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Virulence and resistance genes profiles among *Pseudomonas aeruginosa* isolated in 2015 from patients with cardiovascular diseases in Bucharest, Romania

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ABSTRACT

The purpose of this study was to evaluate the resistance and virulence profiles of *Pseudomonas (P.) aeruginosa* clinical strains isolated in 2015 from patients with cardiovascular diseases hospitalized in the Institute for Cardiovascular Diseases Prof. C.C. Iliescu, Bucharest. Materials and Methods: The strains identification was performed in the hospital unit using automated VITEK2 compact system. Seven virulence genes encoding for protease IV, 3 exoenzymes (exoS, exoT, exoU), two phospholipases plcH- haemolytic phospholipase Cand plcN- nonhaemolytic phospholipase C) and alginate as well as the genes involved in the beta-lactams resistance were investigated by PCR. The molecular analysis through PCR showed that 54.9% of the analyzed strains revealed the *plcH* and *plcH* genes, 50.9% presented *ExoT* and *AlgD* genes, 39.21% of the isolates harbored the protease IV (*TCF/TCR)* gene, 17.64% of the strains the *ExoS* gene and only 1.9% of *P. aeruginosa* expressed *ExoU*. Regarding the carbapenemase genetic resistance, 21.56% of *P. aeruginosa* analyzed strains revealed the bla_{IMP} gene. Conclusions**:** This study reveals that correlating virulence and resistance profiles with the clinical outcome of infection is very useful for setting up efficient preventive and therapeutic procedures for hospitalized patients with cardiovascular diseases, which are colonized or infected with *P. aeruginosa* strains.

Keywords: *Pseudomonas aeruginosa*, virulence factors, resistance, genetic determinants.

1. INTRODUCTION

Pseudomonas aeruginosa possesses an "arsenal" of cell-associated and extracellular virulence factors. Many extracellular virulence factors have been shown to be controlled by a complex regulatory circuit involving cell-to-cell signaling (quorum sensing) systems that allow the bacteria to produce these factors in a coordinated, cell-density–dependent manner [1].

P. aeruginosa is one of the most important bacteria with documented resistance to multiple antimicrobial classes, including β-lactams, carbapenems, aminoglycosides, fluoroquinolones, and polymyxins [2].

The carbapenem-resistant *P. aeruginosa* causes serious infections, such as nosocomial pneumonia which based on the scientific reports is increasing in the hospitalized patients [3]. Resistance to carbapenems is often associated with production of metallo-ß-lactamases [3]. Nosocomial infections caused by *P. aeruginosa* remains the major cause of mortality, particularly because of emergence of multidrug-resistant strains. Carbapenem resistance occurs because of decrease in antibiotics absorption due to lack of an outer membrane porin, as OprD, exclusion from the cell by efflux pump, decrease in outer membrane permeability and production of MBL [4]. OprD is a substrate-specific outer membrane porin of *P. aeruginosa*, which allows the diffusion of basic amino acids, small peptides, and imipenem into the cell. For imipenem, OprD loss can push the MIC above the resistance breakpoint [5]. MBLs are divided into two categories: chromosomally mediated enzymes and those encoded by mobile genetic elements, such as plasmids and transposons [6]. The VIM, IMP and SPM types are the most clinically significant carbapenemases which are encoded by bla_{IMP}, bla_{VIM} and bla_{SPM} genes [7]. At least 14 different VIMs and 23 different IMP MBLs have been identified so far. MBLs are also divided into several families as follows: IMP, VIM, SPM, GIM, SIM, DIM, AIM, KHM, NDM and KPC. Most of them, if not all genes encoding IMP, VIM and SPM types as well as GIM are found as gene cassettes in class 1 integrons, although IMP MBL genes are also found on class 3 integrons [7, 8].

The purpose of this study was to investigate the phenotypic and genotypic antibiotic resistance and virulence profiles of *P. aeruginosa* strains isolated from patients with cardiovascular diseases in Romanian hospitals.

2. EXPERIMENTAL SECTION

This study was conducted on a total of 51 *P. aeruginosa* clinical strains isolated during July -August 2015, taken from patients hospitalized for cardiovascular diseases in hospital „Prof. C.C. Iliescu" – Bucharest. The strains identification was performed in the hospital unit using automated VITEK2 compact system.

