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Original Article

Antimicrobial Resistance Profiles and Virulence Genes of *Pseudomonas aeruginosa* Isolates Originated from Hospitalized Patients in Shiraz, Iran

Maryam Motevasel[1], Masoud Haghkhah[1*]

[1]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

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Introduction: Multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates are among the common cause of Nosocomial infections. In *P. aeruginosa* infections, several genes, *mex*A, and *mex*B are involved in resistance to antibiotics and *psl*A, *pel*A and *brl*R contribute to biofilm formation. This study aims to investigate the prevalence of these genes in *P. aeruginosa* isolates and to determine their relationship with biofilm formation, antibiotic resistant, pigment production, and source of infection. **Methods:** We collected 63 specimens out of 90 samples from patients hospitalized in a hospital affiliated to Shiraz University of Medical Sciences. The specimens belonged to 42 men and 21 women and included urine, sputum, wound, skin, blood, body fluid, and central venous blood (CVB). The samples were cultured on solid media and diagnosed according to standard phenotypic characteristics. Disk diffusion method was used to identify the clinical MDR *P. aeruginosa* isolates, and the genes *psl*A, *pel*A, *brl*R, *mex*A, and *men* were detected by PCR. **Results:** about 25.4% of the clinical isolates were MDR, *i.e.*, resistant to three or more antibiotics. The prevalence of the genes in the clinical isolates was as follows: *psl*A (92.1%), *pel*A (68.3%), *brl*R (93.7%), *mex*A (95.2%) and *mex*B (50.8%). The highest and lowest prevalence of drug resistance belonged to ceftriaxone and amikacin, respectively. The highest MDR *P. aeruginosa* isolates originated from wound, urine and sputum specimens. **Conclusion:** The presence of MDR isolates correlated significantly with the patients' gender, the origin of specimens, and bacterial pigment production. In this study, the detected genes did not significantly correlate with the MDR features of the isolates. *J Med Microbiol Infec Dis, 2018, 6 (2-3): 72-76*.

Keywords: *Pseudomonas aeruginosa*, Multidrug resistance, Biofilm genes, Shiraz, Iran.

INTRODUCTION

Pseudomonas aeruginosa is a non-fermenter Gram-negative rod-shaped bacterium. This bacterium is the most critical human opportunistic pathogen among the causative agents of nosocomial infections, and cause infections in urinary and respiratory tracts, soft tissues, bones, and joints [1].

Treatment of *P. aeruginosa* infections by conventional antibiotics is often challenging due to the development of antibiotic resistance. The bacterium is naturally resistant to narrow-spectrum penicillins, first and second-generation cephalosporins, sulfonamides, and trimethoprim. Resistance against antibiotics is mediated through different mechanisms including impermeability to antibiotics, the efflux pump system, or the production of metallo-beta-lactamases (MBLs) [2]. Presence of the transcriptional regulator *brl*R (biofilm resistance locus regulator) is one of the contributing factors to drug resistance in *P. aeruginosa* isolates [3]. Another mechanism that affects the tolerance of *P. aeruginosa* against antimicrobial agents is a multidrug efflux pump encoded by *mex*AB-*opr*M. The cytoplasmic proteins like MexA and MexB and outer membrane proteins like OprM are categorized as non-ATPase efflux pumps.

MexA is a lipoprotein and a member of the membrane fusion proteins (MFP). The family MexB pumps the antibiotics out of the cell by proton motive force manner. These genes encoding these proteins are involved in the efflux of the drugs across the cytoplasmic membrane and out of the cell [4].

An extracellular matrix consisting of polysaccharides, proteins, nucleic acids, and lipids constitute the biofilm of *Pseudomonads* bacteria. The biofilm function is the attachment to the surface and other cells and protection of the cells from antimicrobials and the host immune system [5, 6].

Attachment of bacteria to the damaged tissues or medical devices like catheter may lead to biofilm formation, which is commonly resistant to antibiotics. In biofilms, the mechanisms of drug resistance depend on multicellular interactions. These communities are the primary cause of many persistent bacterial infections. Within biofilms, bacteria are protected from destructive effects of chemicals and become less sensitive to antibiotics than the similar planktonic forms [7, 5, 8].

