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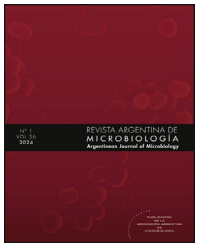

ORIGINAL ARTICLE

High virulence and genetic heterogeneity in *Pseudomonas aeruginosa* isolates from lower respiratory tract infections in neutropenic patients

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Respiratory infections;
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Virulence genes;
β-Lactamase genes;
Molecular epidemiology

Abstract The aim of this study was to determine the antimicrobial susceptibility, virulence genotypes, presence of extended spectrum β-lactamase (ESBL) and carbapenemase genes, and clonality of *Pseudomonas aeruginosa* isolates responsible for lower respiratory tract infections in patients admitted to the National Bone Marrow Transplant Center (NBMTC) of Tunisia. During the 11-year study period, a total of 50 *P. aeruginosa* isolates were responsible for lower respiratory tract infections. Isolates were studied by determining their serotypes, antimicrobial susceptibility, genes encoding ESBL/carbapenemases and virulence factors, and genetic relatedness by ERIC-PCR. High to moderate resistance rates were observed for ticarcillin, ticarcillin–clavulanic acid, piperacillin–tazobactam, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, amikacin, and fosfomycin. One isolate showed an ESBL phenotype encoded by the $bla_{SHV\text{-}2a}$ gene. Of 13 isolates resistant to both imipenem and meropenem, the bla_{GES} gene was amplified in nine isolates. Isolates exhibited 19 virulence genotypes, of which five were prevalent: V1 (14 genes; n = 14), V2 (14 genes, n = 6), V3 (all genes, n = 6), V4 (13 genes, n = 5), and V5 (13 genes, n = 5). ERIC-PCR showed high genetic heterogeneity. These findings highlight the high genetic heterogeneity of *P. aeruginosa* isolates in these neutropenic patients, as well as their remarkable pathogenic power, raising significant concern.

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PALABRAS CLAVE
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Infecciones respiratorias;
Pacientes neutropénicos;
Genes de virulencia;
Genes de β-lactamasa;
Epidemiología molecular

Alta virulencia y heterogeneidad genética en aislamientos de *Pseudomonas aeruginosa* de infecciones del tracto respiratorio inferior en pacientes neutropénicos

Resumen El objetivo de este estudio fue evaluar un conjunto de aislados de *Pseudomonas aeruginosa* responsables de infecciones de las vías respiratorias bajas en pacientes ingresados en el Centro Nacional de Trasplante de Médula Ósea (CNTMO) de Túnez, focalizando en la sensibilidad a los antimicrobianos, los genotipos de virulencia, la presencia de genes de β-lactamasa de espectro extendido (BLEE) y carbapenemasa y la clonalidad. Durante un período de estudio de 11 años, se recuperaron 50 aislados de *P. aeruginosa* a partir de infecciones respiratorias bajas. Se determinaron los serotipos y el parentesco genético se estableció mediante la técnica ERIC-PCR. Se observaron tasas de resistencia de moderadas a altas frente a ticarcilina, ticarcilina-ácido clavulánico, piperacilina-tazobactam, ceftazidima, cefepima, imipenem, meropenem, ciprofloxacina, amikacina y fosfomicina. Solo un aislado presentó el fenotipo BLEE codificado por el gen $bla_{SHV\text{-}2a}$. Entre las 13 cepas resistentes a imipenem y meropenem, 9 dieron positiva la amplificación del gen bla_{GES}. Los aislados exhibieron 19 genotipos de virulencia, de los cuales 5 fueron predominantes: V1 (14 genes; n = 14), V2 (14 genes; n = 6), V3 (todos los genes; n = 6), V4 (13 genes; n = 5) y V5 (13 genes; n = 5). La ERIC-PCR reveló una elevada heterogeneidad genética. Estos hallazgos evidencian la gran heterogeneidad genética de los aislados de *P. aeruginosa* en estos pacientes neutropénicos, así como su alto potencial patógeno, lo que constituye un importante motivo de preocupación clínica.