The antibiotic susceptibility testing was performed by Kirby-Bauer standard disk diffusion method (panels of antibiotic disks recommended by CLSI, 2015).

The genetic support of the virulence, resistance (carbapenem and quinolones), efflux pumps and aminoglycosides was investigated by simplex and multiplex PCR, using a reaction mix of 20 or 25 µl (PCR Master Mix 2x, Thermo Scientific) containing 1 µl of bacterial DNA extracted using the alkaline extraction method. In this purpose, 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 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 of 7 virulence genes encoding for protease IV, three exoenzymes – exoS, exoT, exoU, two phospholipases - plcH (haemolytic phospholipase C) and plcN (non-haemolytic phospholipase C) and for alginate; for carbapenemases bla IMP; blaVIM; bla NDM, bla SPM; bla SIM; for aminoglycosides (aac3Ia); and for efflux pumps (OprD). The PCR reactions were initiated with 1 cycle at 95°C for 2 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final elongation step at 72°C for 7 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

3.1. Antibiotic susceptibility profiles.

In the present study with a total of 51 strains isolated from cardiovascular diseases of hospitalized patients, 37.25% of the isolates were multidrug resistant (MDR). The *P. aeruginosa* strains isolated from cardiovascular diseases were resistant in high proportions to ticarcillin, to third and fourth generation cephalosporins and to ciprofloxacin. 21.56% of the strains demonstrated resistance to imipenem and meropenem (Fig. 1) and 11.76% of the isolated strains demonstrated resistance to colistin, a "last resort" antibiotic in Romania for parenteral treatment of infections produced by XDR strains. A recent study in Romania [9] identified 4 strains of *P. aeruginosa* showing a comparable resistance to antibiotics, one strain being susceptible only to colistin.

Concerning the virulence profiles the molecular analysis through PCR arrays showed that 54.9% of the analyzed strains revealed the *plcH* and *plcH* genes, 50.9% present *ExoT* and *AlgD* genes, 39.21% of the isolates expressed the protease IV (*TCF/TCR)*, 17.64% of the strains revealed the *ExoS* gene and only 1.9% of *P. aeruginosa* expressed *ExoU* gene (Fig.2).

The two phospholipases could act synergistically, first plcH would promote degradation of the erythrocyte membrane (phospholipids components of the outer leaflet: phosphatidylcholine and sphingomyelin), exposing the inner leaflet and Plc-N could then hydrolyze phospatidylserine present in the inner leaflet. The genotyping results showed that 54.9% of the *P. aeruginosa* isolates possess the two phosposlipases (Fig. 2 and Fig. 4) compared with other studies from our country in which only the plcH gene was revealed in the strains isolated from blood cultures and wound secretions [10].

The results of PCR analysis concerning the presence of *algD* gene showed that 50.9% of the isolates express this gene, an aspect which demonstrates the involvement of these strains in infections with biofilm formation [11] (Fig.2 and Fig.5). One of *P. aeruginosa* virulence factors, the exoenzyme S (ExoS), has been proposed to act as an anti-phagocytic factor [12], thus enabling the bacteria to evade the host immune system. More recently, ExoS was found to include a GTPase-activating protein (GAP) activity that targets the low-molecular-weight proteins Rho, Rac1, and Cdc42, which affect eukaryotic cell cytoskeletal structure [13]. From this study, 17.64% of the analyzed strains expressed the ExoS gene (Fig. 2).

Expression of exoenzyme U is known to greatly increase the virulence of *P. aeruginosa in vivo* generally [14, 15], and specifically in lung infections [16]. In our study only 1.9% of the analyzed strains revealed the ExoU gene (Fig. 2). It is known that 90% of ExoU-producing *P. aeruginosa* strains are associated with

severe infections [17]. Of the type III secretion proteins, ExoU is the most cytotoxic. Secretion of ExoU is a marker for highly virulent *P. aeruginosa* isolates obtained from patients with hospital-acquired pneumonia [18].

Regarding the carbapenemase genetic support, 21.56% of *P. aeruginosa* analyzed strains revealed bla_{IMP} (Fig. 3). IMP enzymes were described 34 years ago [19] in Japan and quickly spread worldwide, in representatives of the family *Enterobacteriaceae* and also in non-*Enterobacteriaceae* strains, like in *P. aeruginosa*. Until now 20 variants of the bla_{IMP} gene have been described [19], the gene was reported in Romania, in 2007 [20] with IMP -1 variant in *A. baumannii* isolates from patients from Cardiology Institute in Bucharest isolated between 2003-2006; and in 2013 from clinical isolates of *P. aeruginosa* from patients from five hospitals in Iasi [21] with the IMP- 13 variant.

