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Prevalence of *Pseudomonas aeruginosa*'s Virulence Genes Isolated from Human Infection in Abidjan, Côte d'Ivoire

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Authors' contributions

This work was carried out in collaboration between all authors. Author CKE designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AN and AY managed the analyses of the study. Author CND managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study was to evaluate the distribution of virulence genes among clinical isolates of *P. aeruginosa* and to compare these factors with the localisation of clinical infection and the serotypes. 151 isolates of *P. aeruginosa* were collected from various biological fluids: The strains were identified based on the standard bacteriological characteristics. The serotyping of the strains was done. The Detection of virulence genes was done by polymerase chain reaction (PCR). The genes sought were: *PilB, lasB, nan1, algD, plcH, exoS, and exoU genes.*

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The most common virulence factor found in clinical strains of *P. aeruginosa* was, alg D (encoding for alginate) with a prevalence of 90.7%, plch (haemolytic phospholipase C precusor) with 84.1%, las B (elastase B) 72.8%, pilB (type IV fimbrial biogenesis protein pilB) 70.2%, nan1 (neuraminidase) 37.1%, exoS (exotoxin S) 31.1% and exoU (exotoxin U) 17.9 %. Infections with the highest virulence levels were sepsis and lung infections. The exoS gene was more frequently found in serotypes O2 (43%), O4 (39%) and O3 (25%). As for the exoU gene, the prevalent serotypes were O11 (28.8%) and O1 (20%). The multidrug resistant *P. aeruginosa* isolates were most frequently associated with the presence of algD (78.6%), plch (71.4%), lasB (64.3%), pilB (42%), and nan 1 (42%).
P. aeruginosa of invasive infections like septis and lung infections had the highest level of virulence.

Keywords: P. aeruginosa; virulence; serotype; infection.

1. INTRODUCTION

Pseudomonas aeruginosa (P. aeruginosa) is an opportunistic pathogen that is a common cause of hospital-acquired infections, particularly infecting patients with predisposing factors, such as burn victim, immune compromised hosts, or those with metabolic disorders. In cystic fibrosis (CF) patients, *P. aeruginosa* is believed to be a major contributory factor to chronic lung infections, which could form biofilm and adhere to human mucin in the lower respiratory tract [1]. It can cause acute and chronic infections in human. *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, ear and also cause other blood infections [2]. A major problem in its infections is the resistance to relatively high levels of most antibiotics in use [3] and also a multidrug resistance [4]. These resistance is due to three classes of antibiotic resistance: intrinsic resistance, acquired resistance and genetic resistance [5].

P. aeruginosa possesses a large number of cell-associated and extracellular virulence factors, which are tightly regulated by cell-to-cell signalling systems (quorum sensing) that allow the bacteria to produce these factors in a coordinated, cell-density–dependent [6]. The formation of mucoid colonies of *P. aeruginosa* composed of alginates, involving algD genes, protects the bacterium from the host's immune response and from antibiotics [6, 7]. Other virulence factors produced by this bacterium which are important in its pathogenicity are exotoxine, Exoenzyme U, las B elastase and phospholipase C. Exoenzyme S and U, encoded by the exoS and exoU gene, are secreted by a type-III secretion system in to the cytosol of epithelial cells [8]. Elastase, a zinc metalloprotease encoded by the lasB gene, has an elastolytic activity on lung tissue, attacks eukaryotic proteins such as collagen and elastin and destroys the structural proteins of the cell [9]. In addition, the phospholipids contained in pulmonary surfactants may be hydrolysed by two phospholipases C encoded by plcH and plcN (PLC-H and PLC-N, respectively) [10]. An extracellular neuraminidase (encoded by the nan1) is thought to play an important role in the implantation of the bacterium and to contribute to the formation of biofilm [11].

The aim of this study was to evaluate the distribution of virulence genes encoding for GDP mannose 6-dehydrogenase (alginate) (algD), elastase (lasB), type IV fimbrial biogenesis protein (pilB) and neuraminidase (nan1) among clinical isolates of *P. aeruginosa* and to compare these factors with the location of clinical infection and the serotypes.

