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

Raad Ajam Sayal1, Najat Mohammed Faleeh Alkharasani2, Aqeel A Alsadawi3, Zahraa Hameed Oda Alquraishi4

1Pathological analysis Department/ College of Health and Medical Technology/ Kufa/ Al-Furat Al-Awsat Technical University31003 Al-Kufa, 2Pathological analysis Department/ College of Health and Medical Technology/ Kufa/ Al-Furat Al-Awsat Technical University31003 Al-Kufa, 3Department of Biology, Faculty of Science, Kufa University, 4Pathological analysis Department/ College of Health and Medical Technology/ Kufa/ Al-Furat Al-Awsat Technical 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)-Illa, 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)-Illa 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 Enterobacteriaceae, 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. Enterobacteriaceae 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 micro-organisms (6).

There are several technique for microbial identification such as microscopic, biochemical methods , API 20Enterosystem 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, Amoxillicin, Amikacin, Ciprofloxacan, Cefoxitin , Chlorofomphic, 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°C (11) and then incubated overnight at 37°C.

Identification by Api Enterosystem

The identified *Enterobacteriaceae* 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)-Illa, 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)-Illa, 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 µL of 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 transilluminator (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:

I- 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)

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

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Sequence</th>
<th>Bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aph(3)-Illa</td>
<td>F 5’-GGCTAAAAATGGAATATCACCAGG-3’ R 3’-CTTTAAAAATCATACTAGCTTCGGC-5’</td>
<td>523</td>
<td>13</td>
</tr>
<tr>
<td>ParC</td>
<td>F 5’-TGTATAGGCGATATCAGCTTCGGC-3’ R 3’-CTCAATAGCAGCTCGGAATA-5’</td>
<td>264</td>
<td>14</td>
</tr>
<tr>
<td>Tet/tet(M)</td>
<td>F 5’-GAACTGTATCTAATAGTTGT-3’ R 3’-GATACGCTTACCGAGATCCTTCG-5’</td>
<td>377</td>
<td>15</td>
</tr>
<tr>
<td>aac(6’)-Ib-cr</td>
<td>F-3’TTGGCGATGCTCCTATGTGCTG-5’ R-3’CTCGAATGCTCGGCGTGGTT-5’</td>
<td>490</td>
<td>16</td>
</tr>
<tr>
<td>Esp</td>
<td>F 5’-TTGCTAATGCTATGTCACACCACC-3’ R 5’-GGTCACCACCATTTGCAATGCGC-3’</td>
<td>955</td>
<td>17</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Initial Denaturation</th>
<th>Temperature (°C) / Time</th>
<th>Cycling Conditions</th>
<th>Final Extension</th>
<th>Cycles Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aph(3)-Illa</td>
<td>95°C/ 5min</td>
<td>95°C/ 30 std</td>
<td>Denaturation</td>
<td>72°C/30sec</td>
<td>30 cycles</td>
</tr>
<tr>
<td>ParC</td>
<td>95°C/ 5min</td>
<td>95°C/ 30 std</td>
<td>Annealing</td>
<td>72°C/30sec</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Tet/tet(M)</td>
<td>95°C/ 5min</td>
<td>95°C/ 30 std</td>
<td>Extension</td>
<td>72°C/30sec</td>
<td>30 cycles</td>
</tr>
<tr>
<td>PBP2B</td>
<td>95°C/ 5min</td>
<td>95°C/ 30 std</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esp</td>
<td>95°C/ 5min</td>
<td>95°C/ 30 std</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table (3): Oxidase, Catalase, Urease, Citrate, VP, MR, Motility, Klebsiella iron agar, H2S, Indole, Lactose fermentation, MacConkey agar

<table>
<thead>
<tr>
<th>Test</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Urease</th>
<th>Citrate</th>
<th>VP</th>
<th>MR</th>
<th>Motility</th>
<th>Klebsiella iron agar</th>
<th>H2S</th>
<th>Indole</th>
<th>Lactose fermentation</th>
<th>MacConkey agar</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteus spp</strong></td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+/-)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
<td>(-)</td>
<td>(-)</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>Type of antibiotic</th>
<th>NO(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>17(42.5%)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>25(62.5%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>10(25%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0(100%)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>13(25%)</td>
</tr>
<tr>
<td>Chloromphenicol</td>
<td>17(42.5%)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>14(35%)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>20(50%)</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>35(87.5%)</td>
</tr>
</tbody>
</table>

(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 Ampicillin, 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 (1,2,3,4,7,8,9 and 10) show positive results with tet(m) gene, Lanes (2,3 and 6) show negative results with tet(m) gene. (1.5% agarose gel, 75 V , 1.20 hours)

2-Detection of the Aph(3)-Illa gene
Aph(3)-Illa 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)-Illa gene tested isolates represented 27(67.5%) in proteus mirabilis.

(Figure-3) Amplification of Aph(3)-Illa 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)-Illa gene, Lanes (5,6 and 10) show negative results with Aph(3)-Illa gene. (1.5% agarose 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% agarose 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 (1,2,3,4,5,6,7,9,10 and 11) 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% agarose 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 adenylyltransferases (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 topoisoase 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).

**REFERENCES**