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Virulence and resistance features of *Pseudomonas aeruginosa* strains isolated from patients with cardiovascular diseases

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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 2014 from patients with cardiovascular diseases hospitalized in the National Institute for Cardiovascular Diseases Prof. C.C. Iliescu, Bucharest. The phenotypic screening evaluated eight soluble virulence factors (haemolysins, lecithinase, lipase, caseinase, gelatinase, amylase, DNase, aesculin hydrolysis) and the resistance profiles have been determined by using disk diffusion and the automated Vitek II system. Seven virulence genes encoding for protease IV, three exoenzymes (exoS, exoT, exoU), two phospholipases (plcH- haemolytic phospholipase C and 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 38.8% of *P. aeruginosa* strains expressed the gene codifying for protease IV, 22.22% the ExoS gene, 16.16% the plcH and ExoT genes and 11.11% of the analyzed strains harbored the ExoU and plcN genes. Regarding the resistance genes our study revealed 22.22% of the isolates positives for imipenemase gene (bla_{IMP}) which confers β-lactams resistance. This study reveals that correlating the phenotypic and genotypic 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, resistance, genetic determinants.*

1. INTRODUCTION

Pseudomonas aeruginosa, one of the most common bacteria isolated from chronic wounds [1], is an opportunistic pathogen with innate resistance to many antibiotic classes, including antipseudomonal penicillins, carbapenems, aminoglicosides and ciprofloxacin [2].

Besides intrinsic and acquired resistance to many antibiotics, *P. aeruginosa* expresses many extracellular virulence factors (exotoxins S and T with ADP-ribosylating activity which cause disruption of the host cell cytoskeleton, cytotoxin ExoU with phospholipase activity and cytotoxin ExoY with an adenylate cyclase activity, phospholipases, protease IV and elastase) and surface associated components, as alginate. It is known that the effectors ExoS, ExoY, and ExoT inhibit invasion, while ExoU confers cytotoxicity [3, 4]. *P. aeruginosa* elaborates two known phospholipases C, i.e. PlcH (hemolytic) and PlcN (nonhemolytic). While PlcN has no demonstrated pathogenic activity, PlcH may be an important virulence factor. Indeed, purified PlcH causes vascular permeability, organ damage, and death when injected into mice in high doses [5]. Protease IV is a lysine-specific endoprotease and a significant virulence factor for pathogenesis of *P. aeruginosa* in the eye and lung [6], which degrades important host immunological proteins, such as complement and IgG [7]. Protease IV also compromises the integrity of structural proteins, such as elastin, causing tissue damage and facilitating bacterial infection. All these factors contribute to massive tissue damage, blood infection dissemination, and progression of the disease, and also limit treatment options with currently available antibiotics [8, 9]. The pathogenesis of *P. aeruginosa* infections is multifactorial, the first step in *P. aeruginosa* infections being the colonization of the altered epithelium. After colonization, *P. aeruginosa* produces several extracellular virulence factors (alkaline protease and staphylolytic protease, elastase, protease IV, heat-labile and heat-stable hemolysins, phospholipases C and exotoxins A, S, T, U, Y), responsible for extensive tissue damage, bloodstream invasion, and dissemination [10].

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 [11]. Recent evidence demonstrates that cardiovascular disorders are usually associated with increased level of stress hormones [12, 13].

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

2. EXPERIMENTAL SECTION

2.1. Isolation and identification of bacterial strains.

This study was conducted on a total of 18 *P. aeruginosa* clinical strains isolated during 2014, from patients with cardiovascular diseases hospitalized 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. The antibiotic susceptibility testing of the respective strains was performed by diffusion method (Kirby -Bauer), following the recommendations of CLSI editions 2014.