Introduction

Pseudomonas aeruginosa is commonly found in hospitals and contributes significantly to the occurrence of several acute and chronic nosocomial infections[1]. Life-threatening healthcare-associated infections, such as bloodstream infections, urinary tract infections, burn wound infections, ventilator-associated pneumonia, and cystic fibrosis morbidity, are more common among immunocompromised patients and those confined to intensive care units[2,3].

P. aeruginosa carries multiple virulence factors contributing to successful infection and causing disease, which are highly controlled by cell-to-cell signaling systems called quorum sensing (QS) system (lasR/lasI and rhlR/rhlI). Toxin A (ToxA, also known as ExoA), exoenzymes (ExoT, ExoY, ExoS, and ExoU), alkaline protease AprA and the elastases *LasA* and *LasB* are the principal virulence factors[3,4].

Pseudomonas spp. exotoxin A (ToxA or PE), the most toxic virulence factor, secreted by the majority of clinical isolates, is encoded by the chromosomally located *toxA* gene (also known as *exoA*). This gene inhibits the host cell's ability to synthesize proteins by inactivating the eukaryotic elongation factor 2[3]. The exoenzymes ExoS, ExoT, ExoU, and ExoY are effector exotoxins of the type III secretion system. The cytotoxic phospholipase ExoU disrupts the host cytoplasmic membrane causing cell lysis, while the cytotoxins ExoS, ExoT, and ExoY disrupt the actin cytoskeleton[5,6].

The alkaline protease, also known as aeruginolysin, encoded by the *aprA* gene, facilitates phagocytic evasion by disrupting two endothelium constituents, fibronectin and laminin, and breaking down complement proteins as well as cytokines. Additionally, it cleaves free flagellin monomers and reduces mucocilliary bacterial clearance by activating the epithelial sodium channel[7]. The elastases LasA and LasB destroy elastin, an important component of the pulmonary tissue and blood vessels, impairing lung function and causing pulmonary hemorrhage[8]. The alginate capsule, encoded by the *algD* gene, protects *P. aeruginosa* against host phagocytosis and scavenging ROS released by activated macrophages and neutrophils during the chronic infection[9]. It plays a significant role in chronic lung infections[10]. Moreover, *P. aeruginosa* produces two phospholipases C (PLCs) hydrolyzing various phospholipids of the host cell membrane, namely, hemolytic PlcH and nonhemolytic PlcN[11].

In addition to the potential virulence of *P. aeruginosa*, infections caused by this bacterium are particularly problematic in health care settings, as the bacterium is becoming increasingly resistant to antibiotics, making it more difficult to treat[12]. Indeed, the World Health Organization lists carbapenem-resistant *P. aeruginosa* among the ''critical'' group of bacteria that require immediate and innovative treatment in clinical settings[11].

The aims of this study were to determine the antimicrobial susceptibility, virulence genotypes, occurrence of extended spectrum β-lactamase (ESBL) and carbapenemase genes, and clonality of *P. aeruginosa* isolates responsible for lower tract respiratory infections in patients admitted to the National Bone Marrow Transplant Center (NBMTC) of Tunisia.

Materials and methods

Selection, identification and serotyping of isolates

All non-duplicated *P. aeruginosa* strains isolated from lower respiratory tract samples, with a bacterial enumeration greater than 10^7 UFC/ml, in patients admitted to the NBMTC

