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ORIGINAL ARTICLE

Immunological and molecular detection of biofilm formation and antibiotic resistance genes of *Pseudomonas aeruginosa* isolated from urinary tract

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ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa (P. aeruginosa)* is one of the most common causes of hospital-acquired infections. It is associated with high morbidity and healthcare costs, especially when appropriate antibiotic treatment is delayed. Antibiotic selection for patients with *P. aeruginosa* infections is challenging due to the bacteria's inherent resistance to many commercially available antibiotics. This study investigated antibiotic-resistance genes in isolated bacteria, which play a key role in disease pathogenesis.

Materials and Methods: 100 samples out of the 140 samples collected from urinary tract infections (UTIs) cases between December 15th, 2022, and April 15th, 2023, were included in the study. Identification of bacterial isolates was based on colony morphology, microscopic examination, biochemical tests, and the Vitek-2 system. Antibiotic resistance genes; *Aph(3)-llla, ParC, Tet/tet(M),* and *aac(6´)-Ib-cr* were tested by polymerase chain reaction (PCR).

Results: The obtained results were based on bacterial identifications of 81 clinical samples. Only 26 (32%) of these isolates were *P. aeruginosa,* 21 (26%) were *Escherichia coli*, and 18 (22.2%) were other bacteria. These isolates were used to detect four genes including *tet(M), Aph(3)-llla, Par-c,* and *aac(6´)-Ib-cr.* Four types of primers were used for PCR detection. The results showed that 11/14 (78.57%) carried the *tet(M)* gene, 10/14 (71.42%) carried the *Aph(3)-llla* gene, 14/14 (100%) carried the *Par-c* gene, and 10/14 (71.42%) of the isolates carried the *aac(6´)-Ib-cr* gene. The biofilm formation examining the *esp* gene, showed that 9 (64.28) isolates carried this gene.

Conclusion: The inability of antibiotics to penetrate biofilms is an important factor contributing to the antibiotic tolerance of bacterial biofilms.

Keywords: *Pseudomonas aeruginosa*; *Pseudomonas* infections; Antibiotic resistance; Biofilms

INTRODUCTION

Pseudomonas aeruginosa is a non-spore-forming, non-fermentative Gram-negative bacilli belonging to the *pseudomonadaceae* family. *Pseudomonas* cells possess a single polar flagellum (1). *P. aeruginosa* has been found to cause a variety of infections in clinical practice besides respiratory infections, including common acute septicemia from burn or surgical wound infection, urinary tract infection, septi-

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cemia and endocarditis (2).

Antibiotics are specific chemical compounds derived from or produced by microorganisms that even in small amounts can selectively inhibit the growth or life processes of the microorganisms (3). Antibiotic resistance in pathogens is increasing worldwide in both outpatients as well as hospitalized patients, which are considered as a focus of infection. These resistances can be acquired by mutation or by the acquisition of resistance genes from other organisms. Since the discovery of naturally occurring antibiotics from microbial sources, resistance has rapidly emerged, often soon after their introduction into clinical use (4). The most important mechanisms of action of antibiotics includes inhibition of cell wall remodeling, interference with protein synthesis, interference with nucleic acid synthesis and inhibition of metabolism (5). Some plasmids carry as many as six or seven genes that confer resistance to different antibiotics (6). Mutations occur spontaneously in most bacteria, and because of their high reproduction rate mutant strains will always arise. If a mutant strain is resistant to an antibiotic present in its environment, the antibiotic will select by inhibiting susceptible strains (7).

Antibiotic activity and resistance are influenced by some differences in the structure of Gram-positive and Gram-negative bacteria (8). Generally, Gram-negative bacteria develop four major mechanisms of resistance to antibiotic treatment: enzymatic modification of the antibiotic, outer membrane (OM) permeability, target modifications and efflux of antibiotics from bacteria (9).

The study aimed at investigating trends of urinary tract infections caused by Gram-negative bacteria. We analyzed the prevalence of four virulence factor genes in bacteria strains causing different diseases in patients. We also studied the antibacterial effect on biofilm, when exposed to antibiotics during logarithmic phase growth. The results were correlated with morphological changes in the bacteria following exposure to the antimicrobial agents (10). The present study aimed to survey the antibiotic resistance in Iraq.

MATERIALS AND METHODS

The study was conducted in the laboratories of Bacteriology and Molecular Sciences, College of Biotechnology, Al-Qasim Green University, Iraq. The current study protocol was reviewed and approved by the Ethics Committee of the Shahid Beheshti University, Tehran, Iran (IR.SBU.REC.1403.001).

