Scientific Research Publishing

Advances in Microbiology, 2024, 14, 589-604
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Virulence Factors and Biofilm Formation in Multidrug-Resistant Metallo-β-Lactamase-Producing Clinical Strains of *Pseudomonas aeruginosa*

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How to cite this paper: Traore, A., Benie, C.K.D., Diarrassouba, A., Tuo, W.-T.A., Wayoro, O.Z., Dadié, A., Guessennd, N. and Dosso, M. (2024) Virulence Factors and Biofilm Formation in Multidrug-Resistant Metallo-β-Lactamase-Producing Clinical Strains of *Pseudomonas aeruginosa*. *Advances in Microbiology*, **14**, 589-604.
https://doi.org/10.4236/aim.2024.1412041

Received: November 4, 2024
Accepted: December 15, 2024
Published: December 18, 2024

Abstract

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium, responsible for nosocomial infections, with a complex arsenal of pathogenicity. The aim of this study was to simultaneously characterize the potential for resistance, virulence and biofilm formation in clinical strains. A total of 104 clinical *P. aeruginosa* strains (blood (26), stools (26), pus (26) and urine (26) were the subject of this study. The Mueller-Hinton diffusion method, agglutination test and combined disk diffusion test respectively made it possible to phenotypically determine the resistance profile, serogroups and metallo-β-lactamase production. Virulence, resistance and biofilm formation supports were detected by PCR. *P. aeruginosa* strains were resistant to aztreonam (76.4%), ticarcillin (62.4%), piperacillin (32.4%), imipenem (17.1%), cefepime (14%) and Ceftazidime (8.3%). The serogroups O11 (22.1%), O7 (18.3%), O16 (16.3%), and O9 (14.4%) were mainly determined in clinical strains. The total prevalence of metallo-β lactamase genes was 12.5% (*blaIMP*) and 11.5% (*blaVIM*). In descending order, the virulence genes *exoS* (55.8%), *plcH* (48.1%), *LasB* (47.1%), *pilB* (42.3%) and *algD* (41.3%) were detected ($p < 0.05$). The median biofilm formed in 48 hours varies from 0.4 to 3.3. Clinical strains harbored the biofilm genes *pelA* (28.8%) and *pslA* (23.1%). In conclusion, this study highlights the significant resistance, virulence, and biofilm-forming capabilities of

clinical *Pseudomonas aeruginosa* strains. By profiling 104 strains, we found high resistance rates to multiple antibiotics, with notable serogroups and a considerable prevalence of metallo-β-lactamase genes, which pose a challenge for treatment. Additionally, key virulence genes and biofilm-associated genes were prevalent, underscoring the pathogenic potential of these strains. These findings underscore the importance of characterizing pathogenicity factors as a valuable strategy for monitoring and managing *P. aeruginosa* infections, especially in healthcare settings where such infections are common and difficult to treat.

Keywords

P. aeruginosa, Serogroups, Metallo-β-Lactamase, Virulence, Biofilms

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium considered to be an opportunistic pathogen [1]. *Pseudomonas aeruginosa* is a versatile, opportunistic Gram-negative bacterium known for causing severe infections, particularly in immunocompromised individuals and in hospital settings. It possesses a broad arsenal of virulence factors, including toxins and enzymes that damage host tissues and evade immune responses. Additionally, **P. aeruginosa** is notable for its intrinsic resistance to many antibiotics and its ability to acquire further resistance, making infections challenging to treat [2]. This pathogen's ability to form biofilms on surfaces, such as medical devices, further contributes to its persistence and resistance in clinical environments [3]. In fact, this bacterium preferentially infects around 50% to 60% of immunodeficient or weakened hospitalised patients. *Pseudomonas aeruginosa* is a major pathogen commonly associated with biofilm formation on medical devices, making it one of the most widespread causes of device-related infections. In Europe, nosocomial infections lead to approximately 175,000 deaths annually among 1.75 million hospitalized patients, with *P. aeruginosa* accounting for 10% - 20% of these cases. This bacterium is notoriously challenging to eliminate, particularly in cystic fibrosis patients, where it forms dense, antibiotic-resistant biofilms in the lungs that shield it from both antibiotics and the immune system, worsening long-term outcomes for affected patients [4]. Like *Escherichia coli* and *Staphylococcus aureus*, *Pseudomonas aeruginosa* is responsible for many nosocomial infections [5]. *P. aeruginosa* is implicated in 16% of nosocomial pneumonia, 12% of hospital-acquired urinary tract infections, 10% of bloodstream infections and 8% of surgical-associated infections [6]. The prevalence of *P. aeruginosa* infections is 11.5% in Europe and 17% in developing countries [7]. Despite various therapeutic advances, mortality from *P. aeruginosa* infections remains high at around 30% [6] [7].

