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Original Article

Occurrence of virulence genes and carbapenemase genes in multidrug resistant clinical isolates of *Pseudomonas aeruginosa*

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Abstract
Introduction: Multi-drug resistant (MDR) *Pseudomonas aeruginosa* strains with virulence characteristics have high morbidity and mortality rates. This study aimed to examine the prevalence of antibiotic resistance, virulence factors, and carbapenemase genes in clinical samples.
Methodology: 400 clinical samples (200 UTI+200 wound samples) were collected from hospitalized patients, targeting *P. aeruginosa.*
Results: Out of 400 samples, 162 (40%) were found positive for *P. aeruginosa,* and they revealed significant resistance, with 55% classified as multidrug-resistant. Ceftazidime and Imipenem showed the highest resistance rate. Notably, 90% isolates were resistant to Tobramycin and Amikacin, while only 15-25% of isolates were sensitive to several antibiotics. The samples showed that 101 isolates (62% of the total) had the *algD* gene. Of these, 78% were found to be resistant to multiple drugs. Carbapenemase gene prevalence varied, with *IMP* (77.27%) being the most common, followed by *TEM* (63.63%) and *NDM* (43.18%). The detected exo enzyme genes in 67.9%-88.88% of isolates, with *toxA* being the most prevalent at 88.88%. Wound isolates showed higher virulence gene frequencies compared to urine isolates.
Conclusions: The misuse/overuse of antibiotics, poor hygiene, and errors of health care personnel were the main factors that significantly increased the prevalence of MDR *P. aeruginosa.* Hospitals should craft and implement a specific control strategy to reduce the distribution of MDR *P. aeruginosa*. The MDR isolates expressed exoenzymes-encoding genes as compared to the drug-susceptible isolates. The prevalence of these virulence genes was higher in wound isolates as compared to UTIs. This study indicates that exoenzymes-encoding genes are associated with drug resistance in *P. aeruginosa.*

Key words: Multi-drug resistant; *Pseudomonas aeruginosa*; virulence factors; exo-enzymes; carbapenemase.

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Introduction

Pseudomonas aeruginosa, being an opportunistic pathogen, causes infections in immunocompromised patients and is responsible for about 10 to 20% of nosocomial infections worldwide [1]. In immunocompromised individuals, it can lead to either acute or chronic infections, affecting multiple systems, including the respiratory, urinary tract, and soft tissues [2]. Moreover, this pathogen has developed resistance against all potent antimicrobial drugs, resulting in the emergence of multi-drug-resistant (MDR) strains [3], which are caused by the bacteria's inherent, acquired, and adaptive resistance to antibiotics [4]. This limits the treatment options for *P. aeruginosa* infections and complicates the therapy process. As a result, there are significant rates of morbidity and mortality in addition to the pathogen's adaptable virulence characteristics [5].

P. aeruginosa's genome contains a wide range of virulent genes, including *exoS, oprL, lasB, toxA*, and *nan*, the expression of which is linked to disease severity and adverse treatment results after infection [6]. For *P. aeruginosa* to remain attached to the surface of the host mucosa, the *nan* gene, a neuraminidase, is necessary [7]. The *lasB* gene codes for an elastase involved in the proteolytic cleavage of collagen and elastin in lung injury is encoded by the antibiotic efflux from *P. aeruginosa,* and membrane integrity depends on the *oprL* gene, a peptidoglycan-associated lipoprotein L [8]. The *oprL* gene has been linked to multidrug resistance in *P. aeruginosa* in addition to serving as a taxonomic marker to validate the pathogen's identity [9]. Elongation factor 2 of the host cell is the target of the cytotoxic drug *toxA* gene, which inhibits protein synthesis. The virulence factor's expression is also accountable for the harm that *P. aeruginosa* infections cause to tissues and organs [10]. The virulence factors of *P. aeruginosa* are categorized into two groups: factors that are secreted, such as phospholipases C, pyocyanin, alginate, and DNase, and factors that are associated with cells, such as adhesins and lipopolysaccharide [11]. It has been documented that *exoU, exoS, exoT*, and *exoY* genes encode Type III secretion system (TTSS) components that cause cytotoxic and invasive behaviors [12].