2.2. Evaluation of the soluble enzymatic factors.

The virulence phenotypes were assessed by performing enzymatic tests for the expression of some virulence soluble factors. Overnight culture of the strains was evaluated for the following virulence factors expression: haemolysins, other pore forming toxins (lecithinase, lipase), proteases (caseinase, gelatinase), amylase and aesculin hydrolysis. Detection of haemolysin production was performed by spotting the fresh cultures on 5% sheep blood agar medium and incubation at 37°C for 24h. The colorless area around the culture revealed the presence of haemolysis activity. For lipase production the strains were spotted on 1% Tween 80 agar as a substrate and followed by incubation at 37°C for 24h. An opaque (precipitation) zone around the spot was registered as positive reaction; for lecithinase production, the cultures were spotted into 2.5% yolk agar and incubated at 37°C for 24h. A clear zone around the spot indicated the lecithinase production. The protease activity (caseinase and gelatinase) was determined using 15% soluble casein agar, respectively 3% gelatin as substrate. The strains were spotted and after incubation at 37°C for 24h, a white precipitate surrounding the growth indicates casein proteolysis, and colorless area around culture due to the gelatine hydrolysis, indicates the positive reaction for gelatinase. Amylase was detected using agar with 1% starch and hydrolysis was revealed after adding Lugol's solution (yellow ring around the culture, while the rest of the plate will be blue. For the aesculin hydrolysis the medium containing Fe^{3+} citrate was used and inoculated by spotting, then incubated for 24h at 37°C. A black precipitate around culture due to esculetol released under the action of beta-galactosidase was considered positive reaction.

2.3. Evaluation of the antibiotic susceptibility.

The antibiotic susceptibility testing was performed by Kirby-Bauer standard disk diffusion method (panels of antibiotic disks recommended by CLSI, 2013, 2014).

2.4. PCR assays for virulence genes detection.

The genetic support of the virulence factors 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. 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. Regarding the carbapenemases (bla_{VIM} and bla_{IMP}) the PCR reactions were performed at 94°C for 10 min, followed by 36 cycles at 94°C for 30 s, 52°C for 40 s, 72°C for 50s and a final elongation step at 72°C for 5 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

The isolated strains expressed between one and three of the seven investigated soluble virulence factors (Table 1). The most frequently expressed soluble virulence factors were beta haemolysins (50%) followed by caseinase, gelatinase and esculinase (83.33%) (Table 1). Although some studies [14] reported an increased expression of DN-ase in the strains isolated from wound secretions, in our study only 2 of the 18 strains of *P. aeruginosa* produced DN-ase (11.11%).

P. aeruginosa strains isolated from patients with cardiovascular diseases were resistant in high proportions to ticarcillin, to third and fourth generation cephalosporins and to ciprofloxacin. Only one strain demonstrated resistance to imipenem and meropenem (Figure 1). None of the isolated strains demonstrated resistance to colistin, a “last resort” antibiotic in Romania for parental treatment. A recent study in Romania [15] identified four strains of *P. aeruginosa* showing a comparable antibiotic resistance profile, the strains remaining susceptible only to colistin.

On the other hand, international data reveal variable resistance rates for *P. aeruginosa* strains: one study revealed strains isolated from chronic wounds resistant to piperacillin/tazobactam and aztreonam [16], while another study found isolates of *P. aeruginosa* 100% resistant only to imipenem and meropenem, followed by ceftazidime (86%) and amikacin, aztreonam, cefepime, ciprofloxacin, and piperacillin tazobactam (57% each) [17].

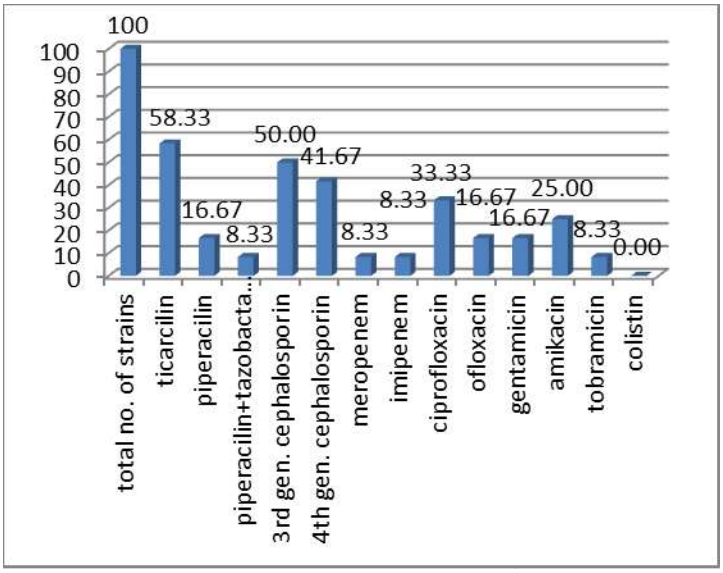

Figure 1. Antibiotic resistance spectrum among isolated *P. aeruginosa* strains.