This opportunistic pyocyanic bacterium is potentially resistant to most commonly used antibiotics. In Africa, despite the different levels of action of beta-

lactam antibiotics, many cases of multi-resistant bacteria have been reported [8]. These multi-resistant strains can harbour and produce certain resistance genes such as metallo-β-lactamase (MβL) [9]. These metallo-enzymes are capable of inactivating β-lactam antibiotics such as carbapenems, penicillins and cephalosporins [8].

In addition to this multi-resistance, the bacterium's pathogenicity relies on a complex arsenal of extracellular factors and cellular attributes [10] [11]. These extracellular factors are mainly exotoxins (exotoxin A), exo-proteases (elastase, staphylolysin, alkaline protease, protease IV), phospholipase C, chromophores and exoenzymes S, T, U.

On the other hand, membrane factors include lipopolysaccharides (LPS), flagella, adhesion factor (type IV pili) and alginate. Virulence factors associated with the membrane are generally involved in the colonisation phase and chronic infection, whereas extracellular factors, which are extremely toxic, are associated with acute infection [1] [10].

Like LPS, alginates are involved in bacterial adhesion to the respiratory epithelium [11]. Exoenzyme S, encoded by the exoS gene, is an ADP-ribosyltransferase that is secreted by the type III secretion system directly into the cytosol of epithelial cells [11]. Injection of exoU exotoxin leads to rapid death (1 to 2 hours) of host cells and the expression of inflammation [1]. ExoU is 100 times more cytotoxic than ExoS according to Benie *et al*. (2017) and Fadhil *et al*. (2016). LasB elastase, a zinc metalloprotease encoded by the LasB gene, has lytic activity in lung tissue.

In addition to these virulence factors, the phospholipids contained in pulmonary surfactant can be hydrolysed by two phospholipases respectively, including plcH (haemolytic phospholipase C) and plcN (non-haemolytic phospholipase C) [12]. This variety of virulence factors not only contributes to the pathogenicity of *Pseudomonas aeruginosa*, but also favours its involvement in the formation of biofilms [10] [13].

Biofilms are structured clusters of bacterial cells embedded in a polymeric matrix and attached to a surface. Various studies indicate that the lifestyle, structure and composition of biofilms lead to increased resistance to antimicrobial agents [1].

In medicine, biofilms are of particular importance because they are involved in a wide range of infections in humans [14]. Around 65% of infections are due to biofilms in so-called developed countries. Moreover, almost 80% of chronic bacterial infections are associated with the presence of biofilms [14]. Infections resulting from biofilms pose real public health problems [15]. Consequently, *P. aeruginosa* infections represent a microbiological, pharmacological and medical challenge.

In Côte d'Ivoire, research has been carried out into the virulence and biofilm-forming potential of multi-resistant Pseudomonas earuginosa strains of animal origin [16] [17]. In Côte d'Ivoire, while a few works have highlighted the characterisation of Pseudomonas earuginosa, data are non-existent relative to the virulence and biofilm-forming potential of clinical multi-resistant metallo-β-

lactamase-producing strains.

The aim of this work is to evaluate the virulence and biofilm-forming potential of multi-resistant metallo-β-lactamase-producing strains of *Pseudomonas aeruginosa* of clinical origin.

2. Materials and Methods

2.1. *Pseudomonas aeruginosa* Isolates

A total of one hundred and four (104) strains of *P. aeruginosa* were obtained from clinical samples (blood (26), stool (26), pus (26) and urine (26). Pseudomonas isolates obtained on Cetrimide were identified using standard bacteriology techniques. Polar mobile strains, producing an oxidase and two types of pigment: pyocyanin and pyoverdine on King A and King B or pyocyanin only on King A, and able to grow at 42˚C, were retained as *P. aeruginosa* and confirmed by PCR.

2.2. Determination of the Resistance Profile of *P. aeruginosa* Strains

The phenotypic resistance profile of *P. aeruginosa* strains was determined using the diffusion method on the Müeller-Hinton (MH) medium. Antibiotics commonly used in human therapy such as penicillins (Ticarcillin, Ticarcillin-clavulanic acid, Piperacillin), cephalosporins (Cefepime, Ceftazidime), carbapenems (Imipenem), quinolones (Ciprofloxacin), monobactams (Aztreonam), fosfomycins (Fosfomycin), polymyxins (Colistin) and aminoglycosides (Kanamycin) were tested.

