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Molecular Study of Biofilm and Some Antibiotic Resistance Gene in *Proteus mirabilis* Isolated from children with UTI Patients in Al-najaf Governorate

Raad Ajam Sayal[1], Najat Mohammed Faleeh Alkharasani[2], Aqeel A Alsadawi[3], Zahraa Hameed Oda Alquraishi[4]

[1]*Pathological analysis Department/ College of Health and Medical Technology/ Kufa/ Al-Furat Al-Awsat Technical University31003 Al-Kufa,*
[2]*Pathological analysis Department/ College of Health and Medical Technology/ Kufa/ Al-Furat Al-Awsat Technical University University31003 Al-Kufa,*
[3]*Department of Biology, Faculty of Science, Kufa University,*
[4]*Pathological analysis Department/ College of Health and Medical Technology/ Kufa/ Al-Furat Al-Awsat Technical University University31003 Al-Kufa,*

Abstract

Introduction: A total of (100) clinical urine specimens collected from patients suffering from urinary tract infection(UTI) during the period from 1, August ,2017 to 9, October, 2017

Methodology : The identification of the *Proteus mirabilis* isolates were depended on colonial morphology, microscopic examination , biochemical tests and Api Enterosystem 20 R (31). In addition antibiotic genes identification was performed by PCR technique with (*tet(M), Aph(3)-llla, ParC, aac(6´)-Ib-cr* and *esp* genes).

Result: Rendering to the results obtained according to the morphological ,cultural characters from 100 sample only 58 (58%) isolate was identify as *Proteus mirabilis* , biochemical characters and API Enterosystem 20 R 40/58(68.9 %) isolate was identify as *Proteus mirabilis* (32). The PCR data showed that *ParC* gene high prevalence 40/40(100%) . Also *tet(M)* gene was positive in 31/40(77.5%) , *Aph(3)-llla* gene was positive in 27/40 (67.5%). While *aac(6´)-Ib-cr* gene showed low prevalence 14/40(35%) in this study. Finally, the PCR data showed moderate prevalence 20/40(50%) for *esp* gene which encode for Biofilm formation.

Conclusion : The study investigated the antibiotic resistance genes of *Proteus mirabilis* which play a major role in pathogenicity (33) .

Keywords: *Proteus mirabilis* , urinary tract infection and antibiotic resistance genes .

INTRODUCTION

Proteus mirabilis belongs to the family Enterobacteriacae, which are gram negative, facultatively anaerobic rods that have the ability to grow in nutrient deficient environments (1). Many species in the family are highly motile, with numerous flagella that allow for several different modes of locomotion. Enterobacteriacae are also known to cause many diseases in both plants and animals(2). Biofilm formation, swarming motility, and ureolytic activity are virulence factors characteristic of *P. mirabilis* strains (4).

Proteus mirabilis is capable of causing symptomatic infections of the urinary tract including cystitis and pyelonephritis and is present in cases of asymptomatic bacteriuria, particularly in the elderly and patients with type 2 diabetes(3).

The most widely investigated *Proteus mirabilis* biofilms are those in the urinary tract, particularly on the catheters surface. The important issues are the crystallized biofilms that lead to catheter incrustation and obstruction. Two main types of crystals may be found inside them: struvite (magnesium ammonium phosphate) and apatite (hydroxyl calcium phosphate). They appear in the urinary tract biofilms and block the urine flow. (5).

Bacterial resistance to antibiotics increasing among patients in both outpatients and in patients, which are considered as a focus of infection. These resistances can be acquired by mutation or by plasmid of resistance genes from other migro-organisms (6). There are several technique for microbial identification ,such as microscopic, ,biochemical methods , API 20Enterosystm method and convential PCR (7).New sensitive and specific diagnostic methods, such as direct PCR analysis of fecal specimens, have been used to identified bacteria and viruses pathogens (8).