[*]**Correspondence:** Masoud Haghkhah
Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran, 7144169155.

Email: mhaghkha@shirazu.ac.ir

Tel: +98 (71) 32286944 **Fax:** +98 (71) 32286940

The *psl* locus is necessary for synthesis and export of polysaccharide [9] and plays a role in biofilm formation at surfaces of the liquid cultures [10]. The primary role of *pel* and *psl* in biofilm structure is maintaining biofilm integrity [1].

This study aims to investigate the prevalence of multidrug-resistant (MDR) *P. aeruginosa* isolates in hospitalized patients and to determine the association of antibiotic resistance in the isolates with several factors including the genes related to the biofilm formation, bacterial pigmentation, the source of infection, and patients' gender.

MATERIAL AND METHODS

Bacterial isolates and culture conditions. We obtained 63 out of 90 clinical isolates of *P. aeruginosa* from patients hospitalized in Shahid Faghihi hospital, affiliated to Shiraz University of Medical Sciences of Iran, between 2015 and 2016. The specimens belonged to 42 men (66.7%), and 21 women (33.3%). The specimens included urine, wound, skin, sputum, body fluid, and blood. The clinical bacterial isolates were cultured on blood agar and MacConkey agar and incubated at 37ºC for 24 hr. The isolates identified by colony morphology, gram staining, and standard biochemical tests, *e.g.*, catalase and oxidase tests, the production of pigments, bacterial motility, glucose fermentation and lactose non-fermentation in the TSI medium, and oxidative in the OF medium [11]. The identity of the biochemically identified *P. aeruginosa* isolates was confirmed by a standard PCR using the species-specific primers that targeted 16S ribosomal DNA (rDNA) sequence [12].

Detection of the genes by PCR. The specific primers for detection of *psl*A (potential exopolysaccharide locus), *pel*A (pellicle locus), *brl*R (biofilm resistance locus regulator), *mex*A&B (multidrug efflux pump) genes were designed in this study (Table 1). The strain *P. aeruginosa* ATCC 27853 and a clinical imipenem resistant isolate of *P. aeruginosa* were used as positive controls. For DNA extraction, the bacterial isolates were cultured on the blood agar and incubated at 37°C for 24 h. Two colonies of bacteria were separately suspended in 200 µl distilled water, centrifuged at 6000 g for 3 sec, and incubated at 95°C in a dry bath incubator (MD-0.2, MS Major Science, Taiwan, Licensed America) for 15 min. The suspensions were exposed to freezing temperature for 10 min and then centrifuged at 13,000g for 10 min. The supernatants containing the DNA were used for PCR assays.

Amplification of genes were carried out in 25µl reaction volumes containing 10x PCR buffer (200 mM Tris/ HCl [pH 8.8 at 25°C], 500 mM KCl), 50 mM $MgCl_2$, 2.5 mM dNTPs, 0.4 pmol/ µl of each primer, 0.2 µl *Taq* DNA polymerase, and 3 µl template DNA.The amplification was programmed for an initial denaturation at 94°C for 5 min , followed by 35 cycles of 94°C for 30s, 52°C for 40 s (except for *mex*A&B gene which was adjusted to 53°C for 30 s), 72°C for 50 s, and a final extension at 72°C for 10 min. The PCR products were resolved on a 1.5% agarose gel for 120 min at 95 V, stained with safe stain, and visualized by a gel documentation instrument (UVI tec, BTS-20 M, England).

Antibiotic susceptibility test. The antibiotic susceptibility pattern of *P. aeruginosa* clinical isolates along *P. aeruginosa* ATCC 27853 as a positive control was evaluated by disk diffusion method according to CLSI guidelines [13]. The tests were performed using ciprofloxacin (5 µg), imipenem (10 µg), gentamicin (10 µg), amikacin (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg) and cefepime (30 µg) disks (PadtanTeb, Iran).