2. MATERIALS AND METHODS

2.1 Isolation of *P. aeruginosa* Strains

From January 2014 to June 2017, 151 isolates of *P. aeruginosa* were collected from various biological fluids: pus (78), sputum (27), urine (16), blood (16), material (12) and Cerebrospinal fluid (6) at the Pasteur Institute of Côte d'Ivoire. Isolation procedures were based on standard isolation. Specimens were inoculated on Nutrient agar, Blood agar, MacConkey agar and were studied for colony. The strains were identified based on the standard bacteriological characteristics: pigmentation of colonies on King A and King B medium (pyocyanine and pyoverdine), growth at 42^{o}C, morphological appearance (Gram negative bacillus, polar mobile), the positive oxidase reaction and other biochemical characteristics using the API 20 NE gallery (Bio-Mérieux®, France) and API database.

2.2 Serotyping of *P. aeruginosa*

The serotyping of the strains was performed using the slide agglutination technique with the

aid of 4 polyvalent specific antisera PMA (O1 + O3 + O4 + O6), PMC (O9 + O10 + O13 + O14), PME + O5 + O15 + O16), PMF (O7 + O8 + O11 + O12)] (Biorad ®, France)) followed by specific monovalent antisera (Biorad®, France)).

2.3 Detection of Virulence Genes by polymerase Chain Reaction (PCR)

2.3.1 Extraction and purification of DNA

Template DNA was extracted from whole organisms by boiling [12]. Bacteria were harvested from an overnight broth culture (Biokar Diagnostics, BK015HA, France), suspended in 1 ml sterile Milli-Q water (Milli-Q™, Millipore Corporation, USA). A suspension of 200 µl was incubated at −20 °C for 15 min and boiled at 95 °C for 15 min. The suspension was immediately cooled at 4 °C for 10 min and then centrifuged at 14,000 rpm for 10 min to pellet the cell debris. The DNA template was purified according to the method described by Zimmermann et al**.** [13].The purity and DNA concentration of the extract were determined by spectrophotometer (Eppendorf Bio Photometer plus, USA).

2.3.2 Amplification of, PilB, lasB, na, algD n1, plcH, exoS, and exoU genes

Multiplex PCRs were used to detect *algD, PilB, lasB, nan1, plcH, exoS,* and *exoU*. PCR mixtures with a final volume of 25 µl consisted of 15.8 µl sterile Milli- Q water (Milli-Q™, Millipore Corporation, USA), 5 µl 5XTP, 1.5 µl MgCl2 (2 mM), 0.2 µl dNTPs (10 mM), 0.1 µl each primer (20 mM) (Integral DNA Technology, France), 0.1 µl Go tag polymerase (Promega Corporation, Madison, WI 53711-5399, USA), and 2 µl DNA template. A quantity of 2 µl of sterile Milli-Q water was used for negative control, and DNA of ATCC reference strain 27853 was used for positive control. The oligonucleotide primers used in this study and the amplification program are listed in *Tables 1*. Each PCR was performed using thermocycleur type T3000 Thermocycler, block type standard 3a (Biometra, Germany). The amplified DNA was separated by gel electrophoresis with 2% agarose containing 0.5 µg/ml with ethidium bromide for 30 min at 130 V, visualised under UV trans-illumination, and photographed (Molecular Imager Gel DocTM XR+, Bio-Rad). A molecular weight marker (TriDye™, 100 bp or 1-kb DNA Ladder, Biolabs) was used. Amplified genes were identified on the basis of fragment size shown in Table 1.

3. RESULTS

3.1 Frequency of virulence genes in *Pseudomonas aeruginosa* isolates

The most common virulence factor found in clinical strains of *P. aeruginosa* was, alg D (encoding for alginate) with a prevalence of 90.7%, plch (haemolytic phospholipase C precusor) with 84.1%, las B (elastase B) 72.8%, pilB (type IV fimbrial biogenesis protein pilB) 70.2%, nan1 (neuraminidase) 37.1%, exoS (exotoxin S) 31.1% and exoU (exotoxin U) 17.9%.

Of 151 isolates of *P. aeruginosa*, 10 had all 7 virulence genes studied, ie a rate of 6.6%.

3.2 Distribution of Virulence Genes by Patient Type

All virulence genes studied were found in both categories of patients. Virulence genes were present in both inpatients and outpatients with varying frequencies (Figure 1). The pilB and exoU genes were found in more than 50% of hospitalised patients. As for the genes alg D, plch, nan1, exoS, they were more frequent in outpatients.

3.3 Distribution of Virulence Genes by Site of Infection

All genes were found in biological products at varying frequencies (Table 2). The algD (alginate) gene had a prevalence that ranged from 80.7 to 100% depending on the biologic product. The highest rates were found in septicemia and invasive materials.