The majority of clinical isolates of *P. aeruginosa* make proteins that are inhibited by exotoxin A. On the other hand, exoenzyme *S* causes various harmful effects on the host cells, ultimately leading to cell death [13]. The mucoid phenotype frequently isolated from cystic fibrosis (CF) patients is caused by alginate, a viscous polysaccharide. Hemolytic and non-hemolytic phospholipases C hydrolyze lung surfactant proteins [14]. Pyocyanin is a blue pigment that is associated with tissue damage through the production of reactive oxygen species and the stimulation of neutrophils [15]. *P. aeruginosa* can break down the extracellular DNA and use it as a source of nourishment to produce DNase. This pathogen also produces hemolysin, which lyses several cell types and promotes the spread of infections [16]. *P. aeruginosa* proteases contribute to the breakdown of the host's tissue and the deactivation of immune system components. Furthermore, *P. aeruginosa* may develop biofilms in both acute and chronic infections, which increases antibiotic resistance and increases the risk of nosocomial persistent infections, which can be fatal. Certain biofilm matrix molecules (like alginate) and cell appendages (like type IV pili) are important for making biofilm and are also considered significant virulence factors [17].

It is important to do epidemiology research on virulence-genotype and resistance-phenotype to learn more about *P. aeruginosa* infection at the molecular or genetic level. Carbapenem-resistant *P. aeruginosa* (CRPA) presents significant difficulties for clinicians, making patient treatment more challenging. Furthermore, studies have demonstrated that specific dominant sequence types could facilitate colonial proliferation of certain CRPA strains worldwide [18]. The present research aims to isolate and identify the clinically important strains of *P. aeruginosa* from infected patients and investigate the expression of key virulence genes associated with the severity of infection, and analyze the correlation between virulence factors and carbapenemase genes in MDR strains.

Methodology

Ethical permission

Before starting the study, ethical permission was obtained from the Ethical Review Committee of Government College University Faisalabad (GCUF/ERC/19/155). Every patient provided written informed consent, and clinical samples were collected in compliance with the Helsinki Declaration [19].

Table 1. List of Primer Sequences for confirmation of *P. aeruginosa*, Carbapenmase resistance and virulence genes.

Gene	Primer sequences (5´-sequence-3´)	Product size	References
oprL	F-ATG GAAATGCTGAAATTCGGC R-CTTCTTCAGCTCGACGCGACG	504bp	[24]
Oprl	F-ATGAACAACGTTCTGAAATTCTCTGCT R-CTTGCGGCTGGCTTTTTCCAG	249bp	[24]
algD	F-CGTCTGCCGCGAGATCGGCT R-GACCTCGACGGTCTTGCGGA	313bp	[25]
blaOXA	5'-GCG TGT ATT AGC TTA TC -3' 5'-CGC GGT TCG GTA GTG TGT TT -3'	438	[24]
blaNDM	5'-GGT TTG GCG ATC TGG TTT TC-3' 5'-CGG AAT GGC TCA TCA CGA TC-3'	621	[24]
blaVIM	5'-GATGGTGTTTGGTCGCATA-3' 5'-CGAATGCGCAGCACCAG-3'	390	[26]
blaIMP	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	232	[26]
BlaKPC	5'-CGT CTA GTT CTGCTG TCT TG-3' 5'-CTT GTC ATC CTTGTT AGG CG-3'	798	[24]
exoS	CTTGAAGGGACTCGACAAGG TTCAGGTCCGCGTAGTGAAT	504	[27]
S1S2imm	F – CACAAGGGAGGGAAGTGA R - CGGCCTTAAAGCCAGGAA	287	[28]
S3RB	F- CGTATCACGAGACAGGCA R- TGCCGCTTCTTCCGCTTT	451	[28]
exoY	CGGATTCTATGGCAGGGAGG GCCCTTGATGCACTCGACCA	289	[27]
exoT	AATCGCCGTCCAACTGCATGCG TGTTCGCCGAGGTACTGCTC	152	[27]