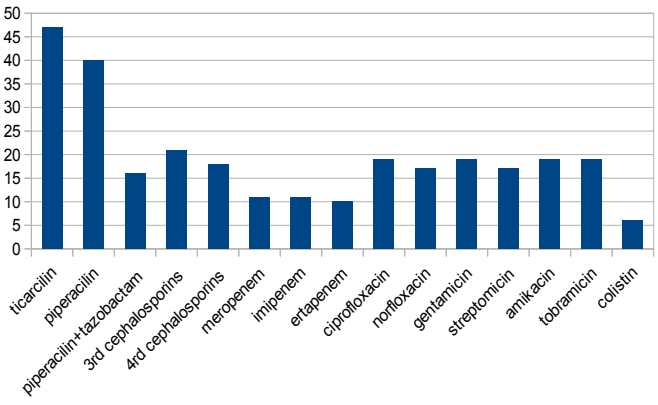

Figure 1. Antibiotic resistance profile among *P. aeruginosa* isolates.

The *aac*(3)-I-a [1.96% of the analyzed strains (Fig. 3, 8)] enzymes confer resistance to gentamicin, sisomicin, and fortimicin (astromicin) and are widespread among *Enterobacteriaceae* and in Gram-negative nonfermentative bacilli [22]. The *aac*(*3*)-*I* alleles are found on mobile gene cassettes inserted into class 1 integrons [22], a location that evidently facilitates their spread among different replicons and eventually among different strains and that likely accounts for the diffusion of these genes in the clinical setting.

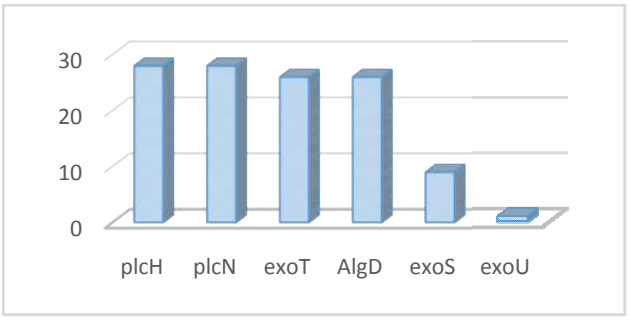

Figure 2. Distribution of the enzymatic virulence factors genes in *P. aeruginosa*.

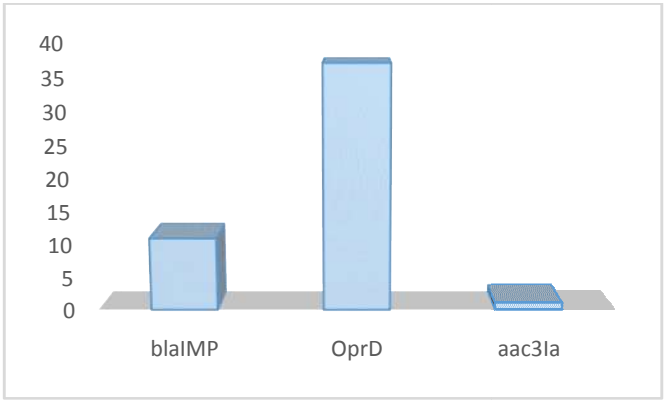

Figure 3. Distribution of carbapenemases, aminoglycosides resistance and efflux pumps genes in the analyzed strains.

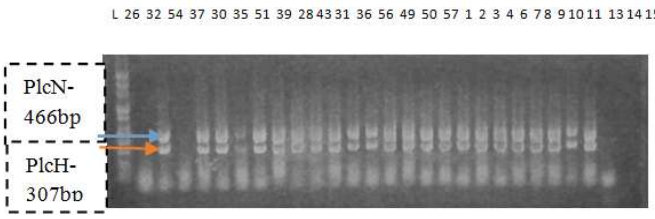

Figure 4. Electrophoresis gel of *PlcH and PlcN* genes amplicons: the figure shows that all the isolates except no. 26 and 54 revealed the two phosposlipases. Well1(top and bottom) markergm: 100pb.