Sample collection. This study included 140 samples collected from patients with urinary tract infections, who visited three major hospitals in Hilla: Hilla General Teaching Hospital, Babylon Maternity and Children Hospital, and Marjan Teaching Hospital during three months from December 15th, 2022 to April 15th, 2023. The ages of the patients ranged from 6 months to 60 years. The sexual distribution of the patients showed a higher frequency among females at 60 (52%) compared to males at 55 (48%).

Sample preparation. These samples were collected by appropriate methods to avoid any possible contamination. The samples were transported by sterile transfer swabs to the laboratory of the Bacteriology Department, and each sample was inoculated using the direct inoculation method on blood agar, MacConkey agar, and then incubated at 37°C under aerobic conditions for 18-24 hours (10).

Identification of bacteria. A single colony was isolated from each primary positive culture from blood agar and MacConkey agar and the cultivation was repeated to obtain a pure culture and then identified based on its morphological and cultural characteristics (hemolysis, lactose fermentation, colony shape, size, color, borders and texture) and the bacteria were smeared, stained with Gram stain and examined under a microscope (11). Biochemical tests were then performed for better identification. Final identification was performed with the automated VITEK-2 compact system using G-ve-ID cards.

Antibiotic profile: disc diffusion method. The antibiotic susceptibility of all *P. aeruginosa* isolates was performed according to the guidelines of the Clinical and Laboratory Standards Institute (12), using the disk diffusion method (Kirby-Bauer method). Single cells were suspended on a standard 0.5 McFarland tube and diffused on the of surface of Mueller Hinton agar. The test was performed using some commercially available antibiotic discs.

Total bacterial DNA extraction. Total bacterial DNA extraction was performed using the Genomic DNA Extraction kit.

PCR amplification and gel electrophoresis. Total extracted genomic DNA of all samples were subjected to PCR to amplify selected antibiotic genes. The specific primers and PCR conditions used are shown in Tables 1 and 2, respectively. Then gel electrophoresis was done on PCR products using 1.5% agarose.

Statistical analysis. The data were analyzed using Microsoft Excel computerized programs 19.

RESULTS

Description of study samples. As shown in Fig. 1, out of 100 UTI samples included in our study, 81 samples (81%) had positive culture and 19 samples (19%) had negative results.

Identification of bacteria specimens. The initial identification of bacterial specimens depended on some criteria including Gram staining, cultural, morphology and biochemical tests. The final identification was performed with the automated Vitek-2 compact system using GN-ID cards which contained 64 biochemical tests and one negative control. Exactly 81 isolates were identified and confirmed by vitek-2 system by (GN-ID cards) to be Gram-negative bacteria.

Out of 81 positive clinical samples, only 26 (32 %) isolates belonged to *P. aeruginosa*, 21 (26%) isolates were *Escherichia coli*, 18 (22.2%) isolates were *Klebsiella pneumonia* and 16 (19.8%) were *Proteus mirabilis.*

In biochemical tests it appeared that 26 (32%) of the isolates were *P. aeruginosa* and it appeared that 21 (26%) of the isolates were *E. coli*, also 18 (22.2%) of the isolated were *K. pneumonia*, and 16 (19.8%) of isolated were *P. aeruginosa* as shown Table 3.

Antibiotic resistance test. The susceptibility of 26 *P. aeruginosa* isolates against 9 selected antibiotics was studied to determine the pattern of isolates' sensi-

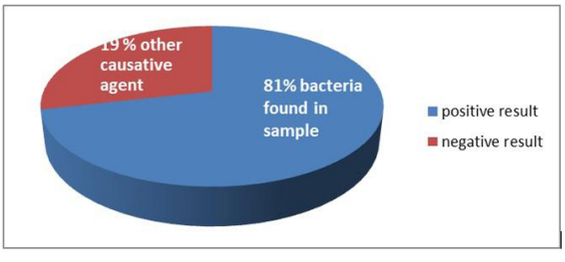

Fig. 1. The frequencies of bacteria isolated from 100 specimens collected from urinary tract infections

Table 1. The primer used in this study

Target Gene	Sequence	Bp	Reference
Aph(3)-llla	F 5′-GGCTAAAATGAGAATATCACCGG -3′ R 5′- CTTTAAAAAATCATACAGCTCGCG -3′	523	14
ParC	F 5′- TGTATGCGATGTCTGAACTG -3′ R 5′- CTCAATAGCAGCTCGGAATA -3′	264	15
Tet/tet(M)	F 5′- GAACTGTATCCTAATGTGTG -3′ R 5′- GATACTCTAACCGAATCTTCG -3′	377	16
aac(6´)-Ib-cr	F 5′-TTGCGATGCTCTATGAGTGGCTA -3′ R 3′-CTCGAATGCCTGGCGTGTTT-5′	490	17