Statistical methods. Distributions and relationship between MDR *P. aeruginosa* and variables such as the genes, specimens, pigment production, and the patients' gender were evaluated by SPSS software, version 16, using Chi-square and Fisher's exact tests at the significance level of $P \leq 0.05$.

RESULTS

Clinical Isolates. In this study, 63 clinical isolates of *P. aeruginosa* were from various specimens including urine (36.5%), sputum (23.8%), wounds (15.9%), skin (7.9%), body fluid (6.3%), blood (6.3%), and CVB (3.2%). The PCR with species-specific 16S rDNA primers yielded a 956 bp band confirming the identity of all isolated as *P. aeruginosa*.

Prevalence of multidrug resistance (MDR) in the clinical isolates. The prevalence of antibiotic resistance among the isolates was as follows: ceftriaxone (50.8%), imipenem (42.9%) and ciprofloxacin (39.7%) (Table 2).

Table 1. Primers used for identification of *P. aeruginosa* isolates and their virulence factors genes

Target	Primer sequence (50–30)	Product size (bp)	Annealing temperature (8°C)	Reference
P. aeruginosa	F: GGGGGATCTTCGGACCTCA R: TCCTTAGAGTGCCCACCCG	956	58	[12]
***psl*A**	F:TGCCTGGAACATAATCACCGT R: GTCGGTAGATAGCCTGTCGC	202	52	This study
***pel*A**	F: GACTGGGTGGTGCTCGAAG R: GCGCTCCTCGGCCTGTAG	312	52	This study
***brl*R**	F:GCAACGACACCAGCACACC R:CGCAGATGCCATAGGAGACC	269	52	This study
***mex*-A**	F: CTGAAGCTGGAGGACGGTAG R:AGGCCTTCGGTAATGATCTTGT	356	52	This study
***mex*-B**	F: TGGGTGATCGCCTTGGTGA R: GGCCAGTTGCAGCTTGTTC	307	52	This study

Table 2. The frequency of antibiotic resistance among the *P. aeruginosa* isolates

Antibiotics	Number of resistance isolates (%)	Number of intermediate isolates (%)	Total, N (%)
CP	25 (39.7)	5 (7.9)	30 (47.6)
IPM	27 (42.9)	3 (4.8)	30 (47.7)
GM	10 (15.9)	2 (3.2)	12 (19.1)
AN	9 (14.3)	2 (3.2)	11 (17.5)
CAZ	12 (19)	4 (6.3)	16 (25.3)
CRO	32 (50.8)	24 (38.1)	56 (88.9)
FEP	10 (15.9)	4 (6.3)	14 (22.2)

CP: ciprofloxacin, IPM: imipenem, GM: gentamicin, AN: amikacin, CAZ: ceftazidime, CRO: ceftriaxone, FEP: cefepim

Table 3. MDR *P. aeruginosa* isolates in relation to type of specimen, the patients' gender and bacterial pigment production property

Variables	general	MDR		*P*-Value
		No	Yes	
Urine	23 (36.5)	19 (40.4%)	4 (25%)	0.002
Sputum	15 (23.8)	13 (27.7%)	2 (12.5%)	
Wound	10 (15.9)	2 (4.3%)	8 (50%)	
Skin	5 (7.9)	5 (10.6%)	0 (.0%)	
Body fluid	4 (6.3)	4 (8.5%)	0 (0%)	
Blood	4 (6.3)	3 (6.4%)	1 (6.2%)	
CVB	2 (3.2)	1 (2.1%)	1 (6.2%)	
Male	42 (66.7)	28 (59.6%)	14 (87.5%)	0.041
Female	21 (33.3)	19 (40.4%)	2 (12.5%)	
Pigment	No	2 (33.3%)	4 (66.7%)	0.032
	Yes	45 (78.9%)	12 (21.1%)	

MDR: multidrug resistant; CVB: central vein blood; N (Percentage)