The reference strain *P. aeruginosa* ATCC 27853 was used for antibiogram quality control. The entire surface of the Müeller-Hinton agar was swabbed sterilely from the bacterial suspension in saline solution with turbidity equivalent to 0.5 McFarland. This inoculum of approximately 108 CFU/mL was produced from 24-hour-old colonies taken on ordinary agar. Fifteen minutes after the antibiotic-impregnated discs were placed on the surface of the inoculated agar, the plates were incubated at 37˚C for 24 hours. After 24 hours, the zones of inhibition were determined using a caliper. The results were interpreted in accordance with the rules of the Antibiogram Committee of the French Microbiology Society [18].

2.3. Serotyping of *Pseudomonas aeruginosa* Strains

Serotyping of *Pseudomonas aeruginosa* strains was carried out by agglutination using polyvalent sera and their corresponding monovalent (Sanofi Diagnostics Pasteur) following the manufacturer's recommendations according to the method of Habs (1957).

2.4. Phenotypic Identification of Strains Producing Metallo-β-Lactamases

Clinical strains of *P. aeruginosa* intermediate or resistant to one or more carbapenems were subjected to Combined Disc Diffusion Tests (CDDT) for the

phenotypic detection of MBL production. A bacterial suspension with a density of 0.5 McFarland obtained from a 24-hour culture was spread with a swab onto a Mueller-Hinton agar plate (Biolife, Milan, Italy). Two discs of imipenem (10 µg) were placed 20 mm from centre to centre on the Mueller Hinton agar. Next, 5 µl of EDTA (0.5 M, pH 8) was added to one of the IMP imipenem discs (10 µg) and the other disc was left without EDTA. As EDTA has some bactericidal activity, another sterile disc not impregnated with antibiotics was also inoculated with 5 µL of EDTA (0.5 M, pH 8). The plates were incubated for 24 hours at 37˚C.

After incubation, the difference ≥ 7 mm between the diameter of the zone of inhibition of the IMP-EDTA disc and that of the IMP disc (10 µg) alone was considered positive and indicated the presence of MBLs. A metallo-β-lactamase-producing strain of *P. aeruginosa* was used as a positive control and *P. aeruginosa* ATCC 27853 as a negative control.

2.5. Molecular Characterisation of *Pseudomonas aeruginosa*

2.5.1. Extraction of *Pseudomonas aeruginosa* Genomic DNA

Genomic DNA was extracted by thermal lysis using the method described by Amutha and Kokila (2014). A few 18h young colonies of each clinical germ were introduced into an Eppendorf tube containing 300 µL of sterile distilled water until cloudy solutions were obtained. The cell suspensions were then frozen at −20˚C for 15 minutes. The frozen Eppendorf tubes were placed in a heating block (96˚C) for 15 minutes (FastPrep (4 m/s)). The supernatant constituting the DNA extract was collected after centrifugation at 12,000 g for 10 minutes. The extracted DNA is stored at −20˚C and used as a DNA template for polymerase chain reactions (PCR).

2.5.2. Reaction Mix and Gene Amplification

1) Reaction mixture

The 25 µL reaction mixture consisted of 13 µL of sterile Milli-Q water (milli-Q™, Millipore Corporation, USA), 5 µL of 5X concentration buffer, 1. 5 µL of Mgcl2, 2 mM (Promega Corporation, Madison, WI 53711-5399, USA), 1µL of d'NTPs, 10 mM, 1µL of each 27F and 1492R primer, 10 mM (TranS, AP111 5U, CHINA), 0.5 µL of Easy Tag® DNA polymerase with a final concentration of 1.5 U (TranS, AP111 5U, CHINA) and 2 µL of DNA template. Sterile Milli-Q water and the *P. aeruginosa* reference strain ATCC 27853 (NCTC 12903; CIP 76110) were used as negative and positive controls respectively for each PCR reaction.

2) Amplification of the 16S rDNA gene

Amplification of the 16S rDNA gene was performed according to the method described by Amutha and Kokila (2014) using primers 27F (5'-AGAGTTT-GATCMTGGCTCAG-3') and 1492R (3'TACGGYTACCTTGTTACGACTT-5'). The amplification programme consisted of an initial denaturation for 5 min at 94˚C followed by 35 cycles of denaturation (94˚C for 30 s), hybridisation (55˚C for 40 s) and extension (72˚C for 30 s), with a single final extension for 10 min at 72˚C.