Materials and Methods

The present study was carried on in the Laboratories of Bacteriology and Molecular in Biology Department, of Sciences, University of Kufa , Iraq , in the period from 1, August ,2017 to 9, October,2017. The study population consist of 100 clinical urine specimens collected from children suffering from urinary tract infection who attended to AL-Zahra Teaching Hospital for children in Al-Najaf province . Isolation of pathogenic bacteria from urinary tract infection specimens and identification to the species level was performed by standard methods (9). The antimicrobial sensitivity testing was done by KirbyBauer disc diffusion method standardized as per (10). Antibiotics were selected according to WHO model list of essential drugs the chosen antibiotics included Ampicilin , Amoxicillin , Amikacin, Ciprofloxacin, Cefoxitin , Chloromphenicol, Nalidixic acid, Gentamicin , Nitrofurantoin and Tetracycline Trimethoprim. Results were interpreted according to NCCLS 2014 standard tables.

Swarming motility. Swarming motility was examined when 5 ml overnight broth culture was inoculated onto the center of brain heart infusion agar plates. The plates were pre-dried for 1 h at 37^{o}C (11) and then incubated overnight at 37^{o}C.

Identification by Api Enterosystem

The identified *Enterobactetriceae* isolates were confirmed with the Api Enterosystem 20 R . This Api system include a strip consisting of 20 microtubes containing dehydrated substrates. Bacteria was suspended in 3 ml of normal saline and adjusted to a turbidity of MacFarland No. 0.5, filled in micro-tubes of strip, during incubation in 37°C° for 18hr API strip was covered by a plastic impermeable chamber to avoid evaporation, some wells (ADH ,LDC, ODC, H2S and URE) were overloaded with oil to provide condition of reduced oxygen tensions; color changes were read visually after the addition of reagents to some wells (TDA, IND, VP). These color changes indicate the presence or absence of the bacteria ability to metabolize a particular substrate. The results entering in the disk to your system gives the name of the isolation of bacterial (Biomerieux – France Company /WWW. Biomerieux .com)

Total DNA Extract

A single colony of cultivated bacteria, which had been incubated overnight, transfer to 2 ml of sterile nutrient broth and incubate at 37 ◦C for 18-24 hours. The DNA extracted and purified using Genomic DNA Mini plasmid kit (Favorgen/Taiwan) according to manufacture instructions . The total DNA was used to detect *tet(M), Aph(3)-llla, ParC, aac(6´)-Ib-cr* and *esp* genes.

PCR Amplification and Gel Electrophoresis

DNA of all isolates were subjected to PCR to detect such as (*tet(M), Aph(3)-llla, ParC, , aac(6´)-Ib-cr* and *esp*). The specific primers and reaction conditions that used in the work are shown in table 1 and 2.

Amplified products were confirmed using 1% agarose gel electrophoresis to estimate the PCR products size. The gel was stained with 4 µLof 10mg/mL ethidium bromide (Sigma, USA) and it run at 80v for 1.5h. A single band was observed at the desired position on ultraviolet light transillumintor (Cleaver, UK); bands were photographed using gel documentation system (Cleaver, UK). A 100bp ladder (Bioneer, Korea) was used to measure the molecular weights of amplified products[12].

RESULT AND DISCUSSION

***E.coli* characterization:**

1- Culturing identification:

The morphological characterization of bacteria revealed that the *proteus mirabilis* isolates were 58/100(58%) which appeared colorless or bale colony on Mac-Concky because non lactose ferment which recorded suspected as *proteus mirabilis***.** Swarming motility *P. mirabilis* possesses some distinctive characteristics within the Enterobacteriaceae family, such as swarming motility, a coordinate multi-cellular behavior dependent on flagella that occurs when cells grow on solid rich media or on viscous surfaces (18). In the present study, typical *P. mirabilis* swarming motility was exhibited by 58 isolates when grown on agar plates.

The biochemical tests which appeared that 40/58(68.9 %) of the isolates were *proteus mirabilis* , as shown (table 3).