Table 2. PCR Conditions for amplification of selected genes

Gene Name	Temperature (°C) / Time					Cycles Number
	Initial	Cycling Conditions			Final	
	Denaturation	Denaturation	Annealing	Extension	Extension	
Aph(3)-llla	95°C/5 min	95°C/30 std	57.8°C/30 sec	72°C/60 sec	72°C/5 min	30 cycles
ParC	95°C/5 min	95°C/30 std	55.2°C/30 sec	72°C/30 sec	72°C/5 min	30 cycles
Tet/tet(M)	95°C/5 min	95°C/30 std	54.4°C/30 sec	72°C/40 sec	72°C/5 min	30 cycles
aac(6´)-Ib-cr	94°C/4 min	94°C/45 std	55˚C/45 std	72°C/45 std	72°C/5 min	40 cycles

Table 3. Scheme of method read of the result of conventional biochemical test

Test Result	Oxidase	Catalase	Urease	Citrate	VP	MR	Motility	Kliglar iron agar	H2S	Indole
E. coli	-	+	-	-	-	+	+	A/A/-		+
Klebsella ssp	-	+	Late+	+	+	+	-	A/A/-		+
Pseudomonas ssp	+	+	-	+	+	+(v)	+	Ak/Ak/-		-
Proteus ssp	-	+	+	-/+	-	+	+	Ak/A/+		-

tivity to various antibiotics depending on the disk diffusion method. The results represent the antibiogram profile of the isolates, indicating that isolates varied in their susceptibility to the antibiotics. The results are shown in Table 4.

In the present study, all the tested isolates were resistant to a minimum of three classes of antibiotics to which they were tested. Therefore, the isolates were considered multidrug resistant.

The usage of antibiotics without antibiotic sensitivity testing is the most important factor promoting the emergence of multi-drug resistance which leads to the selection and dissemination of antibiotic-resistant pathogens in clinical medicine (13).

Molecular detection of bacterial isolates: detection of the *tet(M)* gene. All isolates were tested to detect the *tet(M)* gene, which encodes the enzymes responsible for catalysis od tetracycline antibiotics, using PCR technique with specific forward and reverse primers. The PCR results are shown in Fig. 2. 20 (76.9%) of the isolates were tested positive for the *tet(M)* gene in *P. aeruginosa.*

Detection of the *Aph(3)-llla* gene. *Aph(3)-llla* gene encoding aminoglycoside, which modifies (AMEs) enzymes, was identified in *P. aeruginosa* using PCR technique. Results are shown in Fig. 3, 18 (69.2%) of the isolates were tested positive for the *Aph(3)-llla* gene in *P. aeruginosa.*

Detection of the *Par-c* gene. *Par-c* gene, which encodes enzymes responsible for the catalysis of fluoroquinolone antibiotics, was identified using the PCR technique with specific forward and reverse primers. The PCR results are shown in Fig. 4. 26 (100%) of the isolates were tested positive for the *Par-c* gene in *P. aeruginosa.*

Detection of the *aac(6´)-Ib-cr* gene. *aac(6´)-Ib-cr* gene, which encodes for enzymes responsible for catalysis plasmid-Mediated quinolone resistance, was identified using PCR with specific forward and reverse primers. The PCR results are shown in Fig. 5. 18 (69.2%) of the isolates were tested positive for *aac(6´)-Ib-cr* gene in *P. aeruginosa.*

Table 4. Antibiotics sensitivity of bacteria isolated from different source

	Type of antibiotic	Type of bacteria *P. aeruginosa* (n=26) (%)
1	AK	100%
2	NOR	96.1%
3	CTX	30.7%
4	AMX	11.5%
5	TOB	92.3%
6	NA	76.9%
7	CN	92%
8	TE	3.8%
9	CIP	96.1%

AK; amikacin, NOR; norfloxacin, CTX; cefotaxime, AMX; amoxillin, Tob; tobramicin, NA; nalidixic acid, CN; gentamicin, Tet; tetracycline and CIP; ciprofloxacin

