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Antibiotic Resistance Profiles of *Pseudomonas Aeruginosa* Isolates Containing Virulence Genes

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ABSTRACT

Background: A most common opportunistic pathogen, *Pseudomonas aeruginosa* is present in both humans and animals and responsible for various nosocomial infections and healthcare settings related infections. Different virulence genes like; *oprL* (membrane lipoprotein L) and *toxA* (exotoxin A i.e. ETA) in *P. aeruginosa*, assist in its pathogenicity, toxicity and contribute to high antibiotic resistance. So considering the risk of zoonotic transmission of *P. aeruginosa* through animal-related foods, this study aimed to monitor the prevalence of *oprL* and *toxA* virulence genes and antimicrobial resistance in *P. aeruginosa* isolates, obtained from animal (Cow's milk) and human clinical samples.

Materials and Methods: Of the total 120 collected samples for this study, every 60 samples were collected from animals and humans from respective laboratories. Total 76 isolates of *P. aeruginosa* were isolated and identified by morphological and biochemical tests. The presence of virulence factors like *oprL* and *toxA* were evaluated by PCR analysis and antimicrobial resistance was assessed by antibiotic susceptibility test (Kirby-Bauer method).

Results: From the total 76, *P. aeruginosa* isolates obtained from both animal and human isolates, alone presence and coexistence of both *toxA* and *oprL* genes in *P. aeruginosa* isolates; were detected in PCR analysis. PCR analysis results showed in *P. aeruginosa* isolates, alone distribution of *toxA* and *oprL* genes is, 75% and 54.16% in animals, and 84.61% and 80.76% in humans, respectively. The coexistence of both genes was 37.50% and 40.32% in animals and human isolates, along with high antibiotic resistance in most *P. aeruginosa* isolates.

Conclusion: Therefore, this study suggested PCR analysis can be used for fast and specific detection of *oprL* and *toxA* genes in *P. aeruginosa*. Monitoring of these genes can help to prevent the risk of transmission of multi-drug resistant *P. aeruginosa*, from animals to humans.

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Introduction

Pseudomonas aeruginosa exists ubiquitously in nature and is among the most commonly found opportunistic pathogens. Immunocompromised individuals are more prone to infections caused by *P. aeruginosa* and it is responsible for high morbidity and mortality in them [1, 2]. *P. aeruginosa* is the most common infectious agent in patients with burning wounds, cystic fibrosis, ophthalmic traumas, and immunodeficient people. This bacteria can also cause infections in transplant recipients [3-5]. *P. aeruginosa* is widely distributed in hospitals and the most common causative agent of nosocomial infections among Gram-negative bacilli [6]. Therefore, the accurate and fast identification of *P. aeruginosa* is essential to control its infection and mortality rate. The conventional microbiological and biochemical method for *P. aeruginosa* detection and identification is a time-consuming procedure and cannot cope with the demands of clinical diagnosis. Furthermore, detection by immunological techniques requires complex procedures and it also takes a long time [7]. So, a DNA–based technique, polymerase chain reaction (PCR) is one of the efficient techniques for the rapid and specific identification of the pathogenic *P. aeruginosa* [8]. Due to the promising potential of PCR, the rapid identification of microbial species is made possible by simply amplifying sequences that are unique to this particular microorganism.

Two outer membrane proteins L and I lipoproteins are responsible for the intrinsic resistance of *P. aeruginosa* to antiseptics and antibiotics. These proteins are unique to *P. aeruginosa*, so detection of these proteins aid in the rapid identification of this bacteria in clinical samples. The most abundant lipoprotein *oprL* (outer membrane protein) and the small *oprI* maintain the cell integrity of *P. aeruginosa* by forming interactions with peptidoglycan [9]. *oprL* also protects cells from oxidative stress [10]. Both *oprL* and *oprI* contribute to antibiotic resistance mechanisms in *P. aeruginosa* by altering the membrane permeability and efflux mechanisms [11]. They also interact with other membrane porins like *oprF* to contribute to outer membrane vesicle (OMVs) biogenesis mechanisms which are essential components of biofilm matrixes [12].

