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Resistance features of *Pseudomonas aeruginosa* strains isolated from patients with infectious complications of cardiovascular surgery

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ABSTRACT

The purpose of this study was to evaluate the resistance profiles of *Pseudomonas aeruginosa* clinical strains isolated during 2014-2015 from patients hospitalized in National Institute for Cardiovascular Diseases Prof. C.C. Iliescu, Bucharest with cardiovascular diseases. The strains identification was performed in the hospital unit using the automated VITEK2 compact system. The antibiotic susceptibility testing was performed by Kirby-Bauer disk diffusion method and the genetic support of the resistance to carbapenems, quinolones, aminoglycosides and of porins was investigated by simplex and multiplex PCR. Our study revealed that the *P. aeruginosa* strains isolated from patients underlying cardiovascular surgery were resistant in high proportions to ticarcillin (58.33%), third and fourth generation cephalosporins (ceftazidime=50% and cefepime=41.67%) and to ciprofloxacin (33.33%), but remained susceptible to colistin. 48.48% of the isolates were positive for the OprD gene and 24.24% for imipenemase (blaIMP), both genes encoding for β-lactams resistance mediated by non-enzymatic and respectively, enzymatic mechanisms, and only 3% of the isolates produced the aac3Ia gene, responsible for resistance to aminoglycosides. None of the quinolone resistance genes were found in the investigated strains. The high percentage of carbapenem-resistant strains and the diversity of resistance mechanisms to this last resort class of beta-lactam antibiotic inquire molecular epidemiology studies in order to establish their local or imported origin of the *P. aeruginosa* strains circulating in Romanian hospitals.

Keywords: *Pseudomonas Aeruginosa, Antibiotic Resistance Genes (ARGs), Imipenemase, Porins.*

1. INTRODUCTION

Pseudomonas aeruginosa, one of the most common bacteria isolated from chronic wounds [1], is an opportunistic pathogen with innate resistance to several classes of antibiotics because of the low permeability of its outer-membrane, the constitutive expression of various efflux pumps, and the production of antibiotic-inactivating enzymes (e.g., cephalosporinases) [2]. Resistance to carbapenems is often associated with the production of metallo-ß-lactamases also called carbapenemases [3]. Carbapenemases represent the most versatile family of β-lactamases, which are able to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems [4]. They belong to molecular classes A, D (serine-based hydrolytic mechanism), and class B [metallo-β-lactamases (MBL) that contain zinc in the active site] [5]. Eight MBL acquired enzyme families have been described in clinical isolates of *P. aeruginosa* (VIM, IMP, SPM, GIM, SIM, FIM AIM, NDM) [6], chromosomal/plasmid encoded or integron-born among which VIM, IMP and NDM types are worldwide distributed [5]. The genes encoding IMP-like and VIM-like carbapenemases are located in integrons containing other resistance genes (e.g., aminoglycoside-inactivating enzymes) [7, 8], therefore the respective isolates will reveal a multi-drug resistance phenotype. Enzymes inactivating aminoglycosides are present worldwide, and are detected in up to 20% of clinical isolates in Europe and Latin America [9]. Acting on specific substituents of the aminoglycoside molecule, they do not necessarily confer cross-resistance to all aminoglycosides. Target modification (methylation of 16S rRNA) has also been shown to confer resistance to aminoglycosides [10].

In the absence of acquired carbapenemases, mutational inactivation of OprD is the main mechanism of carbapenem resistance. The outer membrane protein OprD regulates the entry of carbapenems [11]. The loss of OprD function has been shown to play a major role in the acquired resistance to imipenem, with a lesser extent to meropenem [12].

Cardiovascular disease is one of the major causes of mortality and morbidity worldwide and the costs that involve handling this disorder are huge. The 2008 overall rate of death attributable to cardiovascular disease was 244.8 per 100 000 individuals and this rate is critically growing [13]. Recent evidence demonstrates that cardiovascular disorders are usually associated with increased level of stress hormones [14, 15].

Infectious complications after cardiovascular surgery occur in 5% to 21% of cases, increasing postoperative mortality by more than 5 times and increasing the hospitalization time with more

than 14 days, and therefore delaying the patient recovery and increasing the care costs [16]. The most common sites of infection are the respiratory tract, surgical site and catheters or devices infections, usually of bacterial origin, in which P.aeruginosa is the mostcommonly identified Gram-negative bacteria [17].

