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MOLECULAR STUDY OF ANTIBIOTIC RESISTANCE GENE IN *PSEUDOMONAS AERUGINOSA* FROM DIFFERENT SOURCE

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Abstract

A total of (100) specimens collected from patient suffeing from typhoid fever during the period from from 15, December , 2016 to 15, April, 2017. The identification of the bacterial isolates were depended on colonial morphology, microscopic examination ,biochemical tests and Vitek-2 system. The antibiotic genes was used such as (Aph(3)-llla, ParC, Tet/tet(M(, aac(6´)-Ib-cr , Esp). Rendering to the results obtained according to the morphological ,cultural and biochemical characters From the 81 clinical specimen only 26(32 %) isolates belong to *Ps. aeruginosa*, 21(26%) isolates were belonged to *E.coli* , 18(22.2%) isolates were *Klebsella pneumonia* and 16(19.8%) *Proteus mirabilis*. The study investigated the antibiotic resistance genes of bacterial isolated which cooperate a main role in pathogenicity .

Key words : *Salmonella typhi*, typhoid and antibiotic resistance genes.

Introduction

Pseudomonas aeruginosa is a non-spore forming, non-fermentative Gram negative bacilli belong to the *pseudomonadaceae* family .*Pseudomonas* cells possess a single polar flagellum (Kiska and Gilligan, 2003).

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, septicemia and endocarditis (DaSilva *et al*., 2004).

Antibiotics are specific chemical compounds derived from or produced by microorganisms that even in small amounts can selectively inhibit the growth of the life processes or growth of other microorganisms (Ibezim, 2005).

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 (Tenover, 2006).

The most important mechanisms action of antibiotic are includes inhibition of cell wall remodeling, interference with protein synthesis, interference with nucleic acid synthesis and inhibition metabolism (Fluit, 2001). Since the discovery of naturally occurring antibiotics from microbial sources, resistance has rapidly emerged, often soon after their introduction into clinical use began. Antibiotic activity and resistance is influenced by some differences the structure of Gram-positive and Gram-negative bacteria (Thomas, 2007).

The resistance of bacteria to an antibiotic means that bacteria are not susceptible to the action of the antibiotic. Some plasmids carry as many as six or seven genes that confer resistance to different antibiotics. (Cattoir *et al*., 2007). 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 which is present in its environment, the antibiotic will select for it by inhibiting susceptible strains (Black, 2002).

Generally, Gram-negative bacteria develop four major mechanisms of resistance to the antibiotic treatment : Enzymatic modification of the antibiotic , Outer membrane (OM) permeability, Target modifications and Efflux of antibiotics from bacteria (Livermore, 2003; Engelsen, 2209).

Materials and Methods

The study was done at Laboratories of Bacteriology and Molecular in Biology Department, Faculty of Sciences, University of Kufa, Iraq.

Samples collection and bacterial identification

Specimens

This study includes 80 specimens which were collected from patients suffering from urinary tract infections, diarrhea, inflammation bones and inflammation burns and wounds who submitted to three main hospitals in Hilla : Al-Hilla General Teaching Hospital, Babylon Hospital for Maternity and Pediatric and Mergan Teaching Hospital city during a period of three months (from 15, December , 2016 to 15, April, 2017). The patient's age ranged from (6 months-70 years).

Collection of specimens

The proper specimens collected for bacteriological analysis are described below. Those specimens were collected in proper ways to avoid any possible contamination. The specimens were transported by sterile transport swabs to the department of bacteriology laboratory, and each specimen was inoculated using direct method of inoculation on blood agar, MacConkey agar, then inoculated at 37°C under aerobic condition for 18-24 hours (Collee, 1996).

Identification of bacteria

A single colony was taken from each primary positive culture on blood agar and MacConckey agar and repeating growth for gaining pure culture and then it was identified depending on its morphological and cultural characteristics (blood hemolysis, lactose fermentation , colony shape, size, colour, borders, and texture) and then it was examined under the microscope after making smear from pure colony on clean slid and stain with Gram's stain for observation arrangement and reaction bacteria with stain (MacFaddin, 2000), then further identified by biochemical tests. The final identification was performed with automated VITEK-2 compact system using G-ve-ID cards.