Sample collection and isolation

A total of 400 clinical samples were collected from chronic wounds (*n* = 200) and urinary tract infection patients (*n* = 200) using sterile cotton swabs and sterile containers from Allied Hospital, Faisalabad, Pakistan. For the isolation of *P. aeruginosa,* samples were swabbed on Pseudomonas cetrimide agar plates. Isolation and identification of strains were confirmed by Gram staining and biochemical tests like Indole, Catalase, Oxidase, Methyl Red, Citrate, and Voges Proskauer test [20]. Biochemically identified isolates were confirmed via PCR using specific primers.

Antibiotic susceptibility testing (AST)

By using the Muller Hinton agar Kirby-Bauer disk diffusion assay was performed for all clinical isolates to check the susceptibility pattern of *P. aeruginosa* to different antibiotics according to the CLSI guideline 2021 [21]. Antibiotics, including piperacillin, tobramycin, ceftazidime, Gentamicin, ciprofloxacin, ceftriaxone, imipenem, meropenem, aztreonam, norfloxacin, nalidixic acid, and amikacin, were used to assess the susceptibility pattern of isolates.

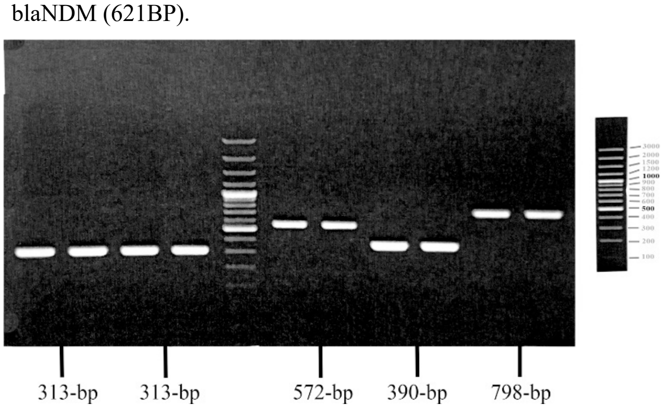
Genomic DNA isolation

Pure colonies of *P. aeruginosa* strains were grown in Luria broth (LB) fluid medium at 37 °C overnight. A DNA Purification Kit was used for genomic DNA extraction [22]. Following that, DNA samples were kept at -20 °C until they were used for the PCR assay that detects virulence genes and carbapenemase.

Detection of Pseudomonas aeruginosa, its virulence genes, and carbapenemase

The polymerase chain reaction was performed for the identification of *Pseudomonas aeruginosa*, its virulence genes (*exoS, exoY*, and *exoT*), which are known to contribute to the pathogenicity of *P. aeruginosa*, and carbapenemase genes (bla*OXA*, bla*NDM*, bla*VIM*, bla*IMP*, and bla*KPC*), which are important in discussing antibiotic resistance to *P. aeruginosa*. Furthermore, the *algD* gene was targeted as it is important for biofilm formation and virulence. The specific primers for each gene are mentioned in Table 1. The conditions used for the polymerase chain reaction (PCR) were as follows: initial denaturing at 94 ºC for 5 min, followed by 30 cycles of denaturing at 94 ºC for 45 seconds, annealing (temperature and time set for each primer), elongation step for 1 minute at 72 ºC, with a final extension cycle for 5 minutes at 72 ºC [23]. By using the gel-doc system on a 1% agarose gel stained with ethidium bromide, the amplified result of PCR was detected.