Considering the above mentioned aspects the purpose of this study was to investigate the phenotypic and genotypic AR of *P. aeruginosa* strains isolated from Bucharest patients with cardiovascular diseases.

2. EXPERIMENTAL SECTION

2.1. Isolation and identification of bacterial strains.

This study was conducted on a total of 33 *P. aeruginosa* clinical strains isolated during 2014-2015, from patients hospitalized for surgery in the National Institute for Cardiovascular Diseases Prof. C.C. Iliescu, Bucharest. The selected strains were isolated from different clinical sources, most of them (94%) from wound secretions, followed by venous blood cultures. The strains identification was performed in the Microbiology Laboratory of the above mentioned hospital with the automated VITEK 2 system.

2.2. Antibiotic susceptibility testing.

The antibiotic susceptibility testing was performed by Kirby-Bauer standard disk diffusion method (panels of antibiotic disks recommended by CLSI, 2014 and 2015) using a *P. aeruginosa* ATCC 27853 as reference strain. The antibiotic disks used were Imipenem (10 µg), Meropenem (10 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Gentamicin (10 µg), Colistin (30 µg), Ceftazidime (30 µg), Cefepime (30 µg), Amykacin (15 µg), Tobramycin (10 µg), Ticarcillin (75 µg), Piperacillin-Tazobactam (30µg), and Piperacillin (100 µg). At first the bacteria were cultured into tryptic soy broth (TSB) agar and incubated at 37°C for 24 hours. After 24 hours, microbial suspensions were prepared, with a density equivalent to the turbidity of the 0.5 McFarland standard and plated with sterile swabs on Muller Hinton (MH) agar. The antibiotic disks were placed on the plate and incubated at 37°C for 24 hours. Following incubation, the diameters of the growth inhibition zone were measured.

2.3. Polymerase Chain Reaction (PCR) assays for antibiotic resistance genes (ARGs) detection.

The genetic support of the ARGs was investigated by simplex and multiplex PCR, using a reaction mix of 25 µl (PCR Master Mix 2x, Thermo Scientific) containing 1 µl of bacterial DNA extracted using the alkaline extraction method. For PCR reaction, 1-5 colonies of bacterial cultures were suspended in 1.5 ml tubes containing 20 µl solution of NaOH (sodium hydroxide) and SDS (sodium dodecyl sulphate) and heated on a thermoblock at 95°C for 15 min. for the permeabilization of the bacterial wall. The following step was the addition of 180 µl of TE buffer (TRIS+EDTA) 1X and centrifugation at 13000 rpm for 3 minutes. All PCR reactions were performed using the Thermal Cycler machine Corbet. Genomic DNA was used as a template for the PCR screening for carbapenemases genes (blaIMP; blaVIM; bla NDM, bla SPM; bla SIM); aminoglycosides inactivating enzymes genes (aac3Ia); quinolones resistance (GyrB, parE, QnrA, QnrS) and for porins (OprD). The PCR reactions were initiated with 1 cycle at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 55-58°C for 30 s, 72°C for 1 min and a final elongation step at 72°C for 7-10 min. The amplification products were visualized by electrophoresis on a 1% agarose gel, stained with the specific weight marker (100pb, Ladder Bench Top, Promega, USA).

3. RESULTS SECTION

P. aeruginosa strains isolated from patients submitted to cardiovascular surgery were resistant in high proportions to ticarcillin (58.33%), to third and fourth generation cephalosporins (Ceftazidime=50% and Cefepime=41.67%) and to ciprofloxacin (33.33%) (Figure 1). None of the isolated strains demonstrated resistance to colistin, a “last resort” antibiotic in Romania for parenteral treatment. A recent study in Romania [18] identified 4 strains of *P. aeruginosa* showing resistance to all tested antibiotics and remaining susceptible only to colistin. Regarding the antibiotic resistance genes our study revealed that 24.24% of the analyzed strains resistant to ticarcillin and meropenem/imipenem produced imipenemase (Figure 2 and Figure 3), 48.48% of the analyzed strains revealed the presence of OprD gene (Figure 4) and only 3% of the isolates produced aac3Ia which confers aminoglycosides resistance. No quinolone resistance genes were found in the investigated strains. IMP-type MBLs were the first acquired carbapenemases identified in *P. aeruginosa* [19]. They are most common in the South-East Asian regions and have been occasionally detected in many other regions worldwide [7, 5], while in Europe there are sporadic reports from Italy, Portugal and central European countries, such as France, the Czech Republic, Slovakia and Austria (http://www.lahey.org/studies) [19, 5, 20]. IMP-type β-lactamases hydrolyse efficiently and confer resistance to most β-lactam to date, antibiotics, 42 IMP-type including variants carbapenems, have been except assigned aztreonam. They usually consist of 246 amino acid residues and, (http://www.lahey.org/studies), which differ from each other by 1-54 amino acid residues and exhibit important structural differences [19]. Most blaIMP genes are carried by gene cassettes in class 1 integrons also harbouring other resistance genes, such as aac, aad or blaOXA, within plasmid- or chromosome-borne integron/transposon structures [19]. Recently a pilot study from three Romanian hospitals located in Iasi and Targu-Mures (December 2014-May 2015) demonstrated the presence of carbapenemase VIM-2 in *P. aeruginosa* clinical or faecal isolates [21]. Similar to our results, Porumbel et al., in 2016 revealed that 22.22% of *P. aeruginosa* nosocomial strains isolated from patients in the same units revealed the blaIMP gene [22]. Gheorghe et al., in 2015 revealed that the carbapenemase VIM-2 is chromosomally located and is associated with a common class 1 integron (aacA7-