Furthermore, exotoxin A (ETA) is the most toxic virulence factor of *P. aeruginosa*. Exotoxin A is an ADP-ribosyl transferase, it transfers an ADP-ribosyl to elongation factor 2, leading to protein synthesis inhibition in mammalian cells [13]. The ways of secretion of exotoxins A are either active or passive, actively secreted by three types of secretion systems involving type 1, type 2 and type 3 secretion systems; or passively secreted through the cell [14]. *toxA* (Exotoxin A) gene is specific and conserved for *P. aeruginosa* species and it is absent in other species of Pseudomonas. The *toxA* is a well-characterized gene and it contributes to the pathogenicity of *P. aeruginosa*, this is because the deletion of the *toxA* gene from the strains of this species resulted in decreased pathogenicity than parental strains [15, 16]. Exotoxin A alone or in combination with other hydrolases cause cell death, severe damage to tissues leading to tissue necrosis and can also cause resistance to antibiotics [17, 18].

The major issue in the detection of *P. aeruginosa* through PCR is that usually only one gene is targeted and this is insufficient for reliable and extensive diagnosis [19]. There is a high diversity in the genotype of *P. aeruginosa* strains [20], and through various studies, it is evident that one or more than one; virulence gene/s are not found in some strains [21]. So, the detection of more than one virulence gene in *P. aeruginosa* isolates can increase the confidence of correct detection and can minimize the risk of false positives. Also, these microorganisms exist in natural sources like; soil and water as well as in animals. Humans consume many animal-related foods and hence there is a risk of the transfer of *P. aeruginosa* related infections [22]. Also, the presence of *toxA* and *oprL* genes show different intensities of the virulence and pathogenicity characteristics in *P. aeruginosa* isolates [23]. Therefore, the purpose of this study was to determine the prevalence of *oprL* and *toxA* virulence genes in *P. aeruginosa* isolates taken from animal and human sources and to evaluate the frequency of antimicrobial resistance in *P. aeruginosa* isolates.

Materials and Methods

Sample collection

Total 120 samples were used for this study, and samples were collected in Ardabil city, Iran, between May 2016 and April 2018. From 120 samples, 60 samples were cow milk sources; which are obtained from the veterinary microbiology laboratory, and the remaining 60 were obtained from human clinical samples including wound, urine, blood, and sputum that are collected from hospital laboratories (Emam Reza, Alvi Ardabil Educational and Medical Hospital, Imam Khomeini). In this descriptive cross-sectional study, based on previous studies and 95% confidence level, 60 samples were intended for each animal and human sources (Totaly 120 samples). This study aims to evaluate the presence of

Table 1. Primer sequences used in PCR assays for virulence genes detection in *P. aeruginosa* strains

Gene	Primer	Nucleotide Sequence (5' →3')	Amplicon Size (base pair)
toxA	Forward	GACAACGCCCTCAGCATCACCA	397
	Reverse	CGCTGGCCCATTCGCTCCAGCG	397
oprL	Forward	ATG GAAATGCTGAAATTCGGC	504
	Reverse	CTTCTTCAGCTCGACGCGACG	504

toxA and *oprL* genes in *P. aeruginosa* isolates isolated from different samples (i.e., cow milk samples and human clinical samples), hence equal number of samples were selected for the assessment.

Isolation and identification of the *P. aeruginosa* from samples

The obtained samples were cultured on MacConkey (MAC) media (HiMedia, India) and Eosin Methylene Blue (EMB) agar (HiMedia, India), incubated overnight at 37 °C [24]. After incubation, plates were examined for bacterial growth and colony formation. Isolation of *P. aeruginosa* was performed with the help of gram staining techniques and various biochemical tests including oxidase, catalase, SIM (sulfide, indole, motility), TSI (triple sugar iron), MR/VP (methyl red/voges-proskauer), ODC (ornithine decarboxylase), SCA (simmons citrate agar), AD (arginine dehydrogenase), LD (lysine decarboxylation), OF (oxidation-fermentation) test (Merck, Germany) [25]. After microbiological and biochemical evaluation, 76 isolates of *P. aeruginosa* were detected and identified, which were further used for subsequent tests. Isolates were identified by conventional methods by standard laboratory techniques like; gram-staining and biochemical tests. Also, an automatic method like; VITEK 2 (bioMérieux) that uses fluorescence-based technology, was used for the rapid identification and susceptibility testing [26].

Antibiotic susceptibility test

Antibiotic susceptibility testing in Müller-Hinton agar medium was examined according to CLSI (Clinical Laboratory Standards Institute) guidelines, by using Kirby-Bauer's standard disk diffusion methods. CLSI guidelines related to testing of multidrug-resistant *P. aeruginosa* were followed and based on that different types of antibiotics were selected which include, beta-lactam, cephalosporin antibiotics and antibiotics which have inhibiting mechanisms for beta-lactamase, DNA, protein synthesis etc were used. The following antibiotic discs (Padtanteb, Co) were used: Levofloxacin (5ug), Ofloxacin (5mg), Ticarcillin (75ug), Cefepime (30ug), Norfloxacin (10ug), [27, 28]. As a control, *P. aeruginosa* ATCC27853 strain was used. By comparing results with CLSI document (M100), P. aeuruginosa isolates were evaluated for the respective antibiotics, as susceptible (S), resistant (R), and intermediate (I) accordingly [29].