Antibiogram Profile

Disk Diffusion Method

Antibiotic susceptibility was carried out for all isolates of *Ps. aeruginosa* according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2014), using disk diffusion method (Kirby-Bauer method). Individual cells were suspending adjusted to a 0.5 McFarland standard tube and spreading surface of Mueller Hinton Agar. The test was conducted using some disks commercially obtainable antibiotics (Bioanalyse, Turkey).

Total DNA extract

DNA extraction from bacteria by the Genomic DNA Extraction kit (Favorgen/Taiwan).

PCR Amplification and Gel Electrophoresis

DNA of all isolates were subjected to PCR to detect antibiotic genes such as (Aph(3)-llla, ParC, Tet/tet(M),

Table 1 : The primer were used in this study.

Target gene	Sequence	Bp	Reference
Aph(3)-llla	F 5'-**GGCTAAAATGAGAATATCACCGG**-3' R 5'- **CTTTAAAAAATCATACAGCTCGCG**-3'	523	Vakulenko *et al.* (2003)
ParC	F 5'-**TGTATGCGATGTCTGAACTG**-3' R 5'-**CTCAATAGCAGCTCGGAATA**-3'	264	Everett *et al.* (1996)
Tet/tet(M)	F 5'-**GAACTGTATCCTAATGTGTG**-3' R 5' -**GATACTCTAACCGAATCTTCG**-3'	377	Guardabassi *et al.* (2000)
aac(6´)-Ib-cr	F 5'-**TTGCGATGCTCTATGAGTGGCTA-3'** R 3'-**CTCGAATGCCTGGCGTGTTT-5'**	490	Park *et al.* (2006)

Table 2 : Programs of PCR thermocycling conditions of primers.

Gene name	Temperature (°C)/Time					Cycles number
	Initial Denaturation	Cycling Conditions			FinalExtension	
		Denaturation	Annealing	Extension		
Aph(3)-llla	95°C /5min	95°C /30 std	57.8°C/30sec	72^{0}C/60sec	72°C/5min	30 cycles
ParC	95°C /5min	95°C /30 std	55.2°C/30sec	72^{0}C/30sec	72°C/5min	30 cycles
Tet/tet(M)	95°C /5min	95°C /30 std	54.4°C/30sec	72^{0}C/40sec	72°C/5min	30 cycles
aac(6´)-Ib-cr	94°C /4 min	94°C /45 std	55ÚC/45std	72^{0}C/45std	72°C/5min	40 cycles

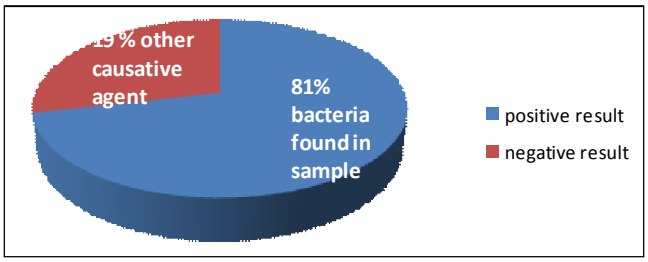

Fig. 1 : The occurrence of bacteria isolated from 100 specimens collected from burns, wound inflammation and urinary tract infections.

aac(6´)-Ib-cr, Esp). The specific primers and reaction conditions that used in the work are shown in tables 1 and 2 (Chaudhary and Payasi, 2014).

Statistical analysis

The data were analyzed using Microsoft Excel computerized programs (Paulson, 2008).

Results

Description of study samples

In this study, a total of (100) specimens were collected, were obtained from patients suffering from burns, wound inflammation and urinary tract infections (UTIs). The patients who attending to Hilla Teaching Hospital, the period of collection extended from October 2010 to January 2011. 81(81%) specimens gave positive culture and the other 19(19%) specimens were considered negative results . These isolates were obtained from burns 12(14.8%), wound 21(25.9 %) and urinary tract infections 48 (59.3%). The results were shown in fig. 1.

Identification of bacteria specimens

The initially identification of bacterial specimens depended on some criteria which included Gram stain ,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 performed identification and confirmed by vitek-2 system by GN-ID cards to Gram negative bacteria.