Figure 1. PCR-based analysis of P. aeruginosa and carbapenemase genes encoding Oprl (249bp), blaIMP (232bp), blaVIM (390), blaNDM (621BP).



Results

Occurrence of Pseudomonas aeruginosa

A total of 400 clinical samples were collected. Among the wound samples, 97 (48%) samples were positive, while in the case of UTIs, 65 samples (32%) were found positive, making the overall prevalence 40% (162/400).

Molecular identification of Pseudomonas aeruginosa

All strains of *P. aeruginosa* (n = 97 from wound samples) and (n = 65 from urine samples) were confirmed positive for the *Oprl* gene. Figure 1 displays the *Oprl* gene gel electrophoresis picture.

Antibiotic Susceptibility Test (AST)

An antibiotic susceptibility test was performed for a total of 162 isolates of *P. aeruginosa*. Guidelines of the Clinical Laboratory Standard Institute were followed to measure the zone of inhibition of tested antibiotics against *P. aeruginosa*. The susceptibility patterns of each antibiotic are shown in Table 2. Among the 162 isolates, 90 (55%) were MDR (exhibited resistance to at least three or more classes of antibiotics that are typically effective against this *P. aeruginosa).*

Table 2. Antimicrobial susceptibility pattern of *P. aeruginosa isolates.*

Antibiotic	Resistance %	Sensitive %
Piperacillin	138 (85%)	24 (15%)
Tobramycin	146 (90%)	16 (10%)
Ceftazidime	162 (100%)	0 (0%)
Gentamicin	130 (80%)	32 (20%)
Ciprofloxacin	122 (75%)	40 (25%)
Imipenem	122 (75%)	40 (25%)
Meropenem	122 (75%)	40 (25%)
Aztreonam	138 (85%)	24 (15%)
Norfloxacin	122 (75%)	40 (25%)
Nalidixic acid	130 (80%)	32 (20%)
Amikacin	146 (90%)	16 (10%)

Table 3. Occurrence of alginate genes in multidrug resistance in *P. aeruginosa*.

Sample no	Positive isolate	MDR%	Alginate	
			Positive isolates %	MDR %
Clinical wound n = 200	97 (48%)	63 (64%)	75 (77%)	59 (78%)
Urine sample n=200	65 (32%)	26 (40%)	26 (40%)	20 (76.9%)
Total n= 200	162 (40%)	89 (55%)	101 (62%)	79 (78%)

Detection of carbapenemase and virulence genes among MDR Pseudomonas aeruginosa

The results showed a total of 101 (62%) isolates of *Pseudomonas aeruginosa,* confirmed to have the algD gene of alginate. Out of these 101 positive isolates of *algD*, 79 (78%) were multidrug-resistant (Table 3). The prevalence of the carbapenemase gene was recorded and mentioned for urine and wound samples in Table 4 (Figures 1, 2).

Detection of Exo enzymes genes in MDR Pseudomonas aeruginosa

Out of the total of 162 isolates of *P. aeruginosa*, 97 were positive from the wound and 65 were positive from urine. The presence of virulence genes *exoS, exoY, exoT* & *toxA* was detected in 67.9%, 61%, 57.4%, and 88.88% of clinical isolates, respectively. In wound specimens, the prevalence of virulent genes *toxA, exoS*, *exoT, and exoY* was 90.7%, 75%, 62.8%, and 62.8% respectively. While in urine isolates, the distribution of virulent genes *toxA, exoS, exoT,* and exoY was 87.69%, 56.9%, 53.8%, and 58.46% respectively. The frequency of the virulent genes was found to be higher in wound isolates as compared to the isolates collected from urine. The *ToxA* gene was frequently higher in clinical isolates (Figure 3).