API Enterosystem 20 R method:-

The Api 20 R is a group of chemical tests differential these tests depend on 20 test. The diagnosis of isolated *proteus mirabilis* were confirmed using APi20R system accomplished according to manufacturer's instructions which explained ; 40 isolates *proteus mirabilis* gave positive results , as show in figure (1)

(Figure-1) API Enterosystem 20 R method for diagnosis of isolated *proteus mirabilis*

This result show that enteric bacteria appear MacConkey agar is a differential media for Enterobacteriaceae and related enteric gram negative bacteria because contain bile salts and crystal violet which inhibit the growth of gram positive bacteria . *Proteus mirabilis*, a motile Gram-negative bacterium, represents a common cause of complicated urinary tract infections. These infections typically occur in patients with functional or anatomical abnormalities of the urinary tract or patients subjected to long-term catheterization [19]. Several virulence factors have been identified and characterized for *P. mirabilis*. These factors include a potent urease that catalyzes formation of ammonia from urea and leads to urinary stone formation, a poreforming hemolysin, ZapA metalloprotease which cleaves both immunoglobulin G (IgG) and IgA, a capsular polysaccharide, four distinct fimbrial types, and peritrichous flagella for swimming and swarming motility [20].

Table (1): The Primer Were Used In This Study

Target Gene	Sequence	Bp	Reference
Aph(3)-llla	F 5′-GGCTAAAATGAGAATATCACCGG -3′ R 3′- CTTTAAAAAATCATACAGCTCGCG -5′	523	13
ParC	F 5′- TGTATGCGATGTCTGAACTG -3′ R 3′- CTCAATAGCAGCTCGGAATA -5′	264	14
Tet/tet(M)	F 5′- GAACTGTATCCTAATGTGTG -3′ R 3′- GATACTCTAACCGAATCTTCG -5′	377	15
aac(6')Ib - cr	F-5′ TTGCGATGCTCTATGAGTGGCTA-3′ R -3′ CTCGAATGCCTGGCGTGTTT-5′	490	16
Esp	F 5′- TTGCTAATGCTAGTCCACGACC-3′ R 5′- GCGTCAACACTTGCATTGCCGAA-3′	955	17

Table (2): Programs of PCR Thermocycling Conditions of Primers

Gene Name	Temperature (°C) / Time					Cycles Number
	Initial Denaturation	Cycling Conditions			Final Extension	
		Denaturation	Annealing	Extension		
Aph(3)-llla	95°C/ 5min	95°C/ 30 std	57.8°C/30sec	72˚C/60sec	72°C/5min	30 cycles
ParC	95°C/ 5min	95°C/ 30 std	55.2°C/30sec	72˚C/30sec	72°C/5min	30 cycles
Tet/tet(M)	95°C/ 5min	95°C/ 30 std	54.4°C/30sec	72˚C/40sec	72°C/5min	30 cycles
PBP2B	95°C/ 5min	95°C/ 30 std	55.5°C/30sec	72˚C/30sec	72°C/5min	30 cycles
Esp	95°C/ 5min	95°C/ 30 std	61°C/30sec	72˚C/100sec	72°C/5min	30 cycles

Table (3)

Test / Result	Oxidase	Catalase	Urease	Citrate	VP	MR	Motility	Kliglar iron agar	H2S	Indole	Lactose fermentation MacConkey agar
Proteus ssp	-	+	+	-/+	-	+	+	Ak/A/+		-	-

(-) negative ; (+) positive ; (AK) alkaline and (A) acid

Susceptibility Test

The antibiotic susceptibility test was performed for all isolates(40) when give positive result in API Enterosystem 20 R test against 10 antibiotics , the results showed in the table(4) .The resistance rates were occurred between 0.00% and 100.00%.