DNA extraction

DNA was extracted from all *P. aeruginosa* isolates by using a commercial DNA extraction kit (Cinna Gen. Co), according to the manufacturer's protocol. Extracted DNA was examined by running DNA samples in 1% agarose gel electrophoresis then stained with ethidium bromide and observed under UV transilluminator [30].

DNA estimation

DNA estimation was performed by NanoDrop at 260/280nm and then preserved at -20˚C until used for further experiments [30].

PCR assay

PCR assay was performed for detecting virulence genes, *toxA* and *oprL*, in *P. aeruginosa* isolates, by using specific primers (Bioron Co, Germany). 0.5ul of the selected primers (with a concentration of 10 picomoles), and 2ul (100ng) DNA templates were used for this assay. Desired genes of *toxA* and *oprL* were amplified by PCR using thermal cycler (Table 1). The conditions of PCR amplification were set as follows: initial denaturation at 95°C for 15 minutes, 35 cycles, denaturation at 95°C for 1min, annealing at 60°C for 45sec, primer extension at 72°C for 1min and final extension at 72°C for 10 min. Multiple water samples were used as the negative controls [30].

Table 2. Antibiotic susceptibility of *P. aeruginosa* isolates

Antibiotic	No. (%)		
	Resistant	Sensitive	Intermediate
Levofloxacin (5ug)	69(90.78)	3(3.94)	4(5.26)
ofloxacin (5 mg)	61(80.26)	3(2.64)	13(17.11)
Ticarcillin (75ug)	74(97.36)	0	2(2.64)
Cefepime (30ug)	68(89.47)	7(9.21)	1(1.31)
Norfloxacin (10ug)	71(93.42)	4(5.26)	1(1.31)

PCR products analysis

PCR products were examined by performing 1% agarose gel electrophoresis, then stained with ethidium bromide and observed under UV transilluminator [30].

Results

In this study, 120 samples were collected from which 60 samples were collected from cow milk and the remaining 60 were from human clinical samples. From all these samples, total 76 isolates of *P. aeruginosa* were isolated and identified with the help of microbiological and biochemical characterizations. Among total 76 numbers of isolates, 24(40%) identified isolates were obtained from animal milk samples and 52(86.66%) isolates were identified from human clinical samples.

Prevalence of antibiotic resistance among P. aeruginosa isolates

The antibiotic susceptibility tests were conducted for all *P. aeruginosa* isolates. 5 antibiotics were used for susceptibility testing and the resistant strains are found in the range of 80-98% against all tested antibiotics. The maximum resistance was found towards ticarcillin (97.36%) and the minimum resistance is found towards ofloxacin (80.26%). Overall, the isolates of *P. aeruginosa* were found to be multi-drug resistant (MDR) (Table 2).

Distribution of *toxA* and *oprL* gene among *P. aeruginosa* isolates:

The detection of *toxA* gene among *P. aeruginosa* isolates was performed through PCR. The results obtained shows that *toxA* gene is 75% distributed in animal isolates and 84.61% in human isolates. The *oprL* virulence gene of *P. aeruginosa* was detected through PCR. The distribution of *oprL* gene is 54.16% in animal isolates and 80.76% in human isolates. The coexistence of both *toxA* and *oprL* virulence genes were detected among *P. aeruginosa* isolates through PCR. The distribution of co-existed *toxA* and *oprL* genes is 37.50% in animal isolates and 40.38% in human isolates (Table 3).