3) Amplification of virulence, biofilm and metallo-β-lactamase genes

Amplification of virulence (exoS, lasB, algD, plcH and pilB), biofilm (pslA,

pelA) and metallo-β-lactamase (blaIMP, blaVIM) genes was performed according to the method described by Pournajaf *et al.* (2018). The amplification program included an initial denaturation for 5 min at 95˚C followed by a repeated cyclic phase. The cyclic phase repeated 33 times with one amplification cycle comprised of a denaturation step of 30 seconds at 95˚C, a primer attachment (hybridisation) step of 60 seconds at 65˚C and an elongation step of 90 seconds at 72˚C. The amplification reaction was completed by a final elongation of 5 min at 72˚C. Samples were stored at +4˚C until the T3000 Thermocycler, Block type standard 3a, (Biometra, Germany) was stopped. Gene amplification products were revealed on a 1.5% agarose gel and visualised by illumination on a UV plate equipped with an illumination device and photographs (Molecular Imager Gel DocTM XR+, Bio-Rad). The amplification programmes and nucleotide sequences of the primers used are described in Table 1.

Table 1. Variety of genes detected.

Genes	Sequences (5'-3')	Amplification Program	Size (bp)	References
Virulence genes				
exoS	**F: CTTGAAGGGACTCGACAAGG** **R: TTCAGGTCCGCGTAGTGAAT**	95˚C, 5 min 33 × [95˚C, 30 s; 65˚C, 60s; 72˚C, 90 s] 72˚C, 5 min; 4˚C…	504	**Pournajaf *et al.*, 2018**
lasB	F: GGAATGAACGAGGCGTTCTC R: GGTCCAGTAGTAGCGGTTGG		300	
algD	**F: AGAAGTCCGAACGCCACACC** **R: CGCATCAACGAACCGAGCATC**		550	
pilB	F: ATG AAC GAC AGC ATC CAA CT R: GGG TGT TGA CGC GAA AGT CGA T		826	
plcH	**F: GAAGCCATGGGCTACTTCAA** **F: AGAGTGACGAGGAGCGGTAG**		307	
MBLs genes				
IMP	F: TGAGCAAGTTATCTGTATTC R: TTAGTTGCTTGGTTTTGATG	95˚C, 5 min 33 × [95˚C, 30 s; 65˚C, 60 s; 72˚C, 90 s] 72˚C, 5 min; 4˚C…	740	**Pournajaf *et al.*, 2018**
VIM	F: AAAGTTATGCCGCACTCACC F: TGCAACTTCATGTTATGCCG		815	
Biofilms genes				
PelA	F:5'-CATACCTTCAGCCATCCGTTCTTC-3' F:5'-CGCATTCGCCGCACTCAG-3'	95˚C, 5 min 33 × [95˚C, 30 s; 65˚C, 60 s; 72˚C, 90 s] 72˚C, 5 min; 4˚C…	786	**Pournajaf *et al.*, 2018**
PslA	F : 5'-TCCCTACCTCAGCAGCAAGC-3' R : 5'-TGTTGTAGCCGTAGCGTTTCTG-3'		656	

2.6. Biofilm Formation Using the Microplate Method (MP)

Biofilm formation by the MP method was based on the technique proposed by (Radzig *et al.*, 2013) with a few modifications. Different suspensions of final volume 1.2 mL consisting of LB medium diluted 10-fold and Casamino acid of final concentration 0.5% were made in different Eppendorf tubes for each *P. aeruginosa* strain of clinical origin. An aliquot of each 24-hour pre-culture of *P. aeruginosa* in TSB with a turbidity equivalent to 0.5 McFarland (108 UFC/ml) is homogenized in each suspension. A five-well replicate was made for each *P. aeruginosa* strain.

Five different 200 µL volumes of the suspension initially prepared without bacterial aliquots were introduced into each of the first five wells of the first line to prepare a crystal violet staining blank. Different volumes of 200 µL of each of the bacterial suspensions produced are distributed successively into the remaining 91 wells. The plates are then covered, tightly packed and incubated at 37˚C under static conditions for 48 hours. After 48 h, the culture medium and non-adhered planktonic bacteria are removed by three successive washes with distilled water. Different volumes of 200 µl of 1% (v/v) crystal violet are dispensed into each well using an 8-channel pipette.

Microplates are incubated for 30 min at room temperature. Excess crystal violet is removed by 3 to 10 successive manual washes of the plates with distilled water. The dye is then solubilized by adding 300 µL of 95% absolute ethanol solution to each well. The dye concentration or absorbance (OD) is measured spectrophotometrically at 595 nm wavelength with the Cytation™ 5 Cell Imaging Multi-Mode Reader microplate reader (BioTek Instruments, Innovation, CA), linked to Gen5v.2.04™ data analysis software (BioTek Instruments, Innovation, CA).

At the end, strains were grouped into: OD 595 < 0.1, non-biofilm producers (NP); OD 595 = 0.1 - 1.0, weak biofilm producers (WP); OD 595 = 1.1 - 3.0, moderate biofilm producers (MP); and OD 595 > 3.0, strong biofilm producers (SP).