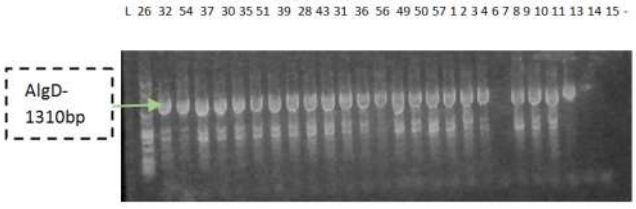

Figure 5. Electrophoresis gel of AlgD genes amplicons: The figure shows that all the analyzed strains except no. 8 are positive.

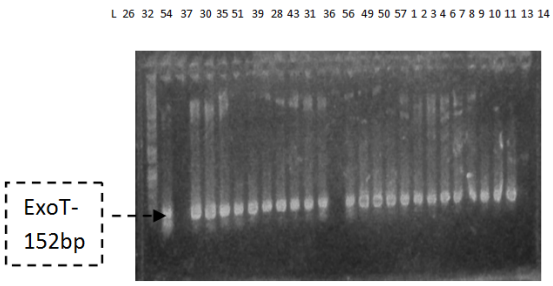

Figure 6. Electrophoresis gel of *ExoU and ExoT* genes amplicons: the figure shows that all the isolates except no. 32 revealed the ExoT gene.

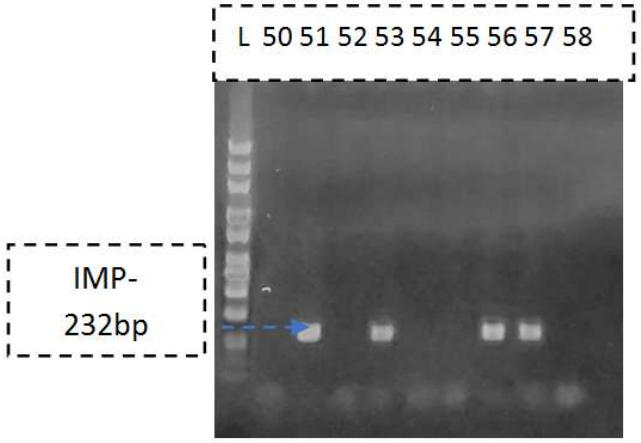

Figure 7. Electrophoresis gel of bla$_{VIM}$ and *bla*$_{IMP}$ genes: the figure showns that the isolates no. 51, 53, 57, 58 expressed the *bla*$_{IMP}$ gene.

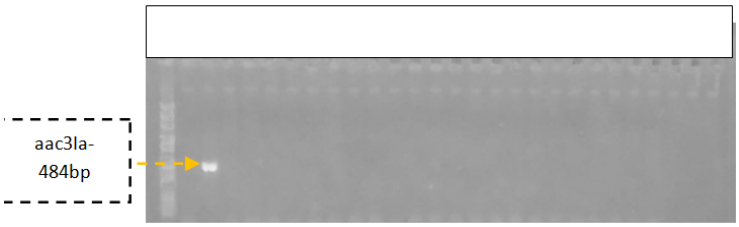

Figure 8. Electrophoresis gel of *aac3Ia* genes amplicons: the figure shows that only the isolate no. 27 isolated in July 2015 revealed the the aminoglycoside resistance aac3Ia gene.

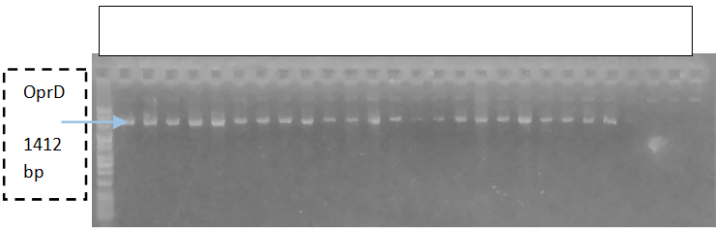

Figure 9. Electrophoresis gel of *OprD* genes amplicons: the figure shows that all the isolates revealed the OprD gene.

4. CONCLUSIONS

Our results show that the analyzed strains expressed a large panel of virulence markers, revealing that the isolates could adapt easily to the microenvironment encountered within the host by modulating the expression of these genes. Furthermore, the diversity of virulence factors associated with multiple drug resistance mechanisms could be responsible of thelarge panel of clinical manifestations of difficult to treat *P. aeruginosa* infections.

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