over an 11-year period (from January 2008 to December 2018) were included in this study. The isolates were identified using conventional methods (oxidase testing, Gram staining) and API20NE (Biomerieux®). Serotypes of isolates were determined using the slide agglutination technique with specific antiserum (Sanofi Diagnostics Pasteur, Marnes-La-coquette, France). All isolates were stored in brain heart infusion broth (Bio-Rad) with 20% glycerol at -20°C for further molecular investigation.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer (disc-diffusion) method on Mueller Hinton (MH) agar (Bio-Rad) in accordance with the standards and recommendations of the Antibiogram Committee of the French Society of Microbiology (CA-SFM/EUCAST.,2017)[13]. The tested antibiotics (Bio-Rad) included ticarcillin (75 μg), ticarcilline–clavulanic acid (75/10 μg), piperacillin (75 μg), piperacillin–tazobactam (75/10 μg), ceftazidime (10 μg), cefepime (30 μg), aztreonam (30 μg), imipenem (10 μg), meropenem (10 μg), gentamicin (10 μg), tobramycin (10 μg), netilmicin (30 μg), amikacin (30 μg), ciprofloxacin (5 μg), and fosfomycin (50 μg). ESBL production was detected by the double-disk method (CA-SFM/EUCAST., 2017)[13]. Isolates were considered multidrug-resistant (MDR) if they were resistant to three or more antibiotics from different families[14].

Detection of virulence-, ESBL- and carbapenemase genes

Genomic DNA extraction was performed using the InstaGene™ matrix (BioRad, California, USA) according to the manufacturer's instructions. Fifteen virulence genes (*lasB*, *algD*, *toxA*, *plcN*, *plcH*, *aprA*, *exoU*, *exoS*, *exoY*, *exoT*, *lasI*, *lasR*, *rhlR*, *rhlI*, and *rhlAB*) were investigated by PCR as previously reported[15–18]. ESBL-encoding genes (bla_{SHV}, bla_{CTXM}, bla_{TEM}, bla_{GES} and bla_{PER}) and carbapenemase genes (bla_{VIM}, bla_{IMP}, bla_{IMI}, bla_{GES}, bla_{NDM}, bla_{OXA-48}, and bla_{KPC}) were investigated in ceftazidime and carbapenem-resistant strains, respectively, by PCR as previously reported[19–23]. Positive and negative control strains of our collection were used[24].

ERIC-PCR typing

The ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus) technique was used to type *P. aeruginosa* isolates. This is a molecular typing method used to study the genetic diversity of *P. aeruginosa* strains. This technique relies on the amplification of specific repetitive intergenic DNA sequences in *P. aeruginosa* using ERIC primer sequences[25]. The PCR products were then analyzed to evaluate the genetic similarity among the strains.

Statistical analysis

The association between virulence genes and MDR isolates was analyzed using the Chi-square test.

Results

P. aeruginosa origin and serotypes

During the 11-year study period, a total of 319 *P. aeruginosa* isolates were collected from various clinical samples: 215 (67.39%) of them were from infection sites, of which 50 (50/319; 15.67%) isolates were responsible for lower respiratory tract infections. Isolates were collected from various hospital units, including external consultation (42%), hematology (28%), day hospital (14%), transplant (12%), and intensive care (4%).

P. aeruginosa isolates mainly belonged to serotype O:6 (n = 16; 32%), followed by serotypes O:3 (n = 6; 12%), O:11 (n = 5; 10%), and O:1 (n = 4; 8%). The remaining six (12%) isolates were untypeable (Fig. 1 and Supplementary data S1).

P. aeruginosa antimicrobial susceptibility

Antibiotic resistance rates were 30% for ticarcillin, 34% for ticarcillin–clavulanic acid, 18% for piperacillin–tazobactam, 16% for ceftazidime, 18% for cefepime, 30% for imipenem, 26% for meropenem, 18% for ciprofloxacin, 18% for amikacin, and 18% for fosfomycin. Only one (2%) isolate showed an ESBL phenotype. A multidrug resistance phenotype was observed in 14% of isolates (Table 1).

bla-gene prevalence

Among the 13 isolates resistant to both imipenem and meropenem, only bla_{GES} was amplified in nine isolates, representing 69.2%. Among the eight ceftazidime-resistant isolates, the bla_{SHV-2a} gene was amplified (n = 1) in the isolate showing an ESBL phenotype.