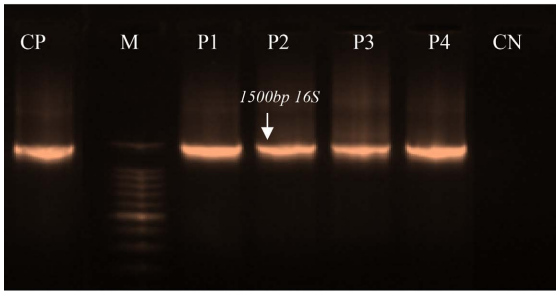
2.7. Statistical Analysis

The distribution of genes in relation to their origin of isolation was compared using the chi-square test. Descriptive statistical methods (frequency, mean, standard deviation) were used for quantitative variables. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical Strains of *P. aeruginosa* Identified

Phenotypic, biochemical and molecular identification using the 16S marker (Figure 1) confirmed that of a total of 110 *Pseudomonas* isolates, 104 (94.5%) belonged to the *P. aeruginosa* species (Table 2).



CP: Positive control: P. aeruginosa ATCC 27853; CN: Negative control; M: Marker Gene Ruler, P1-P4 presence of P. aeruginosa in clinical simples. (Bench Top, 1500 bp DNA Ladder, Promega Corporation, USA).

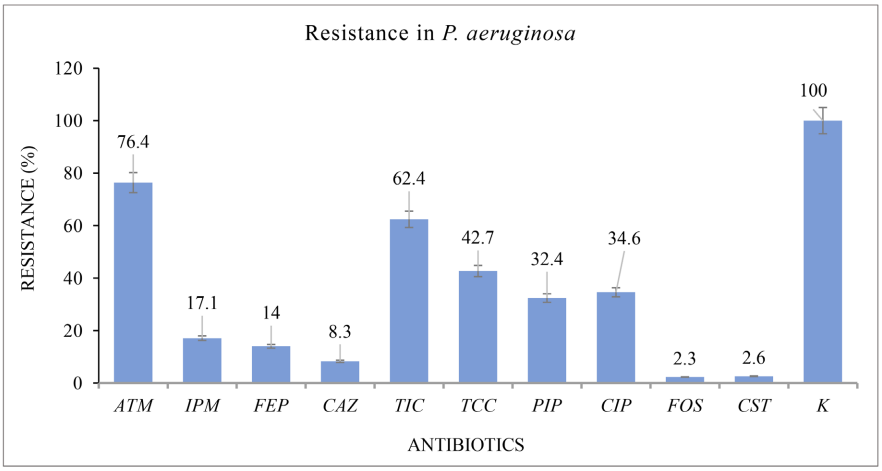
Figure 1. Electrophoretic profile of the 16S rRNA gene in *P. aeruginosa.*

Table 2. Confirmed *Pseudomonas aeruginosa* strains.

Types of Identification	Germs	n	%
Phenotypic	Presumptive isolates	110	100%
Biochemical and molecular	Strain of *P. aeruginosa*	104	94.5%

3.2. Resistance in Clinical Strains of *Pseudomonas aeruginosa*

P. aeruginosa strains of clinical origin showed resistance to penicillins ranging from 32.4% (piperacillin) to 62.4% (ticarcillin). Resistance to cephalosporins ranged from 8.3% (ceftazidime) to 14% (cefepime) (Figure 2). *P. aeruginosa* strains were 17.1% (imipenem) resistant to carbapenems. The majority of *P. aeruginosa* strains were resistant to monobactams, with 76.4% resistant to aztreonam (Figure 2). Total resistance to kanamycin was observed in these clinical strains.



Ticarcillin (TIC), Ticarcillin-clavulanic acid (TCC), Aztreonam (ATM), Cefepime (FEP), Ceftazidine (CAZ), Ciprofloxacin (CIP); Colistin (CST); Imipenem (IPM); Piperacillin (PIP); Fosfomycin (FOS) and Kanamycin (K).

Figure 2. Antimicrobial resistance in clinical *P. aeruginosa* strains.

3.3. Prevalence of Serogroups in *Pseudomonas aeruginosa*

In order of increasing importance, serogroups O11 (22.1%), O7 (18.3%), O16 (16.3%), O9 (14.4%) were detected predominantly in clinical strains. Serogroups O5, O1 and O12 had prevalences of less than 10% (Table 3).

Table 3. Prevalence of serogroups in *Pseudomonas aeruginosa.*

Clinical Strains of *P. aeruginosa* (n = 104)		
Serogroups	**Number**	**Prevalence**
O_{11}	**23**	**(22.1%)**
O_7	**19**	**(18.3%)**
O_{16}	**17**	**(16.3%)**
O_9	**15**	**(14.4%)**

Continued

NS	11	(10.6%)
O_5	10	(9.6%)
O_1	6	(5.8%)
O_{12}	4	(3.8%)

NS: Not serotypeable. O: serogroups.

