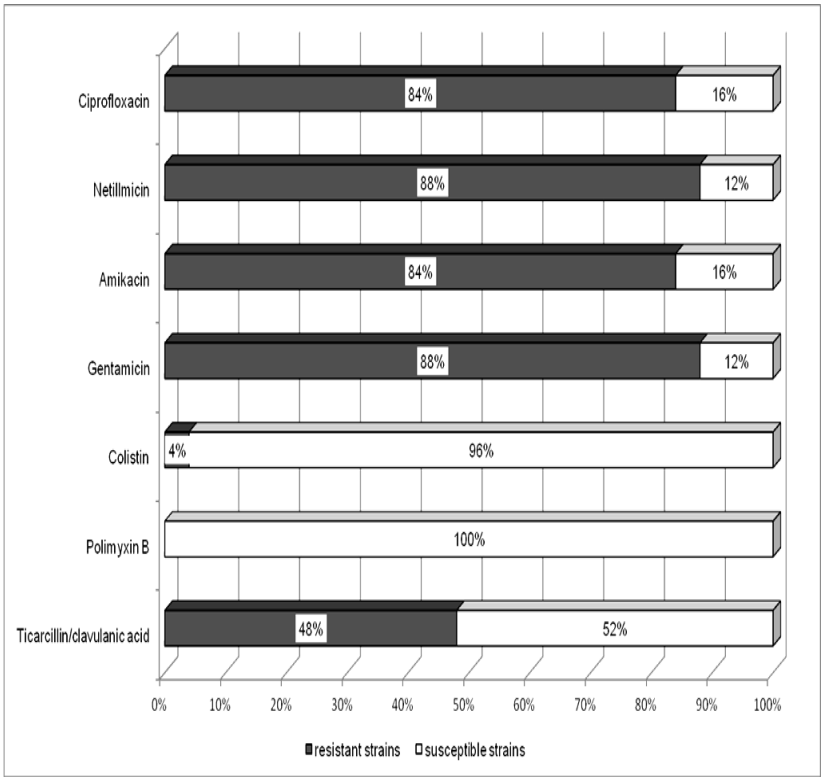


**Fig. 2.** Susceptibility to antibiotics of *Pseudomonas aeruginosa* strains susceptible to imipenem

## DISCUSSION

In recent years the studies of resistance of *Pseudomonas aeruginosa* to meropenem and imipenem showed a worrying increase resistance to these antibiotics [11-13]. Studies carried in Poland by *Patzer et al.* [14] have shown the occurrence of resistance to imipenem in 15% strains of *Pseudomonas aeruginosa*. Resistance of *Pseudomonas aeruginosa* to antibiotics (including carbapenems) may depend on several mechanisms such as: loss of porins (OprD), presence of active systems removal of drugs, modification of receptor binding antibiotic (PBP3 and PBP4) and the ability to produce β-lactamases [3-5, 15].

The prevalence of resistance to amino-glycosides, cephalosporins, fluoroquinolones and combination β-lactam antibiotics with β-lactamase inhibitors is commonly observed among *Pseudo-monas aeruginosa* resistant to carbapenems [7, 12-13]. This is confirmed by our observations resulting from study of resistance to amino-glycoside antibiotics (from 68% to 88% of amikacin-resistant strains), fluoroquinolones (84% of strains). Performed studies using of screening methods (CD and DDST) to detection of metallo-β-lactamases showed this feature in several tested strains. Probably the tests used for detection MBL are not sufficiently specific [15]. The studies of the genetic material of these strains in the PCR reaction did not confirm the presence of $bla_{IMP}$ and $bla_{VIM}$ genes. Our results regarding the specificity these methods in the detection of MBL production are confirmed by opinion of other researchers [16].

Results of our study can be assumed that the frequently reason of resistance to imipenem among multiresistant *Pseudomonas aeruginosa* strains occurring in the hospital environment is probably the loss of porins (OprD) than the ability to produce metallo-β-lactamases [17].

## CONCLUSIONS

1. Resistance to imipenem of tested strains was not associated with ability for producing metallo-β-lactamases (absence of genes $bla_{IMP}$ and $bla_{VIM}$).
2. Antibiotics with high activity against *Pseudomonas aeruginosa* strains resistant to imipenem were polymyxin B (100% susceptible strains) and colistin (96% susceptible strains).

**Conflicts of interest**

We declare that we have no conflicts of interest.

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