Table (4)

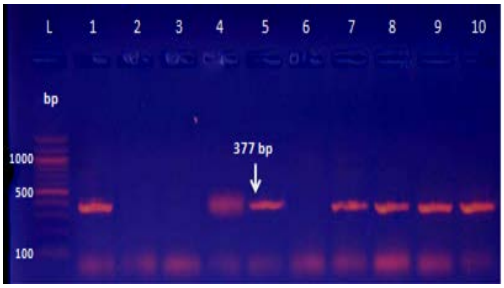
Type of antibiotic	NO=40(%)
Ampicilin	17(42.5%)
Amoxicillin	25(62.5%)
Amikacin	10(25%)
Ciprofloxacin	0(100%)
Cefoxitin	15(25%)
Chloromphenicol	17(42.5%)
Nalidixic acid	14(35%)
Gentamicin	20(50%)
Nitrofurantoin	35(87.5%)

(The table show the No. of resistant isolate ,what about the sensitive and intermediate)

The results of table (4) showed that *Proteus mirabilis*, were resistant to Amoxicillin , Cefoxitin and Chloromphenicol and Nalidixic acid (62.5% , 25%, 42.5% and 35%) ,respectively. This result is near-link with (21)who found that (75%, 50%, 50% and 25%) isolates were resistance for similar antibiotic , respectively.
The frequency of antibiotic resistance of the 40 *Proteus mirabilis* isolates was determined Table (4). *Proteus mirabilis* isolates were showed that resistance to Ampicilin , Amikacin, Gentamicin and Nitrofurantoin (42.5% , 25%, 50% and 87.5%) ,respectively. This result is dissimilar with (22)who found that (23.5%, 70.5%, 82.3% and 70.5%) isolates were resistance for similar antibiotic , respectively
The results of table (4) showed that *Proteus mirabilis*, were resistant to Ciprofloxacin (0%). This result is similar with result obtained by **Al-Jebouri M**, 2013 (21)who found that (0 %) isolates were resistance for similar antibiotic .
The reason of high resistance of antibiotics in area of study might be to the overuse and misuse of antibiotics in the treatment of diarrhea could lead to an increase of antibiotic resistance.

1-Detection of the *tet(M)*gene

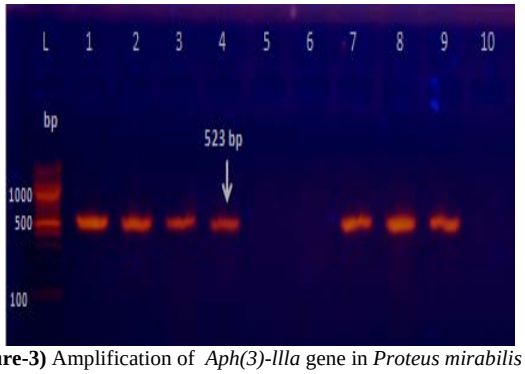
All isolates were investigated to detect genes *tet(M)* which encode for enzymes responsible for catalysis tetracycline antibiotics using PCR technique with specific forward and reverse primers . Seen from the results shown in figure(2) of the current study to *tet(M)* gene tested isolates represented 31(77.5%) in *proteus mirabilis*.



(Figure-2) Amplification of *tet(m)* gene in *Proteus mirabilis* with product 377 bp . Lane L: DNA ladder. Lanes (1,4,5,7,8,9 and 10) 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)

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

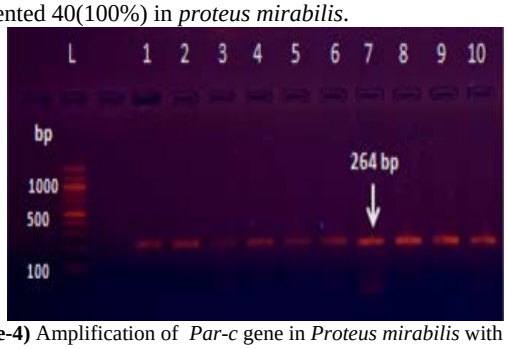
Aph(3)-llla genes encoding amino-glycoside modifying enzymes(AMEs) in *Proteus mirabilis* using PCR technique. Seen from the results shown in figure(3) of the current study to *Aph(3)-llla* gene tested isolates represented 27(67.5%) in *proteus mirabilis*.