Table 4. Frequency (percentage) of *psl*A, *pel*A, *brl*R, *mex*A and *mex*B genes among *P. aeruginosa* isolates

genes	No. (%)
***psl*A**	54 (92.1)
***pel*A**	41 (68.3)
***brl*R**	55 (93.7)
***mex*A**	56 (95.2)
***mex*B**	30 (50.8)

By inclusion of the intermediate patterns as resistant, the resistance prevalence increased to 88.9% for ceftriaxone, 47.7% for imipenem, and 47.6% for ciprofloxacin. The prevalence of multidrug resistance, *i.e.*, resistance to 3 or more antibiotic classes among the clinical isolates are shown in Table 3. The highest frequency of MDR isolates belonged to organisms originated from wounds (50.0%), urine (25.0%) and sputum (12.5%). There was a significant relationship between specimens and detection of MDR-*P. aeruginosa* isolates (*P*=0.002). Also, there was a significant correlation between pigment production and MDR character in the isolates (*P*= 0.032).

PCR detection of the genes in clinical isolates. PCR detected five genes in the clinical isolated with *mex*A exhibiting the highest frequency (95.2%) followed by *brl*R (93.7%), *psl*A (92.1%), *pel*A (68.3%) and *mex*B (50.8%) (Table 4).

DISCUSSION

The bacteria *P. aeruginosa* is an opportunistic nosocomial pathogen with a potential to develop multidrug resistance (MDR). Hospitalized patients are at high risk of acquiring this infection. The combinations fluoroquinolones, carbapenems, and aminoglycosides are the routine antibiotics prescribed for the treatment of *P. aeruginosa* infections. A study conducted by Obritsch and colleagues (2004) revealed that 32% of the isolates from intensive care unit patients were resistant to ciprofloxacin and 23% to imipenem [14]. In a similar study from Iran, 77.3% of clinical isolates from an intensive care unit showed resistance to ceftriaxone [15], which is in agreement with the results of the present study exhibiting the same prevalence of drug resistance for the tested antibiotic. In Germany, in pneumonia patients, the prevalence of resistance to gentamycin, amikacin, cefepime, and ceftazidime was reported as 22%, 9.7%, 16.6%, and 22.7%, respectively [16], but in the present study, the lowest resistance belonged to ceftazidime, followed by amikacin, gentamycin, and cefepime.

In the present study, most of the *P. aeruginosa* isolates were originated from urine specimens, but the MDR isolates were from the wounds reflecting the difficulty in treatment of the *P. aeruginosa*-wound infections compared to urinary tract infections. This research showed the highest resistance to antibiotics ceftriaxone, imipenem, and ciprofloxacin, while the resistance to amikacin was at the minimum level. Hence, administration of this combination might be the more useful for treatment for *P. aeruginosa* infections.

The MDR isolates identified in this study showed a positive correlation with the type of the specimen and bacterial pigment production (Table 3).

Two separate studies in Iran showed that 30.1% and 45.3% of the isolates were multi-drug resistant [17, 18]. Two other studies in the USA and Iran revealed the prevalence of MDR isolates ranged from 31.5% to 58.65% [19, 20]. Our results revealed a lower rate of MDR *P. aerugonosa* isolates. The difference between our study and previous ones is possibly due to the variation of the tested antibiotics and type of the specimens.

In the present study, 7 (11.1%) of MDR isolates were resistant to the all tested antibiotics and might be defined as extensively drug-resistant (XDR), *i.e.*, the organism with no susceptibility to none of the tested agent [21]

The variation in the distribution of all the five genes among the clinical isolates showed no correlation with the drug resistance (P= 0.7) and pigment production (P= 0.404) of the isolates. Our result and a previous study in Iran [20] indicated a high prevalence of *psl*A in the isolates. The difference between the prevalence of *pel* genes in this study and the other studies [20] might be attributed to the deployed primers.

We detected a significant correlation between pigment production and drug resistance with the type of infection (P=0.032). This finding suggests that pyocyanin, pyoverdin, and other present *pseudomonad* pigments may contribute to drug resistance and pathogenesis phenomenon. Also, the site of infections may have an essential role in the development of drug resistance.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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