Table 1. Phenotypic and molecular virulence characteristics of *P. aeruginosa strains.*

Strain lab. code	Strains	Virulence markers					
		TCF\TCR	ExoS	plcH	plcN	ExoU	ExoT
15	*P.aeruginosa-729*						
16	*P.aeruginosa-353*	X	X	X	X	X	
17	*P.aeruginosa-164*	X	X				
	P.aeruginosa-524						
23	*P.aeruginosa-729*						
24	*P.aeruginosa-53*	X		X	X	X	X
22	*P.aeruginosa-425*						
28	*P.aeruginosa-855*						
36	*P.aeruginosa-1101*	X			X		
46	*P.aeruginosa-253*						
57	*P.aeruginosa-311*						
58	*P.aeruginosa-361/1*	X	X				X
59	*P.aeruginosa-262*						
31	*P.aeruginosa-238*						
40	*P.aeruginosa-649*	X	X				X
44	*P.aeruginosa-428*						
45	*P.aeruginosa-296*						
25	*P.aeruginosa-940*						
26	*P.aeruginosa-31*	X					

Strain lab. code	Strains	Virulence markers							
		Amylase	*Lipase*	*Lecitinase*	DN- ase	Esculinase	Gelatinase	Hemolysinea, β	Caseinase
15	*P.aeruginosa-729*							β	
16	*P.aeruginosa-353*								
17	*P.aeruginosa-164*						X	β	
	P.aeruginosa-524							β	X
23	*P.aeruginosa-729*							β	X
24	*P.aeruginosa-53*				X			β	X
22	*P.aeruginosa-425*							β	
28	*P.aeruginosa-855*		X		X			β	
36	*P.aeruginosa-1101*							α	
46	*P.aeruginosa-253*					X	X	β	
57	*P.aeruginosa-311*					X	X		
58	*P.aeruginosa-361/1*					X			
59	*P.aeruginosa-262*					X			
31	*P.aeruginosa-238*			X				β	
40	*P.aeruginosa-649*		X			X		α	
44	*P.aeruginosa-428*		X	X				β	
45	*P.aeruginosa-296*		X	X				β	
25	*P.aeruginosa-940*		X	X	X				
26	*P.aeruginosa-31*		X	X	X				

The molecular analysis through PCR arrays showed that 38.8% of *P. aeruginosa* expressed the gene codifying for protease IV (*TCF/TCR*), 22.22% of the strains expressed ExoS gene, 16.16% of *P. aeruginosa* isolates revealed plcH and ExoT genes and 11.11% of the analyzed strains revealed the ExoU and plcN genes (Figure 2, Table 1).

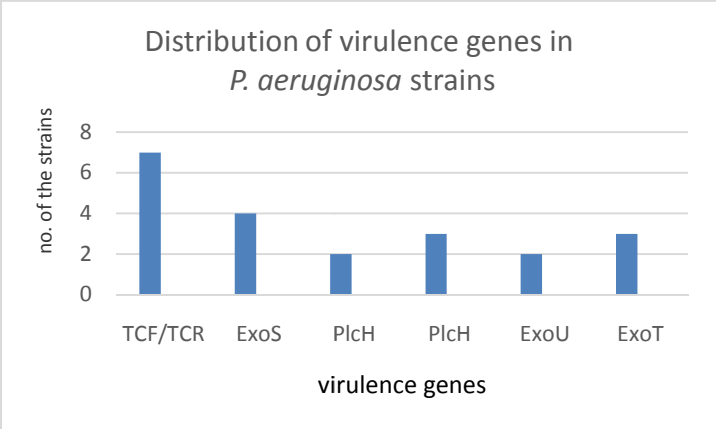

Figure 2. Distribution of virulence genes in the *P. aeruginosa* analyzed strains.

P. aeruginosa strains produce three soluble proteins which are responsible for invasion, i.e.: a rhamnolipid and two phospholipases C (haemolytic phospholipase C (plcH), and non-haemolytic phospholipase C (plcN)) [18]. These two phospholipases could work synergistically, i.e.: the 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 the phospatidylserine present in the inner leaflet. Our results of genotyping analysis showed that 16.16% of the *P. aeruginosa* isolates possess haemolytic phospholipase C and 11,11% of the strains revealed non-haemolytic phospholipase C (Figures 2, 3) 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 [14]. The phospholipase gene expression is sustained at phenotypic level, the same strains being positive for lipase production.