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

3-Detection of the *Par-c* gene

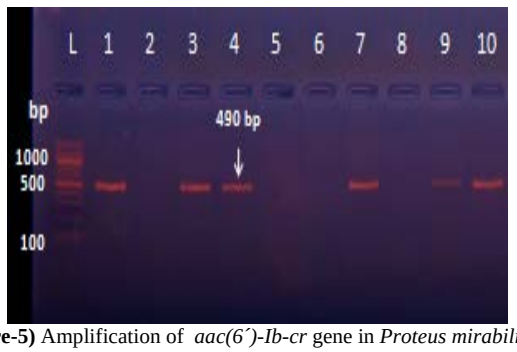
Par-c gene which encode for enzymes responsible for catalysis fluoroquinolone antibiotics using PCR technique with specific forward and reverse primers . Seen from the results shown in figure(4) of the current study to *Par-c* gene tested isolates represented 40(100%) in *proteus mirabilis*.



(Figure-4) Amplification of *Par-c* gene in *Proteus mirabilis* with product 264 bp . Lane L: DNA ladder. Lanes (1,2,3,4,5,6,7,8,9 and 10) show positive results with *Par-c* gene not show negative results with *Par-c* gene. (1.5%agrose gel , 75 V , 1.20 hours)

4-Detection of the *aac(6´)-Ib-cr* gene

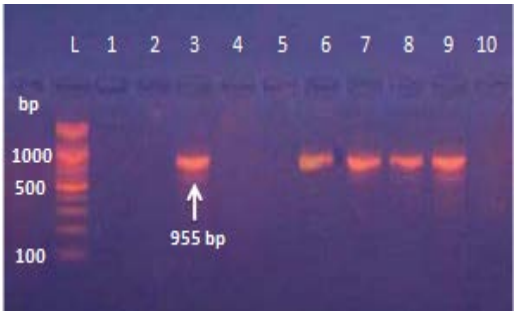
aac(6´)-Ib-cr gene which encode for enzymes responsible for catalysis plasmid -Mediated Quinolone Resistance Genes using PCR technique with specific forward and reverse primers . Seen from the results shown in figure(5) of the current study to *aac(6´)-Ib-cr* gene tested isolates represented 40(100%) in *proteus mirabilis*.



(Figure-5) Amplification of *aac(6´)-Ib-cr* gene in *Proteus mirabilis* with product 490 bp . Lane L: DNA ladder. Lanes (1,3,4,7,9 and 10) 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).

5-Detection of the *esp* gene

esp gene which encode for enzymes responsible for Biofilm formation in *proteus mirabilis* using PCR technique with specific forward and reverse primers . Seen from the results shown in figure(6) of the current study to *Par-c* gene tested isolates represented 20(50%) in *proteus mirabilis*.



(Figure-6) Amplification of *esp* gene in *Proteus mirabilis* with product 955 bp . Lane L: DNA ladder. Lanes (3,6,7,8,9 and 10) show positive results with *esp* gene Lanes (1,2,4,5 and 10) show negative results with *esp* gene. (1.5%agrose gel , 75 V , 1.20 hours)

In Tetracycline resistant *proteus mirabilis*, genes carried on transposons and/or plasmids encode transmembrane proteins that efflux the antibiotic by an energy dependent manner (23) , 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 (24) .

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 (25). 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 (25, 26). 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) (27). These enzymes work together in the replication, transcription, recombination, and repair of bacterial DNA (28). Alterations in quinolone resistance determining regions (QRDRs) via mutations have been recognized as one of the main resistance mechanisms in *Escherichia coli* (29). At the same time, the amino-acid substitution in these regions has significant effects on quinolone and FQ Minimum Inhibitory Concentration (MIC) values (30). 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 (28).

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