blaVIM-2) or with an atypical structure (aacA7-blaVIM-2-dfrB5-tniC), in clinical strains of *P. aeruginosa* from Bucharest hospitals, which belonged to ST233, ST364 and ST1074 clones [23]. Another study from the Infectious Diseases hospital of Cluj-Napoca, performed between 2011-2013 revealed that VIM-2 producing *P. aeruginosa* belonged to ST 2026 clone, which is very close to ST233, while IMP-13 producing *P. aeruginosa* strains belonged to ST 1982 [24] and the IMP-13 gene was associated with class 1 integrons. Data revealed by Gheorghe et al., in 2014 demonstrated the presence of VIM- 4 associated with class 1 integron (two different types of class 1 integrons: aacA7-blaVIM-4 and aadB) in *P. aeruginosa* isolates from Bucharest hospitals (2012-2013) [25]. Mereuta et al., in 2013 revealed that VIM-2 carbapenemase in *P. aeruginosa* isolated from Iasi hospitals is associated with class 1 integrons being demonstrated two different configurations: IntI1-aacA7-blaVIM-2-qacEΔ1 in the case of the majority isolates and less frequent IntI1-aacA7-ΔblaVIM-ΔcmlA1-qacEΔ1 [26].

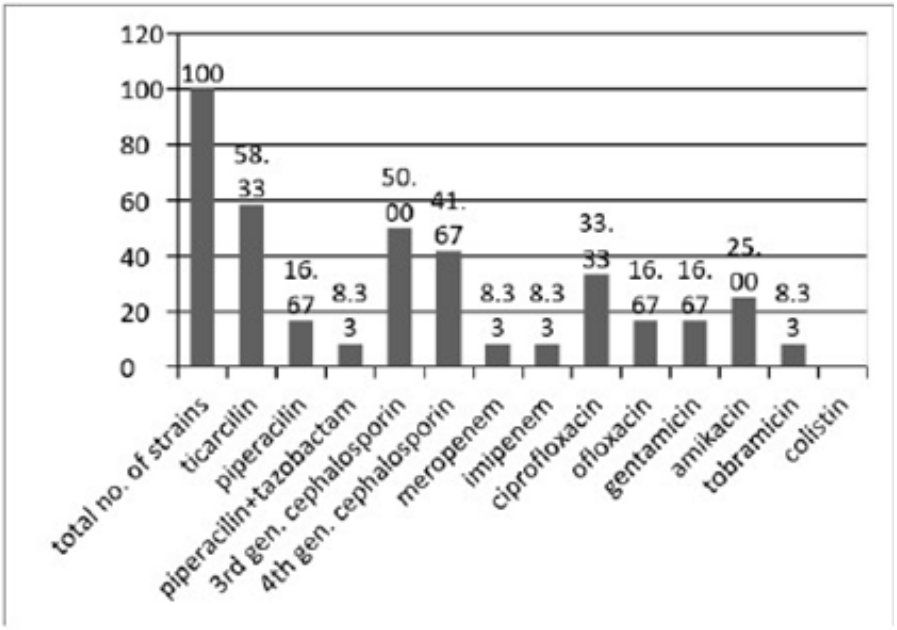

Figure 1. Antibiotic resistance profile among *P. aeruginosa* isolates.