Plch (phospholipase C) genes were detected in strains from all the various biological samples types with frequencies ranging from 66.7% to 84.6%. The highest rates are found in septicemia and pulmonary infections

As for the lasB (elastase) gene, it was present in more than 50% of all biological samples types with a higher frequency of 81.5% in the pulmonary samples. It was the same for the pilB gene.

Regarding the exotoxin, the exoS gene was present in 47% in biological samples types with respective frequencies of 46.1% in the blood and 42.8% in the urine. As for the exoU gene, the rates were the lowest ranging from 7.6% to 18.5%.

The nan1 gene (neuraminidase) detected in less than 50% of the isolates. The prevalence was higher in the blood, urine and pus with respective frequencies of 46.1%, 42.9% and 39.9%.

3.4 Distribution of Type III Virulence Genes by Serotype

Thirteen different serotype were found at varying rates. The serotypes found were: O4 (23.4%), O11 (13.9%), O6 (10.6%), O2 (7.9%), O3 (7.3%), O16 (6.6%), O1 (6.6%), O5 (6%) O10 (6%), O15 (4%), O12 (2.6%), O7 (0.6%) and O14 (0.6%). Three strains were not typeable out of 151.

3.5 Distribution of Exotoxins U and S According to Serotypes

Regarding virulence genes mediated by the type III pathway (exoS and exoU.), it has been found in all serotypes with varying proportions. The exoS gene was more frequently found in serotypes O2 (43%), O4 (39%) and O3 (25%). As for the exoU gene, the prevalent serotypes were O11 (28.8%) and O1 (20%) (Fig. 2).

4. DISCUSSION

Most of the 151 isolates had the virulence genes: 90.7% had alg D (encoding for alginate), plch (haemolytic phospholipase C precusor) with 84.1%, lasB (elastase lasB) at 72.8%, and pilB (type IV). fimbrial biogenesis protein pilB) at 70.2%. Mitov I. et al in Bulgaria in 2010 found similar results with alg (91.1%), plch (91.6) lasB (100%) [16]. But not for PilB which were found at a lower rate (23.8%). The prevalence of nan 1 was 37.1%, Wa'ad M. in turkey found a lower rate for nan1 (15%) [7].

The prevalence of exoS (exotoxin S) is 31.1% and exoU (exotoxin U) is 17.9%. These rates are lower than those of Mitov et al. with exoS (62.4%) and exoU (30.2%).

Alginate gives the bacterium several properties including: the mucosal phenotype, adhesion to tracheal cells, inhibition of phagocytosis and the immune response [17].

The algD gene had a prevalence ranging from 80.7 to 100% at different infectious sites. All strains of *P. aeruginosa* isolated from septicemia and intraorganic material (probe, prosthesis ...) had the algD gene. Similar results were found by Wa'ad M in turkey in 2011.

Phospholipase C plays a role in the lysis of target cells (pulmonary atelectasis) and is involved in acute and chronic infection. [17]. The plch gene was found in all types of infections with prevalence ranging from 66.7% to 84.6%. The highest prevalence was found in sepsis with 84.6% followed by lung infections with 81.5% and then urinary tract infections (78.8%). This frequency at the urinary level is close to that Heidary et al. (70.4%) in 2016 in Iran [18]. Elastase causes the destruction of tissues containing elastin, tissue necrosis and haemorrhage. [19]. The lasB gene was found in isolates from all infectious sites with variable rates. Prevalence were high and ranged from 57.1% to 81.5%. The highest rate was found at the pulmonary level (81.5%). These results are close to those of Mitov I. et al. (100%).

The type IV pilus allows adhesion to respiratory epithelial cells. Pileated strains cause more pneumonia and an increased mortality [19]. The pilB gene frequency ranged from 53.8% to 70.3% depending on the site. The highest rate was found at the pulmonary level.

The neuraminidase of *P. aeruginosa* is involved in the formation of biofilm contributing to the initial colonisation of the airways [11]. The Nan1 gene (neuraminidase) had a prevalence of 23% to 46.1% with the highest level in the blood. This is a factor that affects the prognosis in septicemia. In urinary tract infections rate was 42.9%, this result is similar to the study of Lanotte et al. (40.7%) [20]

Strains of *P. aeruginosa* from bacteremia and pneumopathy that use the type III secretion system, cause a mortality risk 6 times higher. [21]The presence of genes coding for exotoxins (ExoS, ExoT, ExoU and ExoY) is a predictor of a poor prognosis and could help distinguish potentially life-threatening infections. [21]