3.4. Biofilm Formation and Producer Category

The study also showed the phenotypic potential of clinical strains of *P. aeruginosa* to form biofilms. The median biofilm formed in 48 hours ranged from 0.4 to 3.3 (Table 4). Categories of biofilm producers showed that some strains were non-producers and others biofilm producers. Biofilm producer categories were classified as high producer (34%), moderate producer (68%) and low producer (12%) (Table 4).

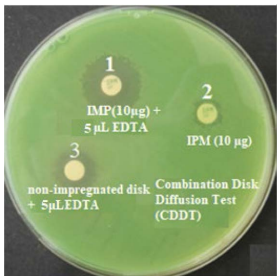
Table 4. Biofilm Median biofilm and producers category.

Clinical Strains	Biofilm Formed after 48 h			Biofilm Producer Category after 48 h			
	Minimum OD	OD Median	Maximum OD	NP	WP	MP	SP
P. aeruginosa (n = 104)	0.4	1.4 ± 0.4	3.3	6%	12%	68%	34%

NP: Non producers; WP: Weak Producers; MP: Moderate Producers; SP: Strong Producers.

3.5. Phenotypic and Molecular Determinants of Metallo-β Lactamase (MβLs)

Phenotypic detection of MBLs by the Combined Disk Diffusion Test (CDDD) showed that 56 (53.8%) of the clinical strains of *P. aeruginosa* intermediate or resistant to one or more carbapenems were MBLs producers Figure 3. The total prevalence of metallo-β lactamase molecular determinants in clinical strains was 12.5% (blaIMP) and 11.5% (blaVIM) respectively. Blood isolates were predominantly blaVIM (30.8%) and blaIMP (26.9%) Table 5. No metallo-β lactamase genes were detected in strains from urinary samples Table 5.



The test was considered positive for MBLs production when the difference between the diameter of the inhibition zone of the IPM-EDTA disc and that of the IPM (10 µg) disc alone was ≥7 mm. 1: IMP (10 µg) discs + 5 µl EDTA (0.5 M, pH 8); 2: IMP (10 µg) discs without EDTA (0.5 M, pH 8); 3: unimpregnated disc + 5 µl EDTA (0.5 M, pH 8).

Figure 3. Metallo-β lactamase (MBLs) producing by clinical *P. aeruginosa.*

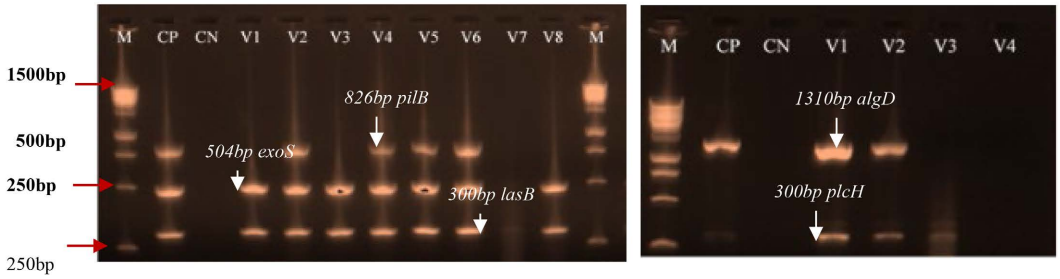
Table 5. Prevalence of molecular determinants of MβLs in *P. aeruginosa.*

Molecular Supports	Number of *P. aeruginosa* Strains per Biological Product (n)				Total (n = 104)
	Blood (n = 26)	Urine (n = 26)	Stool (n = 26)	Pus (n = 26)	
Metallo-β-Lactamase	**Prevalences of Metallo-β-Lactamase Genes (%)**				
blaIMP	07 (**26.9%**)	00 (%)	04 (**15.4%**)	2 (**7.7%**)	13 (**12.5%**)
blaVIM	08 (**30.8%**)	00 (%)	02 (**7.7%**)	2 (**7.7%**)	12 (**11.5%**)

MβL Imipenemase (IMP), Verona Imipenemase (VIM).