Discussion

P. aeruginosa is an opportunistic bacteria that completes its infection by attaching, colonizing, invading locally and spreading as a systemic disease [31]. *P. aeruginosa* is at top of the list among pathogens causing nosocomial infections. It mostly causes severe infections in humans and its treatment is quite challenging as this organism has intrinsic resistance to various classes of drugs (multidrug resistance; MDR) and is also capable of acquiring resistance to all effective antibiotics. For many years, *P. aeruginosa* is known for its significant contribution to surgical site infection throughout the world and these infections rank third in the most com-

Table 3. Distribution of *toxA* and *oprL* genes in animal and human *P. aeruginosa* isolates

Sample Source	Total Isolates	No. (%)		
		Positive for *toxA*	Positive for *oprL*	Positive for Coexistence *toxA* and *oprL* Genes
Animal	24	18 (75.0)	13(54.16)	9(37.50)
Human	52	44(84.61)	42(80.76)	21(40.38)

monly occurring hospital-acquired infection [32]. *P. aeruginosa* cause infections at a higher rate in burn patients than other patients. The absence of intact skin barrier and suppressed immune system in such patients, contribute to patients' extended stay in hospital. Also, invasive procedures for therapy and diagnosis make them vulnerable to infections [33]. *P. aeruginosa* have a group of virulence factors that exhaust the defence system of the host and directly harm host tissues. The crucially important virulence factors of *P. aeruginosa* are exotoxin A, outer membrane proteins (*oprL* and oprI) and quorum-sensing determinants [34]. Exotoxin A is the main weapon of *P. aeruginosa* against the host defence system [35]. Machado et al., used a proteomics approach to explain the adaptive resistance in *P. aeruginosa* isolates and found that adaptive resistance mechanisms against antibiotics involved membrane alterations [35].

oprL and *oprI* serve as the connecting bridge between outer membranes to the peptidoglycan of bacteria. Lipoprotein constituent of *oprL* mainly involves, ejection transporting mechanisms; that leads to disturbance of cell penetrability [36]. The expressions of key membrane-related proteins like; *oprL*, *oprI* and porins like; *oprF* and oprG, found to be down-regulated during higher concentrations of benzalkonium chloride (BC). This down-regulation of the expression of these genes suggested their potential role in the development of resistance mechanism against antibiotics [37].

Amirmozafari et al., studied the association between the prevalence of exotoxin A and exoenzyme S in antibiotic resistance in *P. aeruginosa* isolates [36]. Razaqq et al. study showed that antibiotics like Gentamicin result in induction of *toxA* genes and the development of antibiotic resistance in *P. aeruginosa* isolates obtained from Cow infected mastitis [37]. A study showed that *P. aeruginosa* strain with exotoxin A showed high levels of antibiotic resistance indicating the potential role of *toxA* gene as mediator in exotoxinA (ETA) activity and development of antibiotic resistance [38]. Hence researchers have suggested that exotoxinA can be used as a potential target for developing vaccines [38]. It was reported that highly virulent ETA toxin can be produced by 95% of *P. aeruginosa* clinical isolates [39]. A genetic study of the *toxA* gene isolated from burn wound infections revealed the capability of the *toxA* gene to undergo mutations (by transversion and transitions). While some other studies revealed that the *toxA* gene can undergo point mutations to change the amino acid sequence, frameshift mutations, deletions as well as insertions. Hence, monitoring and detection of this virulence gene in *P. aeruginosa* is important and presence of heavy metals in environments and antiseptics and use of antibiotics contribute in development of resistance in *P. aeruginosa* [40, 41]. Gharieb et al. studied *P. aeruginosa* isolates from different sources like; animals, humans and farm environments [41]. This study showed that human isolates showed more antibiotic resistances than non-human *P. aeruginosa* isolates. Hence study supported the potential role of antibiotic drugs consumption in humans is responsible for the development of multi-drug resistance in *P. aeruginosa* isolates from humans than that of in livestock [42].

A molecular study about the virulence genes like; *oprL* and *toxA* in *P. aeruginosa* isolates from Sea Bream Fish, revealed that these genes can be used for the rapid and specific identification of these microorganisms for this type of fish food. Further analysis for the fluoroquinolone antibiotics resistance revealed the presence of multidrug resistance in animal food is a concerning issue [43]. Mohammed et al. studied the prevalence of different virulence genes like [43]; *oprL*, *toxA*, ExoU, ExoS, rhlR, lasI, and ecfX in *P. aeruginosa* isolates obtained from raw milk samples. Out of the total 6 strains of *P. aeruginosa* obtained, all strains harbored both genes *oprL* and *toxA*. Hence, they suggested a need to monitor and improve the quality of raw milk sold in markets, since it is associated with public health [44]. Neamah et al. studied virulence genes *P. aeruginosa* isolates isolated from human and animal samples from Diwaniya province [44]. The study revealed the presence of different virulence genes like; *oprL*, oprI, *toxA* and exoS. But *toxA* gene showed a higher prevalence in their samples related to wound and burn infections, otitis media inflammation and milk samples than the rest of the genes [45].