From the 81 clinical specimen, only 26(32 %) isolates belong to *Ps. aeruginosa*, 21(26%) isolates were belonged to *E.coli*, 18(22.2%) isolates were *Klebsella pneumonia* and 16(19.8%) *Proteus mirabilis*.

In biochemical tests which appeared that 26(32%) of isolates as *Ps. aeruginosa* and appeared that 21(26%) of isolates were *E.coli*, also appeared that 18(22.2%) of isolated of *Klebsella pneumonia* and 16(19.8%) of isolated *Proteus mirabilis* as show in table 3.

Automation in clinical microbiology started much later than other clinical laboratories. In 1982, the Enterobacteriaceae- plus biochemical card was introduced, providing for automatic identification of the Enterobacteriaceae with in 8 hours of incubation (Otto-Karg *et al*., 2009).

VITEK-2 have contributed to better and more-cost effective management of patients by enabling clinical microbiologists to identify medically relevant bacteria more rapidly and accurately. It was emphasized that an important value for a highly standardized commercial identification system must be the capability of the manufacturer to maintain or even improve the performance of an identification system over time (Funke *et al*., 1998).

The results also raveled that 26 isolates of *Pseudomonas*. This results are in nearlink with (Motoshima *et al*., 2007), who found sensitivity and specificity the diagnostic *Pseudomonas aeruginosa* by biochemical methods relative were 98.1% and 100%, respectively.

Antibiotic resistance

The susceptibility of 26 (*Ps. aeruginosa*) isolates against 9 selected antibiotics was studied to determine the pattern of isolates sensitivity to various antibiotics depending on disk diffusion method.

The results represent the antibiogram profile of the isolates, indicate that isolates varied in their susceptibility to the antibiotics. The result show in table 4.

Antibiotic resistance is a major clinical problem in treating bacterial infections worldwide, all isolates were screened for their antibiotic resistance against selected antimicrobial agents recommended by CLSI (2014). A strain is considered a multidrug resistant (MDR), if an isolate is resistant to representatives of three or more classes of antibiotics (Falagas and Karageorgopoulos, 2008).

In the present study, all the tested isolates are resistant to a minimum of three classes of antibiotics to which they are tested. Therefore, the isolates are considered multidrug resistant. The usage of antibiotics without antibiotics sensitivity testing, is the most important factor promoting the emergence of multi-drug resistance, which lead to selection and dissemination of antibiotic resistant pathogens in clinical medicine (Neu, 1992).

Molecular detection of bacterial isolates

Detection of the *tet(M)* gene

All isolates were investigated to detect genes *tet(M)*, which encode for enzymes responsible for catalysis

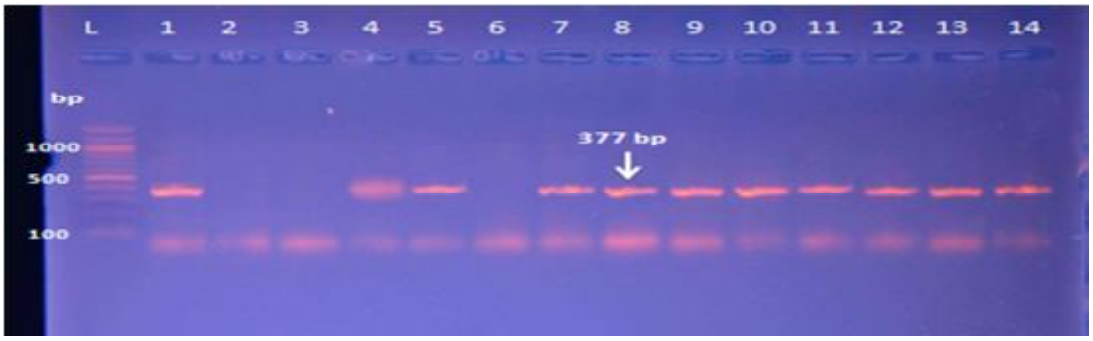

Fig. 2 : Amplification of *tet(m)* gene *Ps. aeruginosa* with product 377 bp. Lane L: DNA ladder. Lanes (1,4,5,7,8,9, 10,11,12,13 and14) show positive results with *tet(m)* gene, Lanes (2,3 and 6) show negative results with *tet(m)* gene. (1.5% agrose gel, 75 V, 1.20 hours).