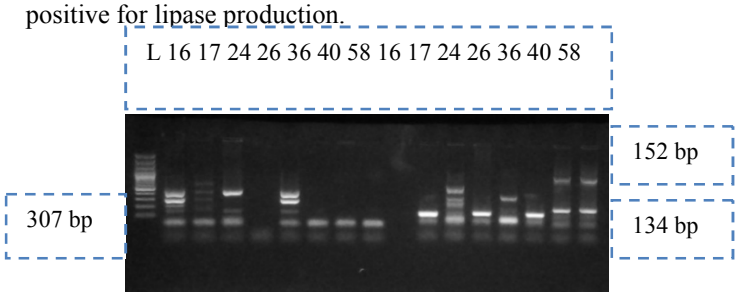

Figure 3. Electrophoresis gel for exotoxins (ExoU and ExoT genes-right side) and phospholipases [PlcH (466pb) and PlcN (307pb) amplicons (left side). Line 1: PCR Marker (Promega) - 100pb; positives isolates for the two phospholypases: no16, 24 and for exotoxins- ExoU-16, 24; for ExoT-24, 58 and 40.

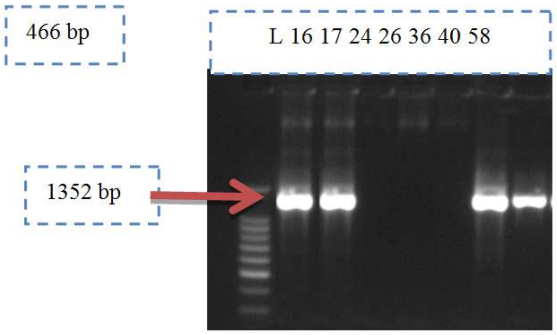

Figure 4. Electrophoresis gel of ExoS gene: the positives strains are no.16, 17, 40, 58.

It is known that the exotoxins ExoS, ExoY, and ExoT inhibit invasion, while ExoU confers cytotoxicity [4, 19]. ExoT and ExoS act on a number of host small G proteins, thus altering the cytoskeleton and signaling pathways [20]. The concomitant presence of ExoU and ExoS was also revealed by one isolate of *P. aeruginosa* (Figures 3, 4).

The isolated strains of *P. aeruginosa* exhibited a high frequency of protease IV (TCF/TCR-38.8% of the isolates), which plays an important role in the induction of tissue injuries (Figure 5, Table 1). Holban et al. [14] reported also a highest frequency of plcH and protease IV genes in their study.

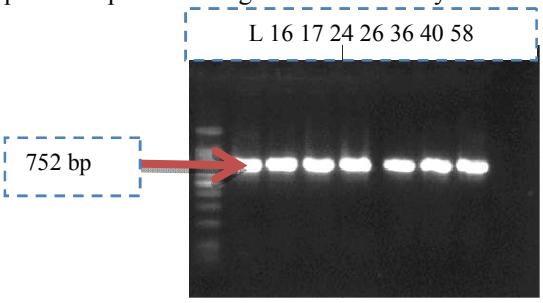

Figure 5. Electrophoresis gel of protease IV (TCF/TCR) amplicons; the positives strains are no. 16, 17, 24, 26, 36, 40, 58.

Regarding the antibiotic resistance genes our study revealed that 22.22% of the analyzed strains resistant to ticarcilin and meropenem/imipenem produced imipenemase (Figure 6).

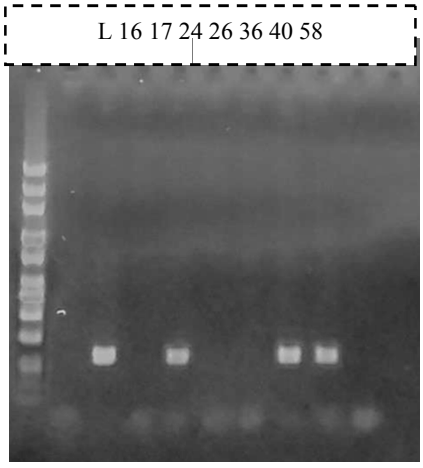

Figure 6. Electrophoresis gel of bla_{IMP} amplicons; the positive strains are no. 17,26,40,58.

4. CONCLUSIONS

All this data could help clinicians to achieve correlations between clinical manifestations and the virulence or resistance features of the involved strain and to adjust the therapeutic approach. The phenotypic virulence markers were correlated with some specific virulence genes profiles, revealing that the isolates could adapt easily to the microenvironment encountered within the host by modulating the expression of these genes.

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6. ACKNOWLEDGEMENTS

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