For exoS, rates ranged from 22.2% to 46.1%. The highest rate was found in septicemia. This means that 46% of patients with *P. aeruginosa* sepsis have a mortality risk that is 6 times higher than normal. For Feltman et al. in the US, the highest rates were found for pulmonary infections (85%) [22]. In Iran somes authors found for ExoS, 66,7 %(sputum) and 33,3% for the sepsis. [23]

The exoenzyme U causes lesions of the epithelial cells responsible for pulmonary infection and bacteremia or even septic shock

[17,19]. Regarding exo U rates ranged from 7.6% to 18.5%. The highest rate was found in lung infections. Feltman et al. found 10% which is close. At the level of sepsis they found 40% against 15.4% in this study. According to the study carried out in Iran the prevalence were higher with pulmonary level 68.6% and 65.7% for septicemia [23].

Table 1. Primers used for amplification of virulence genes in multiplex PCR

Primers	Genes	Sequences (5'-3')	Size (Pb)	T°C	source
algD-F	algD	ATG CGA ATC AGC ATC TTT GGT	1310	62	[14]
algD-R		CTA CCA GCA GAT GCC CTC GGC			
pilB-F	pilB	ATG AAC GAC AGC ATC CAA CT	826	60	[15]
pilB-R		GGG TGT TGA CGC GAA AGT CGA T			
nan1-F	nan1	ATG AAT ACT TAT TTT GAT AT	1317	53	[15]
nan1-R		CTA AAT CCA TGC TCT GAC CC			
lasB-F	lasB	GGA ATG AAC GAG GCG TTC TC	300	60	[14]
lasB-R		GGT CCA GTA GTA GCG GTT GG			
plcH-F	plcH	GAA GCC ATG GGC TAC TTC AA	307	60	[14]
plcH-R		AGA GTG ACG AGG AGC GGT AG			
exoS-F	exoS	CTT GAA GGG ACT CGA CAA GG	504	60	[14]
exoS-R		TTC AGG TCC GCG TAG TGA AT			
exoU-F	exoU	GGG AAT ACT TTC CGG GAA GTT	428	60	[15]
exoU-R		CGA TCT CGC TGC TAA TGT GTT			

Table 2. Distribution of virulence genes according to the nature of the biological sample

Biological samples	*lasB* N (%)	*exoS* N (%)	*plch* N (%)	*nan1* N (%)	*exoU* N (%)	*pilB* N (%)	*algD* N (%)
Pulmonary fluid (n=27)	22 (81,5)	6 (22,2)	22(81,5)	10(37)	5(18,5)	19(70,3)	22(81,5)
Pus cutaneous and mucous (n=78)	52(66,7)	24(30,7)	61(78,2)	28(39,9)	14(17,9)	53(67,9)	63(80,7)
blood (n=13)	8(61,5)	6 (46,1)	11(84,6)	6(46,1)	2(15,4)	8(61,5)	13(100)
cerebrospinal fluid (n=6)	4(66,7)	1(16,7)	4(66,7)	2(33,3)	1(16,7)	3(50)	5(83,3)
Urines (n=14)	8(57,1)	6(42,8)	11(78,6)	6(42,9)	2(14,3)	8(57,1)	13(92,8)
Invasive Matériels (n=13)	9(69,2)	3(23)	10(76,9)	3(23)	1(7,6)	7(53,8)	13(100)