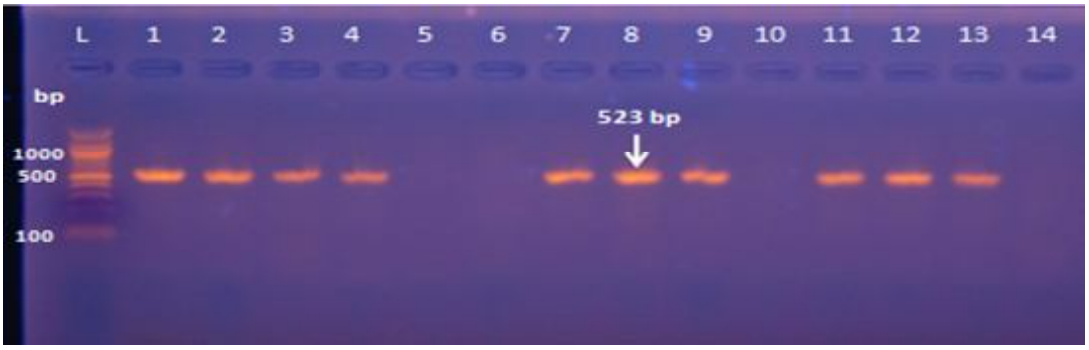

Fig. 3 : Amplification of *Aph(3)-llla* gene in *Ps. aeruginosa* with product 523 bp. Lane L: DNA ladder. Lanes (1,2,3,4,7,8,9 ,11,12 and13) show positive results with *Aph(3)-llla* gene, Lanes (5, 6, 10 and14) show negative results with *Aph(3)-llla* gene. (1.5% agrose gel, 75 V, 1.20 hours).

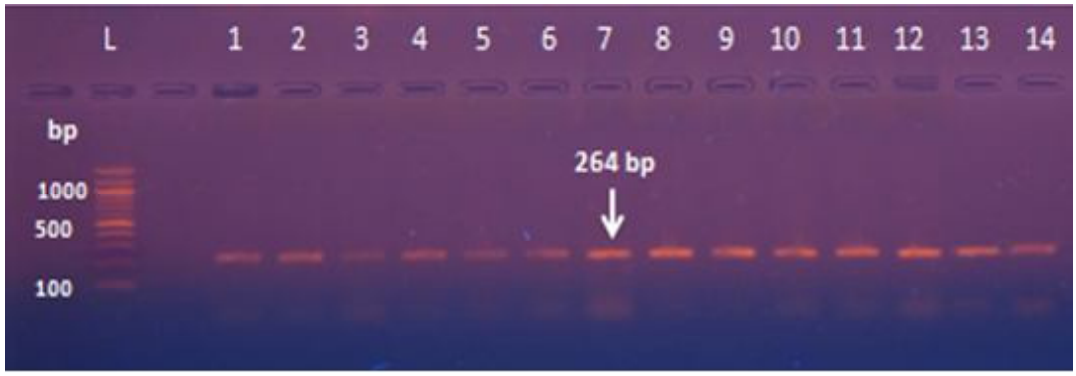

Fig. 4 : Amplification of *Par-c* gene in *Ps. aeruginosa* with product 264 bp. Lane L: DNA ladder. All isolates show positive results with *Par-c*. (1.5% agrose gel, 75 V, 1.20 hours).

tetracycline antibiotics using PCR technique with specific forward and reverse primers. Seen from the results shown in fig. (2) of the current study to *tet(M)* gene tested isolates represented 20(76.9%) in *Ps. aeruginosa*.

Detection of the *Aph(3)-llla* gene

Aph(3)-llla genes encoding amino-glycoside modifying enzymes(AMEs) in *Proteus mirabilis* using PCR technique. Seen from the results shown in figure(3) of the current study to *Aph(3)-llla* gene tested isolates represented 18(69.2%) in *Ps. aeruginosa*.