Prevalence of virulence genes

P. aeruginosa isolates exhibited 19 virulence genotypes (Table 2). Five virulence genotypes were prevalent (present in 36 isolates (72%)): V1 (associating 14 genes; n = 14), V2 (14 genes, n = 6), V3 (all genes, n = 6), V4 (13 genes, n = 5), and V5 (13 genes, n = 5). No statistically significant association between imipenem-resistant isolates and virulence genes was found (Fig. 2).

P. aeruginosa typing

ERIC-PCR showed high genetic heterogeneity; with each isolate displaying a unique ERIC-PCR fingerprint.

Discussion

Only a small percentage (15.67%) of *P. aeruginosa* was responsible for lower respiratory tract infections in hemato-oncology patients in this study. However, this species is highly virulent in neutropenic patients mainly causing bloodstream infections, respiratory infections, urinary tract infections, and skin and soft tissue infections[26].

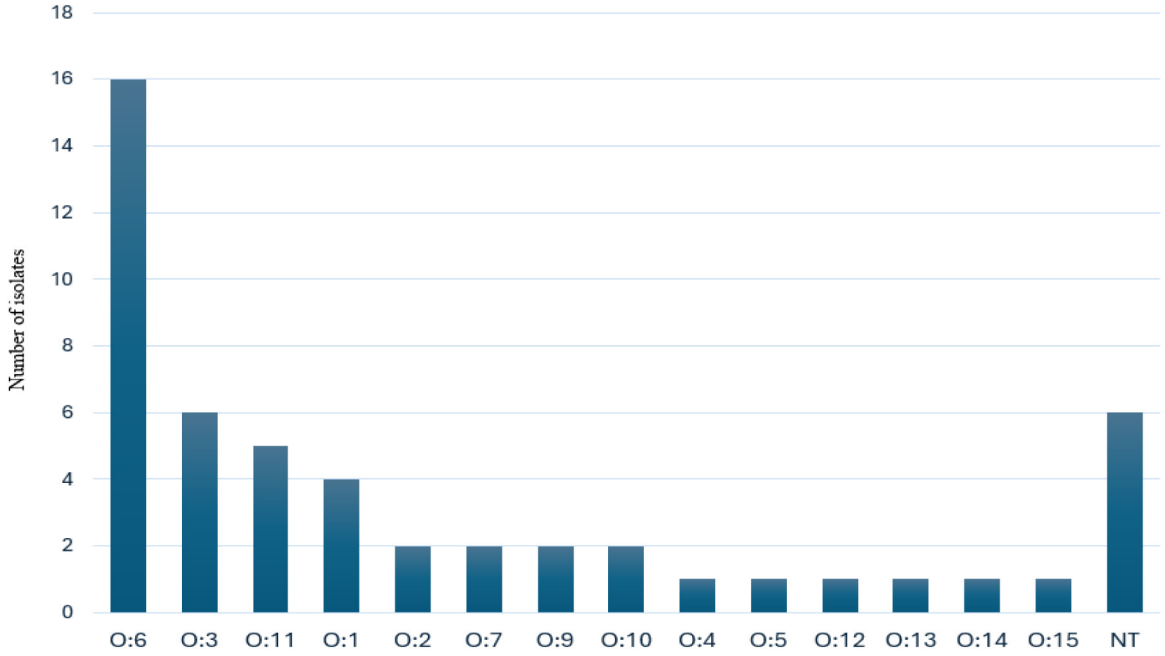

Figure 1 Serotype distribution of *P. aeruginosa* isolates recovered from low respiratory infection. NT: untypeable.

Table 1 Phenotypic and genotypic traits of the imipenem-resistant *P. aeruginosa* isolates recovered from lower respiratory tract infections.