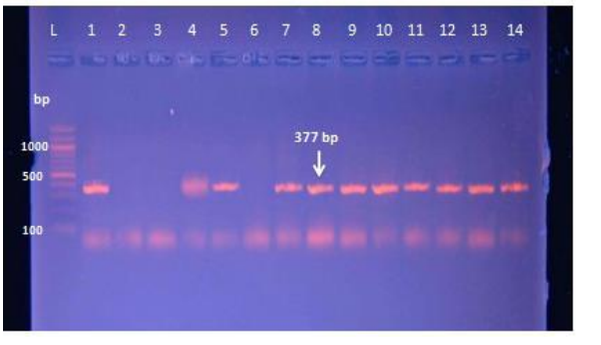

Fig. 2. Amplification of *tet(m)* gene *P. aeruginosa* with product length of 377 bp. Lane L: 100 ladder100. Lanes (1, 4, 5, 7, 8, 9,10, 11, 12, 13 and 14) show positive results for the *tet(m)* gene, Lanes (2,3 and 6) show negative results for the *tet(m)* gene. (1.5% agrose gel, 75 V, 1.20 hours).

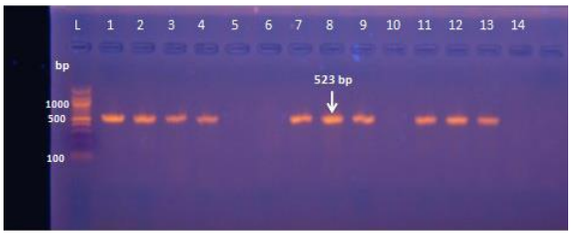

Fig. 3. Amplification of *Aph(3)-llla* gene in *P. aeruginosa* with product length of 523 bp . Lane L: DNA 100. Lanes (1, 2, 3, 4, 7, 8, 9, 11, 12 and 13) show positive results for the *Aph(3)-llla* gene, Lan*es (5,6, 10 and 14) show negative results for the* Aph(3)-llla gene. (1.5% agrose gel, 75 V, 1.20 hours).

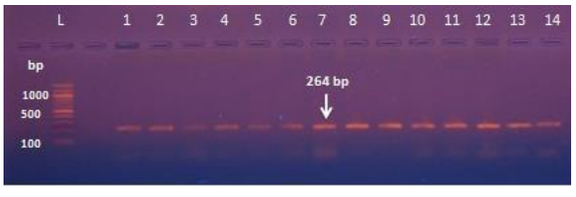

Fig. 4. Amplification of *Par-c* gene in *P. aeruginosa* with product length of 264 bp. Lane L: 100 ladder. All isolates show positive results for the *Par-c* gene. (1.5% agrose gel, 75 V, 1.20 hours).

Fig. 5. Amplification of *aac(6´)-Ib-cr* gene in *P. aeruginosa* with product length 490 bp. Lane L: 100 ladder. Lanes (1, 3, 4, 7, 9, 10, 11, 12, 13 and 14) show positive results for the *aac(6´)-Ib-cr* gene, Lanes (2, 5, 6 and 8) show negative results for the *aac(6´)-Ib-cr* gene. (1.5% agrose gel, 75 V , 1.20 hours).

In tetracycline resistant *P. mirabilis*, genes carried on transposons and/or plasmids encoding transmembrane proteins that efflux the antibiotic by an energy dependent manner (14). Resistance to β-lactams may arise in *P. aeruginosa* as a result of mutations in genes encoding PBPs that reduce the affinity of PBPs for the antibiotics. It was pointed out that the multidrug resistance is mostly due to antibiotics resistance genes which bear on transferable conjugative plasmid, transposons, integrons class 1 or on transconjugants carrying gene cassettes responsible for phenotypic multi antibiotic resistance (15).

DISCUSSION

In this study, 67.5% of aminoglycoside resistance isolates, harbored the *aph (3')- IIIa* gene as the most prevalent gene. Aminoglycosides by inhibiting bacterial protein synthesis show bactericidal activity. This group of antibiotics especially gentamycin and tobramycin in combination with beta-lactam or glycopeptides antibiotics have synergic effects on the treatment of *S. aureus* infection, particularly endocarditis (16). Resistance to aminoglycosides occurs mainly by drug inactivation via bacterial aminoglycoside modifying enzymes (AMEs) that are encoded by the genes located on plasmids or transposons (17). AMEs are classified into four groups according to the modification imposed on aminoglycoside antibiotics: acetyltransferases (AACs), phosphotransferases (APHs), nucleotidyltransferases (ANTs), and adenyltransferases. Another study performed by Tendolkar et al. (2005) aimed to localize the specific domain(s) of *esp* gen that plays a role in *esp*-mediated biofilm enhancement. It was reported by the researchers that an *Enterococcus faecalis* strain expressing only the N-terminal domain of *esp* fused to a heterologous protein anchor formed biofilms that were quantitatively similar to those formed by a strain expressing full-length (18).

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