Discussion

Pseudomonas aeruginosa, a well-known opportunistic pathogen responsible for causing UTIs, wound infections, bloodstream infections, and cystic fibrosis, can form biofilms and secrete bacteriocins for its survival that help this pathogen to further elevate resistance against almost all antimicrobials. *P. aeruginosa* is one of the leading causes of mortality and morbidity. As far as the pathogenicity of *P. aeruginosa* is concerned, this nosocomial pathogen produces a large variety of cell-associated as well as extracellular virulent factors [20].

In the present study, 400 clinical samples (n = 200 wounds and n = 200 urine samples) were taken from Allied Hospital, Faisalabad. Out of these, 40% (n = 162) isolates were positive for *Pseudomonas aeruginosa* after PCR confirmation. The findings reported a larger number of wound samples positive for *P. aeruginosa* as compared to the urine samples. Wounds provide a favorable environment for growth of opportunistic pathogens, especially *P. aeruginosa* [29]. The high prevalence of *P. aeruginosa* in these clinical samples is due to poor sanitation in hospitals and improper wound management [30].

P. aeruginosa displays resistance to presently available numerous classes of antibiotics which

Figure 2. Detection of virulent genes in MDR P. aeruginosa by PCR (313-bp algD, 287- bp S1S2imm, and S3RB).

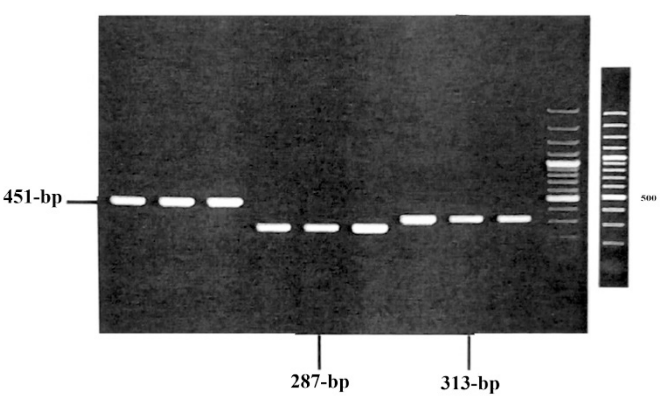


Figure 3. Detection of virulent genes in MDR P. aeruginosa by PCR (504-bp exoS).

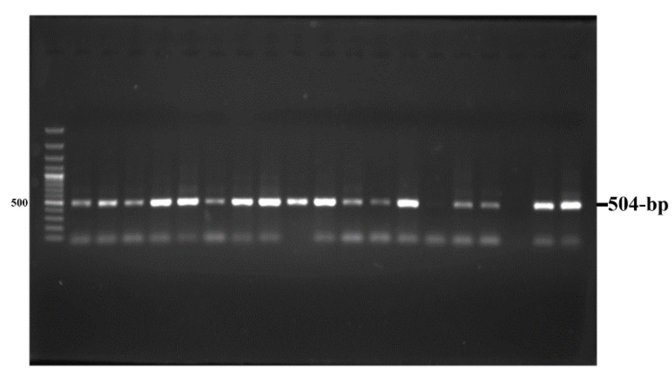


Table 4. Prevalence of carbapenemase genes in multi-drug resistant *Pseudomonas aeruginosa*.

MDR %	blaIMP	blaTEM	blaNDM	blaOXA	blaVIM	blaKPC
Urine sample (n = 26)	17 (65%)	13 (50%)	11 (42%)	9 (34.6%)	7 (26.9%)	1 (3.8%)
Clinical wound (n = 63)	52 (82.5%)	43 (68%)	28 (44%)	16 (25%)	20 (31.7%)	1 (1.5%)
Total (n = 89)	69(77.5%)	56 (62.9%)	39 (43.8%)	25 (28%)	27 (30 %)	2(2.24%)

develops a challenging situation for curing *P. aeruginosa* infections [3]. The foremost mechanisms of resistance against antibiotics, used for the treatment of *P. aeruginosa*, include intrinsic, adaptive, and innate mechanisms, and above all, intrinsic mechanisms display over-expression of efflux pump and the outer membrane permeability [31]. Mutations and horizontal transfer display the acquired resistance. Adaptive mechanisms are displayed due to the biofilm formation and hinder the entry of antibiotics into the internal structure of bacterial cells. *P. aeruginosa* can escape from the host defense by targeting the immune response due to the production of siderophores, pigment production, biofilm's impervious outer membrane, and great environmental tolerance [32].