Unit	Date	Reference	Antimicrobial resistance profile	*bla* genes	Serotype
EC	26/10/2009	6897-2	IMI-MER-FOS	bla_{GES}	O:3
TU	14/06/2010	4101	AZT-CEF-TIC-TIC/AC-PIP-PIP/TAZ-CAZ-IMI-MER-GEN-NET-TOB-AMI-CIP-FOS	$bla_{SHV\text{-}2a}$	O:11
EC	27/12/2010	8398-3	IMI-MER-NET	bla_{GES}	O:6
HU	26/11/2010	7645	AZT-CEF-TIC-TIC/AC-PIP-PIP/TAZ-CAZ-IMI-MER-GEN-NET-AMI-CIP-FOS	–	O:6
EC	11/10/2010	6613	AZT-TIC-TIC/AC-IMI-MER-GEN-NET-TOB-FOS	bla_{GES}	O:3
HU	09/09/2011	5669-1	TIC-TIC/AC-IMI-MER	bla_{GES}	O:3
HU	18/04/2011	2371	IMI	–	O:6
TU	25/12/2012	9553	IMI-MER-GEN-NET-AMI	bla_{GES}	O:11
TU	01/11/2012	8091	TIC-TIC/AC-PIP-PIP/TAZ-CAZ-IMI-MER-NET-CIP	–	O:12
HU	12/09/2015	6057-1	TIC-IMI-MER-FOS	bla_{GES}	O:10
HU	10/02/2016	879	AZT-IMI	–	O:6
ICU	29/04/2016	2977	AZT-CEF-TIC-TIC/AC-PIP-PIP/TAZ-CAZ-IMI-MER-GEN-AMI-CIP	–	NT
HU	28/12/2017	7221	AZT-CEF-TIC-TIC/AC-PIP-PIP/TAZ-IMI-MER-GEN-NET-TOB-AMI-CIP-FOS	bla_{GES}	O:13
TU	19/12/2018	26362	AZT-TIC-AC-PIP-IMI-MER-FOS	bla_{GES}	O:1
HU	13/07/2018	7844	PIP-IMI-MER	bla_{GES}	O:1

AZT: aztreonam; CEF: cefepime; TIC: ticarcillin; TIC/AC: ticarcillin–clavulanic acid; PIP: piperacillin; PIP/TAZ: piperacillin–tazobactam; CAZ: ceftazidime; IMI: imipenem; MER: meropenem; AMI: amikacin; CIP: ciprofloxacin; FOS: fosfomycin; HU: hospital units; EC: external consultation; HU: hematology unit; DH: day hospital; TU: transplant unit; ICU: intensive care unit; NT: untypeable; +: presence; –: absence.

Antimicrobial-resistance is globally a serious problem for human and animal health. Certainly, this phenomenon complicates the care of critically ill patients such as immunocompromised patients with hematological malignancies[27]. Relatively high antimicrobial resistance rates were observed in this study, especially against clinically relevant antibiotics such as ticarcillin–clavulanic acid, piperacillin–tazobactam, ceftazidime, cefepime, carbapenems, and ciprofloxacin. Similar antimicrobial resistance rates have been reported in *P. aeruginosa* isolates collected from various origins in neutropenic patients[28,29]. In addition, a MDR phenotype was observed in 14% of isolates. Infections due to MDR *P. aeruginosa* were associated with recent use of carbapenems and other beta-lactams and fluoroquinolones, ventilator care, and *P. aeruginosa* infection or colonization within the previous year[30].

Carbapenem-resistant isolates accounted for 30% (30% for imipenem and 26% for meropenem) of the total *P. aeruginosa* isolates, of which six were MDR. Over the last two decades, carbapenem-resistant *P. aeruginosa* has emerged as a serious threat to human health, as most of these isolates exhibited multidrug-resistant

Table 2 Virulence genotype profiles detected in the 50 *P. aeruginosa* isolates.