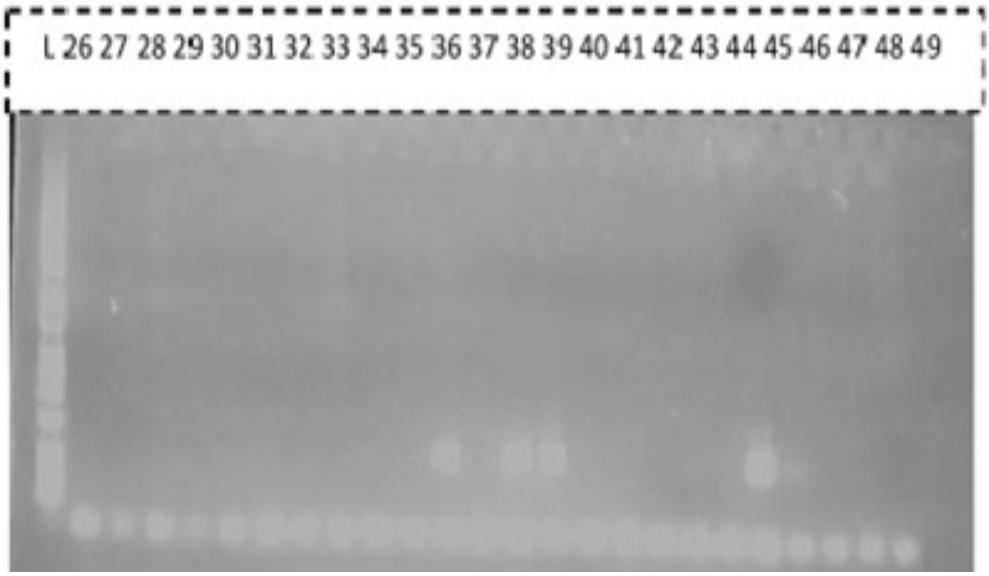

Figure 2. Electrophoresis gel of blaIMP: positive strains –no. 35, 37, 38, 44. 57, 58.

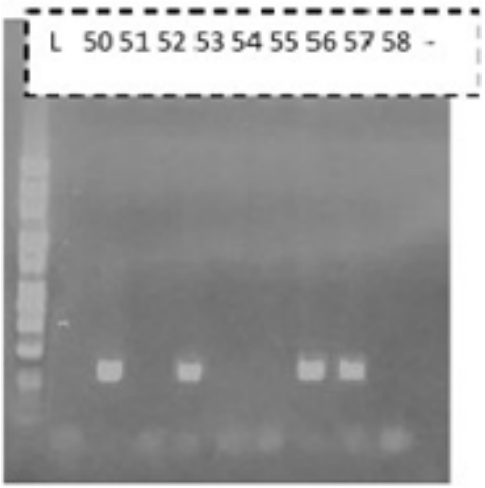

Figure 3. Electrophoresis gel of blaIMP: positive strains - no. 51, 53.

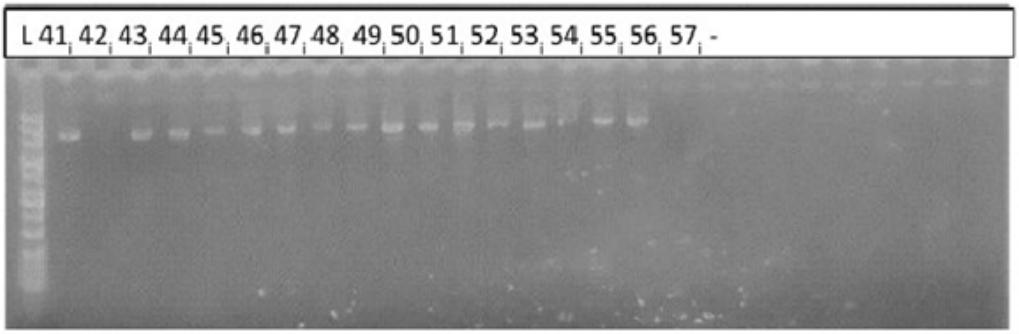

Figure 4. Electrophoresis gel of OprD porin: positive strains - no. 41, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57.

4. CONCLUSIONS

The phenotypic resistance markers were correlated with some specific ARGs, revealing that the *P. aeruginosa* isolates could adapt easily to the high selective pressure exhibited by different antibiotics in the hospital environment. The better knowledge of the epidemiological context in different hospital units could help clinicians to achieve correlations between clinical manifestations of the infection and the presence of certain ARGs and to appropriately adjust the therapeutic approach.

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