Detection of the *Par-c* gene

Par-c gene, which encode for enzymes responsible for catalysis fluoroquinolone antibiotics using PCR technique with specific forward and reverse primers. Seen from the results shown in figure(4) of the current study to *Par-c* gene tested isolates represented 26(100%) in *Ps. aeruginosa*.

Detection of the *aac(6′)-Ib-cr* gene

aac(6′)-Ib-cr gene, which encode for enzymes responsible for catalysis plasmid -Mediated Quinolone Resistance Genes using PCR technique with specific forward and reverse primers. Seen from the results shown in fig. (5) of the current study to *aac(6′)-Ib-cr* gene tested isolates represented 18(69.2%) in *Ps. aeruginosa*.

Fig. 5 : Amplification of *aac(6')-Ib-cr* gene in *Ps. aeruginosa* with product 490 bp. Lane L: DNA ladder. Lanes (1,3,4,7,9,10,11,12,13 and 14) show positive results with *aac(6')-Ib-cr* gene , Lanes (2,5,6 and8) show negative results with *aac(6')-Ib-cr* gene. (1.5% agrose gel, 75 V, 1.20 hours).

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

Test / Result	Oxidase	Catalase	Urease	Citrate	VP	MR	Motility	Kliglar iron agar	H_2S	Indole
E. coli	-	+	-	-	-	+	+	A/A/-		+
Klebsella ssp	-	+	Late +	+	+	+	-	A/A/-		+
Pseudomonas ssp	+	+	-	+	+	+(v)	+	Ak/Ak/-		-
Proteus ssp	-	+	+	-/+	-	+	+	Ak/A/+		-

Table 4 : Antibiotics sensitivity of bacteria isolated from different source (Number (%)).

Type of antibiotic		Type of bacteria *Ps.aeruginosa* (n=26)(%)
1	AK	110%
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.

In Tetracycline resistant *Proteus mirabilis,* genes carried on transposons and/or plasmids encode transmembrane proteins that efflux the antibiotic by an energy depen-dent manner (Shankar *et al*., 2009), resistance to β-lactams may arise in *Proteus mirabilis* as a result of mutations in genes encoding PBPs that reduce the affinity of PBPs for the antibiotics. 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 that expression phenotypic multi antibiotic resistance (Ida *et al*., 2001).

Aminoglycoside modifying enzymes in *S. aureus* isolates are encoded by aac(6')-Ie-aph(2"), aph (3')- IIIa, and ant (4')-Ia genes and confer resistance to aminoglycosides. In this study 67.5% of aminoglycoside resistance isolates, harbored the aph (3')- IIIa gene as the most prevalent gene. Aminoglycosides by inhibiting the bacterial protein synthesis show bactericidal activity. This group of antibiotics especially gentamycin and tobramycin in combination with beta-lactam or glycopeptides antibiotics have synergical effects on treatment of *S. aureus* infection, particularly endocarditis (Schmitz *et al*., 1999). 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 (Schmitz *et al*., 1999; Ida *et al*., 2001). AMEs are classified into four groups according to the modification imposed on aminoglycoside antibiotics: acetyltransferases (AACs), phosphotransferases (APHs), nucleotidyltransferases (ANTs) and adenyltransferases (AADs).

Quinolones and fluoroquinolones (FQs) constitute a family of antibacterial agents that damage bacterial DNA via inhibition of type II topoisomerases. These are heterotetrameric enzymes including DNA gyrase with two subunits A and B (respectively encoded by gyrA and

gyrB genes) and DNA topoisomerase IV with two subunits A and B (respectively encoded by parC and parE genes) (Rodríguez-Martínez *et al*., 2011). These enzymes work together in the replication, transcription, recombination, and repair of bacterial DNA (Jacoby, 2005). Alterations in quinolone resistance determining regions (QRDRs) via mutations have been recognized as one of the main resistance mechanisms in *Escherichia coli* (Ruiz, 2003). At the same time, the amino-acid substitution in these regions has significant effects on quinolone and FQ Minimum Inhibitory Concentration (MIC) values (Shigemura *et al*., 2012). In quinolone resistant gram negative bacteria, such as *E. coli*, mutations occurring in gyrA (mainly at Ser-83 and ASP-87) and parC (mainly at Ser-80 and Glu-84) subunits have been described as a secondary target (Jacoby, 2005).

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