Virulotype	Virulence genotypes															Number of isolates
	lasR	*lasB*	*algD*	*lasI*	*rhlR*	*rhlAB*	*rhlI*	*plcN*	*plcH*	*toxA*	*aprA*	*exoS*	*exoU*	*exoY*	*exoT*	
V.1	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	14
V.2	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	6
V.3	+	+	+	+	+-	+	+	+	+	+	+	+	+	+	+	6
V.4	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	5
V.5	+	+	+	+	+	+	—	+	+	+	+	+	—	+	+	5
V.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	1
V.7	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	1
V.8	+	+	+	+	+	+	+	+	+	+	+	—	+	—	—	1
V.9	+	+	+	+	+	+	—	+	+	+	+	—	+	+	+	1
V.10	+	+	+	+	—	+	+	+	+	+	+	+	—	+	+	1
V.11	+	+	+	+	+	+	+	+	+	+	+	+	—	+	—	1
V.12	+	+	+	+	+	+	+	+	+	+	+	—	+	—	+	1
V.13	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	1
V.14	+	+	+	+	+	+	—	+	+	+	+	+	+	—	+	1
V.15	+	+	+	+	+	+	—	+	+	+	+	+	+	+	—	1
V.16	+	+	+	+	+	+	—	+	+	+	+	+	+	—	—	1
V.17	—	+	+	+	+	+	—	+	+	+	+	+	—	—	+	1
V.18	—	+	+	—	+	+	+	+	+	+	+	+	—	+	—	1
V.19	—	+	+	+	+	+	+	+	+	+	+	+	—	+	+	1

+: presence; —: absence.

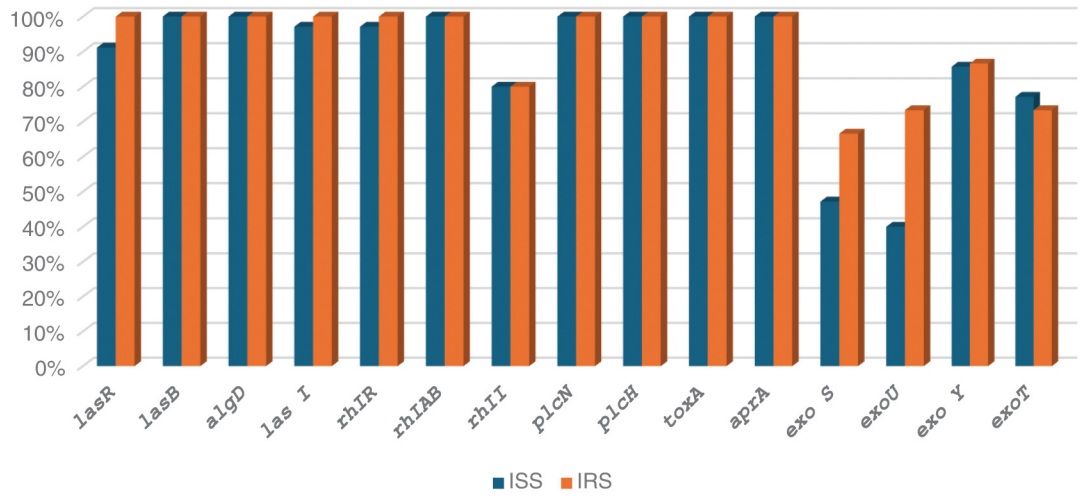

Figure 2 Distribution of virulence genes in *Pseudomonas aeruginosa* strains with respect to imipenem resistance. ISS: imipenem-susceptible strains; IRS: imipenem-resistant strains.

(MDR), extreme drug-resistant – or even pandrug-resistant phenotypes[31].

Among the 13 isolates resistant to both imipenem and meropenem, nine harbored a bla_{GES}-type gene. In Tunisia, bla_{GES} genes such as bla_{GES-5} and bla_{GES-45} have been reported in *P. aeruginosa* from clinical samples[24,32,33]. While these genes remain rare globally, they are often associated with outbreaks[32–35]. In the remaining carbapenem-resistant isolates, other non-enzymatic mechanisms might encode this resistance, such as loss of outer membrane channel OprD or mutations in efflux systems (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM).

The bla_{SHV-2a} gene was detected in only one isolate, the only one exhibiting an ESBL phenotype, among eight ceftazidime-resistant isolates. The SHV-2a type ESBL was frequently detected in *Enterobactereceae* and *P. aeruginosa* before the expansion of CTX-M enzymes; however, it is currently rare[36,37].