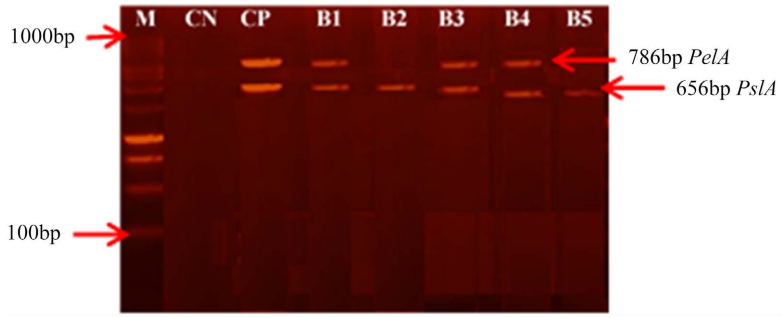
3.6. Virulence and Biofilm Genes in Clinical Strains of *P. aeruginosa*

Some *P. aeruginosa* strains harbored five (5) virulence genes (lasB, exoS, algD, plcH and pilB) studied (line 2), while others harbored fewer than five (5) genes Figure 4. Electrophoretic profiling of biofilm-forming genes showed that some clinical *P. aeruginosa* strains harbored two biofilm-forming genes (V1 and V2) (PelA and PslA), while others harbored a single gene (V3) (PslA) (Figure 5).



pilB type IV fimbriae biogenesis protein gene, gene encoding pili; lasB gene, gene encoding lasB elastase; exoS gene, gene encoding exoenzyme S; algD gene, 6-dehydrogenase algD (alginate) gene, encoding GDP-mannose and plcH gene, encoding hemolytic phospholipase C.

Figure 4. Electrophoretic profile of virulence gene amplification products.



Line B1, B3, B4: presence of two biofilm genes (PelA and PslA), Line B2 and B5: presence of a single gene (PslA). CP: positive control, CN: negative control; M: molecular weight marker.

Figure 5. Electrophoretic profile of genes involved in biofilm formation in *P. aeruginosa.*

3.7. Prevalence of Virulence and Biofilm Molecular Supports

In descending order, the virulence genes exoS (55.8%), plcH (48.1%), LasB (47.1%), pilB (42.3%) and algD (41.3%) were detected with varying prevalence in

clinical strains of *P. aeruginosa* (Table 6). The pelA (28.8%) and pslA (23.1%) genes involved in biofilm formation were detected in clinical strains of *P. aeruginosa*. No biofilm-forming genes were detected in urinary strains.

Table 6. Prevalence of virulence genes and biofilms.

Molecular Supports	Number of *P. aeruginosa* Strains per Biological Product (n)				Total (n = 104)
	Blood (n = 26)	Urine (n = 26)	Stool (n = 26)	Pus (n = 26)	
Virulence	**Prevalence of Virulence Factors (%)**				
exoS	18 (**69.2%**)	16 (**61.5%**)	14 (**53.8%**)	10 (**38.5%**)	58 (**55.8%**)
plcH	15 (**57.7%**)	12 (**46.2%**)	17 (**65.4%**)	06 (**23.1%**)	50 (**48.1%**)
lasB	15 (**57.7%**)	12 (**46.2%**)	15 (**57.7%**)	07 (**26.9%**)	49 (**47.1%**)
pilB	12 (**46.2%**)	10 (**38.5%**)	14 (**53.8%**)	08 (**30.8%**)	44 (**42.3%**)
algD	12 (**46.2%**)	10 (**38.5%**)	12 (**46.2%**)	09 (**34.6%**)	43 (**41.3%**)
Biofilms	**Biofilm Gene Prevalences (%)**				
pelA	11(**42.3%**)	00 (%)	11(**42.3%**)	08 (**30.8%**)	30 (**28.8%**)
pslA	07 (**26.9%**)	00 (%)	10(**38.5%**)	07 (**27.9%**)	24 (**23.1%**)

pilB type IV fimbriae biogenesis protein gene, gene coding for pili; lasB gene, gene coding for lasB elastase; exoS gene, gene coding for exoenzyme S; algD gene, 6-dehydrogenase algD (alginate) gene coding for GDP-mannose and plcH gene, coding for hemolytic phospholipase C; Pel: pellicle (Pel gene coding for pellicles); Psl: polysaccharide synthesis locus.

4. Discussion

Pseudomonas aeruginosa is a nosocomial pathogen responsible for morbidity and mortality in immunocompromised patients [8] [19]. Biological diagnosis of infections caused by this pyocyanic bacterium is most often performed by classical methods such as growth on specific culture media and biochemical identification [20] [21]. In this study, molecular identification using the 16S marker confirmed that of all 110 clinical *Pseudomonas* isolates, 104 (94.5%) belonged to the *P. aeruginosa* species.

These *P. aeruginosa* strains were predominantly resistant to monobactams, with 76.4% resistant to aztreonam. Resistance to penicillins also ranged from 32.4% to 62.4%. Strain resistance to penicillins and monobactams (aztreonam) could be due to acquired resistance (plasmids, transposons). According to Kumar and Schweizer (2005), this acquired resistance could be due to increased impermeability of the outer membrane, or to the production of inactivating enzymes capable of expelling these types of antibiotics.