Multiple studies reported various classes of antibiotics that are used to treat the infections caused by *Pseudomonas aeruginosa* [6]. The present study demonstrated high resistance to Ceftazidime (100%). While the antibiotics found sensitive against 25% isolates were Imipenem, ciprofloxacin, and norfloxacin. Previous study reported the data disc diffusion test, i-e, cefoxitin (100%), cefotaxime (100%), aztreonam (100%), and nitrofurantoin (100%) were the isolates with the highest resistance among ceftazidime-avibactam and ceftolozane-tazobactam resistant strains. Furthermore, fosfomycin had the lowest resistance (62.5%) as reported by Rahimzadeh *et al.* [33] from Iran. Another study revealed that the antibiotics with the highest resistance rates were levofloxacin (98.3%, 58/59), ciprofloxacin (96.6%, 57/59), gentamicin (91.5%, 54/59), ceftazidime (79.7%, 47/59), piperacillin-tazobactam (50.8%, 30/59), and aztreonam (27.1%, 16/18) [34]. The development of MDR *P. aeruginosa* has also increased the burden of morbidity and mortality and decreased the number of therapeutic options that are accessible. In the current study, 55% of the tested *P. aeruginosa* isolates were MDR. However, the prevalence of MDR *P. aeruginosa* isolates in the current study is lower than the study conducted in Egypt by Edward *et al.* [35]. Antibiotic overuse may be the cause of this, and to address the issue, strict prescription guidelines are required.

Extracellular virulence factors include biofilm formation, *LasB* and *LasA* elastases, exotoxin *A*, exoenzymes *S, T*, and *Y*, and exotoxin *U*. The ability to develop adaptive as well as intrinsic resistance against many antimicrobials makes the treatment of *Pseudomonas aeruginosa* infection even more difficult [36]. Membrane impermeability, over-expressed efflux pumps, target site modification, and beta-lactam enzymes are the most common mechanisms used by *Pseudomonas aeruginosa* to confer resistance [37].

A total of 101(62%) isolates of *Pseudomonas aeruginosa* were detected to have the *algD* gene of alginate. Out of 101 positive isolates of *algD*, 79(78%) were multidrug resistant, while 22(22%) were susceptible to antibiotics. Similar studies conducted by Ghadaksaz *et al.* [38] showed the prevalence of *algD* to be 87.5% out of 104 isolates of *Pseudomonas aeruginosa*. Al-Ahmadi and Roodsari [39] determined that the prevalence of *algD* was 96.7% out of 188 clinical samples of *P. aeruginosa*. Benie *et al*. [40] detected a prevalence of *algD* (72.1%) from a total of 204 strains of *P. aeruginosa*. Al Dawodeyah *et al*. [41] detected *algD* (98%) in 284 clinical samples of respiratory tract infections. A prior study revealed that 91.3% of the examined isolates had the *algD* gene, one of the investigated virulence genes examined by Edward *et al.* [35]. The results indicate that *algD* is more prevalent in MDR isolates, which suggests that biofilm formation is associated with drug resistance because it does not allow access of drugs to the organism. Alginate is a polysaccharide that makes up the biofilm matrix. The rate-limiting step in its synthesis is encoded by the gene *algD*. Additionally, it prevents complement protein activation and decreases phagocytosis to shield *P. aeruginosa* from host defense mechanisms. Resistance of MBL and ESBL against *P. aeruginosa* has reached to alarming level. The current study shows that *P. aeruginosa* is highly resistant to β-lactam drugs. The study found 65% cases of *IMP*, 50% *TEM*, 42% *NDM*, 34.6% *OXA*, 26.9% *VIM*, and 3.8% *KPC* gene prevalent in the 26 urine samples. A total of sixty-three clinical wound specimens showed a greater prevalence overall: 82.5% for *IMP*, 68% for *TEM*, 44% for *NDM*, 25% for *OXA*, 31.7% for *VIM,* and 1.5% for *KPC*. A prevalence of 77.5% for *IMP*, 62.9% for *TEM*, 43.8% for *NDM*, 28% for *OXA*, 30% for *VIM*, and 2.24% for *KPC* was found when the combined data of 89 samples were taken into account. The results highlighted a significant difference in the prevalence of antibiotic resistance genes between urine and wound samples, with the latter consistently showing higher rates for the majority of resistance types examined in the study. A previous study conducted by Jalalvand *et al.* [42] showed that out of 45 CREs, 13 (28.88%) had solely the *OXA*-48 gene, 3 (6.66%) the *NDM* gene, and 1 (2.22%) the *KPC* gene. Furthermore, 4 (8.8%) of these isolates co-produced the *OXA*-48 and *KPC* gene; 11 (24.44%) co-produced the *NDM, OXA*-48, and *KPC* gene. 11 (24.44%) of these isolates co-produced the *NDM* and *OXA*-48 gene. Inadequate sterilization, negligence of medical personnel, and poor sanitation are all risk factors that play a key role in the transmission of resistant pathogens.