In this study, all *P. aeruginosa* responsible for lower respiratory tract infections harbored *toxA*, *plcN*, *plcH*, *aprA*, *algD*, *lasB* and *rhlAB* genes. The exotoxin A gene, *toxA*, was detected in 97.2–100% of strains in some studies[38,39]. Notably, the *toxA* and *exoA* genes are synonymous, as both encode exotoxin A, causing confusion in several studies[15,16].

Similarly, *plcH* and *plcN* genes were detected in 100% of isolates in several studies[40,41], but in less than 70% of strains isolated from wound infections in an Iranian study (67.2% for plcN and 50.9% for plcH)[42]. More than 80% of the strains harbored *lasR*, *lasI*, *rhlR* and *rhlI* genes. The high prevalence of these genes has been previously described[43]. They are involved in the regulation of QS type systems and in the induction of biofilm formation, the expression of virulence factors and the production of pigments[44].

More than 50% of isolates possessed *exoU* (50%), exoS (76%), *exoT* (76%) and *exoY* (86%) genes. The presence of exo genes is highly prevalent in *P. aeruginosa* strains once they are part of a type III secretion system (T3SS)[45]. However, the presence of the different effectors is not constant[45]. *P. aeruginosa* isolates with positive T3SS genotypes (*exoS*, *exoT*, *exoU*, and *exoY*) have been linked to an increased mortality rate, particularly in lower respiratory tract and systemic infections[46,47]. In our study, *P. aeruginosa* isolates expressed cytotoxic (*exoU+*/*exoS-*) (14/50) or invasive (*exoU-*/*exoS+*) (25/50) or cytotoxic/invasive (*exoU+*/*exoS+*) (11/50) phenotypes. The frequency of these phenotypes varies depending on the origin of the strain: the *exoU* and *exoS* genes were reported, respectively, in 64.5% and 29% of strains isolated from burn patients[48]; 62.4% and 91.6% of patients with cystic fibrosis[49]; 47.6% and 90.5% of patients with urinary infections[50]; and 18% and 63% of patients with surgical site infections[16].

ExoT and *exoY* have a moderate impact on virulence, whereas *exoS* and *exoU* play a major role in pathogenesis and raise significant concern[47]. The *exoS* and *exoU* genes were co-hosted in eleven isolates (22%). This invasive-cytotoxic genotype had a low prevalence[49]. Nonetheless, a few recent reports have described a high prevalence of isolates simultaneously carrying *ExoS* and *ExoU* genes[39,51]. Generally, the *exoU* and *exoS* genes are mutually exclusive[52]. Given that *ExoS* is chromosomally encoded and that *ExoU* is present inside a mobilizable genomic island, it is difficult to interpret this mutual exclusion. Based on several studies, *exoU* may be selected in anthropic settings, but the presence of *exoS* may favor bacterial survival in non-anthropic environments[53]. This could explain its lower prevalence compared with other virulence genes[54]. Furthermore, ExoU is 100 times more cytotoxic than ExoS[5], which explain s its increasing prevalence among clinical strains[50].

ERIC-PCR has been widely used in the characterization of *P. aeruginosa* strains from various sources, including human infections, cases of hemorrhagic pneumonia, and clinical and environmental settings[55,56]. Heterogeneous DNA fingerprints were detected using ERIC-PCR; each isolate exhibited a specific DNA fingerprint, implying their non-clonality. This finding demonstrated that isolates are endogenous to each patient, and no clonal spread occurred among them.

Conclusion

Taken together, the study findings highlight the high genetic heterogeneity of *P. aeruginosa* strains responsible for lower respiratory tract infections in hemato-oncology patients. A relatively high rate of carbapenem resistance was observed, with the gene detected in resistant strains. Furthermore, a high prevalence of virulence genes was noted, including the presence of *toxA*, *plcN*, *plcH*, *aprA*, *algD*, *lasB* and *rhlAB* genes in all strains.

Ethical approval

This study was conducted with the approval of the Local Medical Committee for National Bone Marrow Transplantation.

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Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version available at https://doi.org/10.1016/j.ram.2026.100714.

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