The results obtained through PCR manifest that in animal isolates, *toxA* gene alone is 75% distributed and *oprL* gene alone is 54.16%. Whereas in human isolates, the distribution of *toxA* gene alone is 84.61% and *oprL* gene is 80.76% distributed alone. This manifests that the *toxA* gene is more widely distributed in both animal and human isolates than the *oprL* gene. Chand et al. reported the presence of the *toxA* virulence gene at a higher frequency of 89% among *P. aeruginosa* isolates from burn patients [46]. The results also revealed that both *toxA* and *oprL* genes are extensively distributed in human isolates as compared to animal isolates. Furthermore, the distribution of coexisted *toxA* and *oprL* genes is found at 37.50% in animal isolates and 40.32% in human isolates. The detection of both *toxA* and *oprL* genes simultaneously can decrease the risk of false detection of *P. aeruginosa* isolates and can increase the probability of correct identification of these isolates.

A cross-sectional study in the government hospital of Nepal showed that, *P. aeruginosa* isolated obtained from different clinical specimens harbored *toxA* and *oprL* genes by 95.40% and 100% and showed a high level of antibiotic resistance against conventional antibiotics [47]. While in another study prevalence of *toxA* and *oprL* genes was found at 63.33% and 100%, in different human clinical samples. The study showed *toxA* gene has a higher presence in burn and pulmonary tract related infections than from blood samples [48]. In our study, the prevalence of the *toxA* gene was found to be 84.61%, while *oprL* by 80.76%. These differences in the levels of virulence genes patterns in different human populations suggest that it might be associated with the variances in the geographical and environmental conditions. This might be associated with the better adaptation capability of some of the *P. aeruginosa* strains to become more infectious in particular conditions. The main factors that contributed to the prevalence of *P. aeruginosa* and the dissemination of its virulence genes are nature of area, extent and type of contamination, level of immunity of individual patients and virulent strains [27].

In this study, the resistant strains *P. aeruginosa* are found in the range of 80%-98% against all tested antibiotics. The strains are most resistant towards ticarcillin (97.36%) and minimum resistance was found to be against antibiotic ofloxacin (80.26%). Overall, all the isolates of *P. aeruginosa* are found to be multidrug-resistant (MDR) in this study. Developing countries are facing great challenge of the emergence of multidrug-resistant *P. aeruginosa* in health care centers [49]. The major route of acquired resistance is the horizontal gene transfer of antibiotic-resistant genes across bacteria. In addition, the loss and gain of functional genes are also responsible for the rapid development of resistance by bacteria [50]. MDR isolates of *P. aeruginosa* are related to high mortality and costs because of extended hospitalization, surgery and treatment with antibiotics for a long period [51]. Moreover, the development of acquired resistance in previously susceptible isolates during an anti-pseudomonal therapy is a serious problem and the emerging MDR strains of *P. aeruginosa* make it difficult to treat the infections [52]. The inappropriate and unnecessary extensive use of antibiotics can select resistant Pseudomonas isolates for multiplication. *P. aeruginosa* resistance to routinely used antibiotics has been increasing and it has reached an alarming situation [53]. The mechanisms of resistance adapted by *P. aeruginosa* against these antibiotics are including; inactivation enzymes, efflux pumps, low permeable membrane and formation of biofilm [54]. Various strains of *P. aeruginosa* can produce many classes of Extended-spectrum beta-lactamase (ESBL) which can manifest high resistance against different antibiotics [55]. The ESBL and Metallo β-lactamase (MBL) mediated mechanisms of resistance are crucially important in the emerging resistance of *P. aeruginosa*. The strains of *P. aeruginosa* producing ESBLs and MBLs have been reported worldwide [56].

An opportunistic pathogen, *P. aeruginosa* is widely distributed in natural environments, animals and human populations and its inherent capability to acquire multidrug resistance with the help of different virulence genes, makes this pathogen a major health concern. This study suggests that simultaneous use of *oprL* and *toxA* genes can increase the confidence in the detection of *P. aeruginosa* by PCR analysis. This study revealed the high resistance of *P. aeruginosa* isolates, from both animal and human sources, and the level of resistance towards commonly used antibiotics has been increased to a dangerous level. Hence study supports the need for the development of preventive measures to stop the zoonotic transfer of this pathogen to humans through animal-related foods or during livestock management. However, further studies are required to find out the other virulence factors which are contributing to drug resistance, in the infectious *P. aeruginosa* isolates obtained from both animals and human clinical samples.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article.

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Conflict of interest

The author declared no conflict of interest.

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