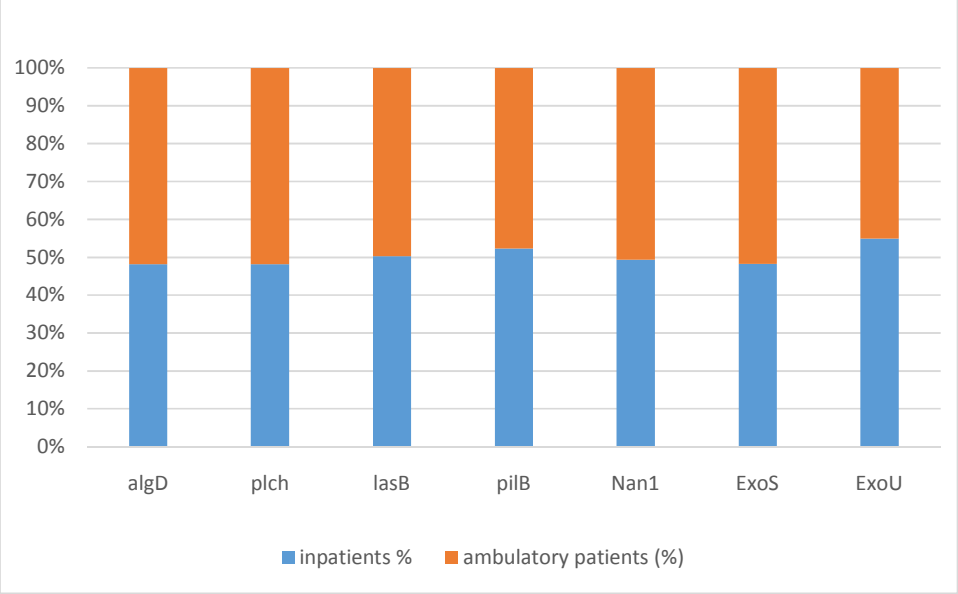

Fig. 1. Distribution of virulence genes by patient type

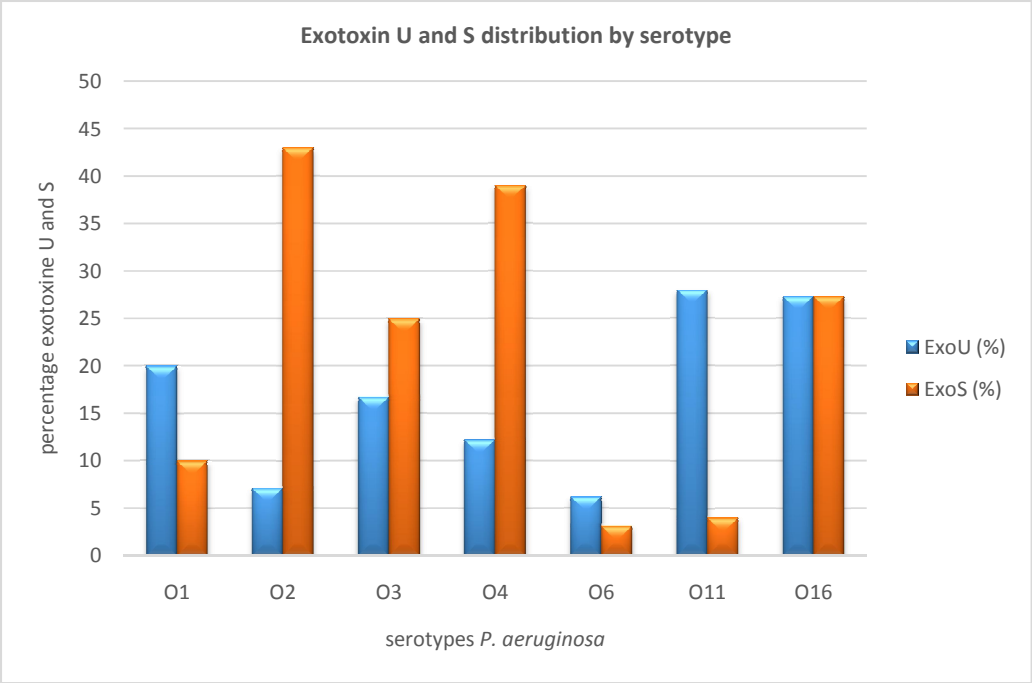

Fig. 2. Exotoxin U and exotoxin S recovery according to serotype

Regarding virulence in relation to serotype, the serotypes with the high levels of exoS genes were respectively O2 (43%), O4 (39%), O16 (27.3%), O3 (25%), %). The prevalence of exoS was similar to the results of Faure K in 2003 in the USA for serotypes O2 (50%) and O4 (25%).

The serotypes among which the exoU gene was the most recovered were respectively: O11 (28%) O16 (27.3%), O1 (20%), O3 (16.7%). The serotype with the highest exoU rate was O11 (28%), which was lower than that of Faure K in 2003 (77.8%) [24].

5. CONCLUSION

The most found virulence genes were algD, plch, lasB, pilB, nan1, exoS and exoU. Most isolates had the algD gene at the different infectious sites. In septicemia 46.1% of *P. aeruginosa* possessed the nan1 neuraminidase gene involved in biofilm formation, as well as the exotoxin S gene. *P. aeruginosa* isolated from pulmonary infections and septicemia had a high arsenal virulence factor. Simultaneous determination of virulence factors and antimicrobial resistance is the contemporary approach for the examination of microbiological aspects of *P. aeruginosa* infections.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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