Resistance to cephalosporins ranged from 8.3% to 14%. Resistance to imipenem was 17.1%. These respective levels of resistance to cephalosporins and carbapenems could be explained by chromosomal mechanisms, in combination with extended-spectrum beta-lactamase (ESBL) resistance mechanisms [1] [8]. This observed level of phenotypic resistance to carbapenems could justify the presence of blaIMP (12.5%) and blaVIM (11.5%) metallo-β lactamase molecular supports detected in this study. Indeed, these metallo-enzymes are capable of inactivating

β-lactam antibiotics such as carbapenems, penicillins and cephalosporins [9] [22]. Various studies have shown that these enzymes could promote the involvement of *P. aeruginosa* in nosocomial infections, sepsis and pneumonia [8].

The total kanamycin resistance of all these clinical strains studied expresses their natural resistance to this antibiotic. The majority of these multi-resistant *P. aeruginosa* clinical strains belonged to serogroups O11 (22.1%), O7 (18.3%), O16 (16.3%) and O9 (14.4%).

This diversity of serogroups could be explained by the fact that these types of serogroups are most often involved in infections of bacterial origin [23]. The same observation was made by Lu *et al*. (2014), who reported that serotypes O11, O7 and O16 were associated with the production of elastase and certain enzymes secreted by the type III secretion system (TTS) itself involved in lung lesions. In their study, they found that O11 serotype strains were not only the most virulent but also associated with severe nosocomial infections [23] [24]. All these serotypes and multiresistance phenotypes observed in these clinical strains could be associated with particular pathogenicity and virulence determinants.

This study also showed that these clinical strains of *P. aeruginosa* possessed different virulence and biofilm-forming factors that could contribute to their pathogenicity. In descending order, virulence genes such as exoS (55.8%), plcH (48.1%), LasB (47.1%), pilB (42.3%) and algD (41.3%) were detected in these *P. aeruginosa* clinical strains.

The prevalences of the predominantly detected exoS and plcH genes, shows that these isolated strains were capable of secreting hemolytic exoenzymeS and phospholipase C. Consequently, these strains could be involved in pulmonary infections [25]. The prevalence of elastase encoding the lasB (zinc metalloprotease) gene and secreted by the type II secretion system indicates that this protease plays an important role in *P. aeruginosa* pathogenesis by cleaving elastin and collagen [25] [26].

In fact, this enzyme is responsible for the destruction of junctions between epithelial cells and a reduction in the innate immune response [25] [26]. Elastase B is also capable of inactivating other proteins such as IgA, IgG and complement compounds, thus modulating the immune response [25] [27].

The presence of the algD gene could justify the conversion of clinical strains of *P. aeruginosa* to a mucoid phenotype overproducing alginate [28]. This alginate presence indicates that the strains could be involved in biofilm formation. Indeed, alginates have been widely considered the major exopolysaccharides of the biofilm matrix [28].

Furthermore, the presence of the pilB gene encoding pili shows that these clinical strains could not only be involved in biofilm formation but in the initiation of infections. The study showed that the pelA (28.8%) and pslA (23.1%) genes involved in biofilm formation were detected in these clinical strains of *P. aeruginosa* with different prevalences. The presence of these PslA and PelA genes indicates that these strains are involved in polysaccharide and film biosynthesis respectively.

Indeed, the production of polysaccharide and pellicles observed in these strains could favor the mechanisms involved in the virulence and pathogenicity of these clinical strains [29]. Simultaneous determination of serogroups, virulence factors, biofilm formation and metallo-βlactamase is an approach of interest for effective surveillance of *P. aeruginosa*-associated infections.

5. Conclusion

The study demonstrated the sensitivity and effectiveness of the molecular tool in characterizing clinical strains of *P. aeruginosa*. It highlighted the diversity of serogroups, virulence determinants, biofilm formation and metallo-β-lactamase in clinical strains of *P. aeruginosa*. This study demonstrates the importance of controlling the factors involved in monitoring *P. aeruginosa*-associated infections.

Author's Contribution

AT, AD, CKDB, DM and NG conceived and designed the study. OWZ performed the experiments. AD, CKDB, and TWTA analysed the data. AD, CKDB, TWTA, and AD contributed reagents/materials/analysis tools. AD, CKDB, and TWTA wrote the paper. All the authors read, reviewed, and approved the final manuscript.

Funding Sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the INRS–Institut Armand Frappier of Canada, the Pasteur Institute of Paris, and the Institut Pasteur of Cote d'Ivoire and the Department of Medical Sciences, University of Alassane Ouattara for their advice and excellent technical assistance.

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