P. aeruginosa's multidimensional harmfulness is caused by its virulence, which is cooperatively controlled by several signaling layers. Out of the total of 162 isolates of *P. aeruginosa*, 97 were positive from the wound and 65 were positive from urine. *ExoS, exoY, exoT*, and *toxA* are virulence genes that were found in 67.9%, 61%, 57.4%, and 88.88% of clinical isolates, respectively. The frequency of pathogenic genes *toxA, exoS, exoT*, and *exoY* in wound specimens was 90.7%, 75%, 62.8%, and 62.8%, respectively. The distribution of virulent genes *toxA, exoS, exoT*, and *exoY* in urine isolates was found to be 87.69%, 56.9%, 53.8%, and 58.46%, respectively. The frequency of the virulent genes was found to be higher in wound isolates as compared to the isolates collected from urine. The *ToxA* gene was frequently higher in clinical isolates. A previous study conducted by Chand *et al.* [43] showed that 95.4% of isolates were positive for the *toxA* gene. In Brazil, an approximately similar study found that 81.82% of *P. aeruginosa* isolates tested were positive for *toxA* [44]. According to a study carried out by Badamchi *et al.* [45] in Iran, the *toxA* gene was present in 69.4% of *P. aeruginosa* isolates. A possible explanation for the differences seen in the distribution of virulence factor genes among the populations is the possibility that certain strains of *P. aeruginosa* are more suited to the environmental and geographic characteristics of infectious areas, where they may reappear. The virulence genes of *P. aeruginosa* and its prevalence are determined by several factors, including the type and severity of contamination, the immunological condition of individual patients, and the virulence of the strains.

Conclusions

It is concluded that the present study estimated the prevalence of MDR *P. aeruginosa* in wounds and urine samples of hospitalized patients. Misuse or overuse of antibiotics, poor hygiene environment, and errors of health care personnel were the main factors that significantly increased the prevalence of MDR *P. aeruginosa.* A specific control strategy should be crafted and implemented in hospitals to reduce the distribution of MDR *P. aeruginosa.* It is concluded that MDR isolates of *P. aeruginosa* mostly expressed exoenzymes encoding genes as compared to the drug-susceptible isolates. The prevalence of these virulence genes was higher in wound isolates as compared to UTIs. This study indicates that exoenzymes encoding genes are associated with drug resistance in *P. aeruginosa*.

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Conflict of interest

No conflict of interest is declared.

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