ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

# Molecular Detection of Some Virulence Factors Genes of Proteus Mirabilis That Isolated From Urinary Tract Infection

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## Abstract

A total of (160) clinical specimen of urine collected from patients suffering from urinary tract infections at different ages and from both sex in different hospitals in Baghdad during the period from September 2017 to February 2018.were cultured on selective media MacConkey agar. Bacterial isolates were identified by a microscopic examination and diagnosed by using biochemical tests. A further identification by vitak 2system.The results showed that (103) samples were positive for bacteriological culture (64.6%), the percentage of *Proteus* spp were (54) urine sample (52.4%) and percentage of *Proteus mirabilis* isolates were (40) isolates (74%) while *Proteus vulgaris* were (14) isolates (25.9%) while the results indicated the isolation rate of *Proteus mirablis from* females was (67%) which was higher than that of males (33%).Selection ten isolates of *P. mirabilis* which show highest urease activity and adhesion to uroepithelia cell. Aderence capability of the isolates was also tested in accordance to epithelial cells isolated from female urine all isolates were capable of adhering to epithelial cells. The genetic study included an extraction of genomic DNA from (10) *p. mirablis*, then detection of virulence genes *ure C* that responsible for urease , *mrpA* for fimbrial and ability to adherence to uroepithelial cell for these isolated by using convential PCR all isolated give positive result. Selection five from *ure c and mrp A* gene amplicons of *P. mirabilis* sent to Korea for direct sequencing. The nucleotide sequences of *ure c, and mrp A* genes were compared with the sequences of the *mrp A and ure c* gene using BLAST option of NCBI (Basic Local Alignment Search Tool, BLAST v. 2.2.15, www.ncbi.nlm.nih.gov. The result was 100% matching with the registrar globally.

## Introduction

The genus *Proteus,* which was described for the first time by Hauser in 1885, belongs to the *Enterobacteriaceae* family. In this family it is placed in the tribe *Proteeae,* together with the genera *Morganella* and *Providencia* [1]. *Proteus spp*. consist of gram-negative, motile, Aerobic rod-shaped bacilli generally range from 0.3 to 1.0 µm in width and0.6 to 6.0 µm in length [2].The genus *Proteus* currently consists of five species which are *P. mirabilis, P.vulgaris, P. penneri, P. hauseri* and *P. myxofaciens* [3]. *P. mirabilis* expresses several virulence factor involved in infection like adhesions, flagella, toxins, quorum-sensing, enzymes and immune invasion [4]. *P. mirabilis,* once attached to urinary tract it infects the kidney more commonly than *E. coli.* It is best known for its ability to form stones in the bladder and kidney, as well as its ability to form crystalline biofilms on the outer surface and in the lumen of indwelling urinary catheters [5].

## Materials and Methods

### Patients and Specimens

160 urine samples were collected from patients suffering from urinary tract infections: these samples taken from Medicine city/Baghdad teaching hospital, and Central Child hospital for the period from September 2017 to February 2018.

### Bacterial Diagnosis

Isolation of *P. mirablis* bacteria was performed by a surface streak procedure on both blood and MacConkey agar using calibrated loops and incubated aerobically at 37°C for 24 hours .Bacterial identification was made using biochemical test ,namely indole, citrate, oxidase, catalase, urea hydrolysis,H2S production ,lactose fermentation

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**Detection of Urease Enzyme** [6]

An amount of 10 µl sample solution was mixed with 990 µl urea determination solution in a small test tube, and the pH of the mixture was adjusted to 5.1 with 0.1 M HCl. The reaction was started by adding 2 µl *proteus mirablis* suspension (about 2 × 105 cells). After 20 min of incubation at room temperature, the color of the reaction solution was read at 588 nm.

**Detection of Adhesion** [7]

### Preparation of the Selected Isolates of P. mirabilis

The cells of *Proteus* centrifuged, washed the pellet twice with PBS and the cells adjusted to contain about O.D 600 about 0.6 giving 1 × 109 cells/ml by using viable count.

### Preparation of Epithelial Cells

Uroepithelial cells were collected from urine of healthy females by centrifugation, then cells were washed three times with PBS before resusapension in PBS [8].

### *In Vitro* Adhesion Test

Aliquot of bacterial suspension, and epithelial cells suspension, were incubated in shaker-incubator. Unattached bacterium were removed by centrifugation 3 times in PBS. The final pellet was resuspended during a drop of PBS, dropped onto a glass slide microscope and air dried. The glass slide was fixed with methanol : acetic acid (3:1) and stained with crystal violate .The number of attached bacteria was counted by light microscope.

### Genomic DNA Extraction

DNA was extracted from ten isolates of *P. mirabilis* by using a commercial purification kit (Presto Mini Genomic DNA Kit, (Gene aid, Thailand).

### F. PCR Amplifications

Detection of virulence genes was performed by amplifying the genes via PCR. Descriptions of the PCR primers used in this study are displayed in Table 1.G- Sequence analysis of *mrpA* and *ureC* genes for genotyping of isolates.

**Table 1: Primers used in this study**

| Gene | Primer sequence ( 5′- 3′) | Product size(bp) | Reference |
|---|---|---|---|
| *Ure C* | **F:** GTTATTCGTGATGGATGGG<br>**R :** ATAAGGTGGTTACGCCAGA | 317bp | 9 |
| *MrpA* | **F**:GAGCCATTCAATTAGGAATCCA<br>**R**:AGCTCTGTACTTCCTTGTACAGA | 648bp | 10 |

F*: Forward R**: Reverse

**Table2: PCR program for amplification of *ure C* gene**

| No. | Stages | Temperature & time | | |
|---|---|---|---|---|
| 1 | Initial denaturation | 95 °C | 2min | |
| 2 | Denaturation | 95 °C | 30 sec | 30 cycles |
| 3 | Annealing | 56.2 °C | 30 sec | |
| 4 | Extension | 72°C | 1 min | |
| 5 | Final extension | 72°C | 5 min | |

**Table3: PCR program for amplification *mrpA* gene**

| No. | Stages | Temperature & time | | |
|---|---|---|---|---|
| 1 | Initial denaturation | 95°C | 5min | |
| 2 | Denaturation | 95°C | 1 min | 30cycle |
| 3 | Annealing | 58°C | 1 min | |
| 4 | Extension | 72°C | 1 min | |
| 5 | Final extension | 72°C | 7min | |

## Results and Discussion

### Isolation and Identification of *Proteus Mirabilis*

Loopful amount from these samples were inoculated on MacConkey agar and blood agar and then incubated overnight at 37°C. A distinguishable swarming which is a unique characteristic for genus proteus was observed, which is considered as confirmatory phenomenon for genus proteus as previously described by [11].

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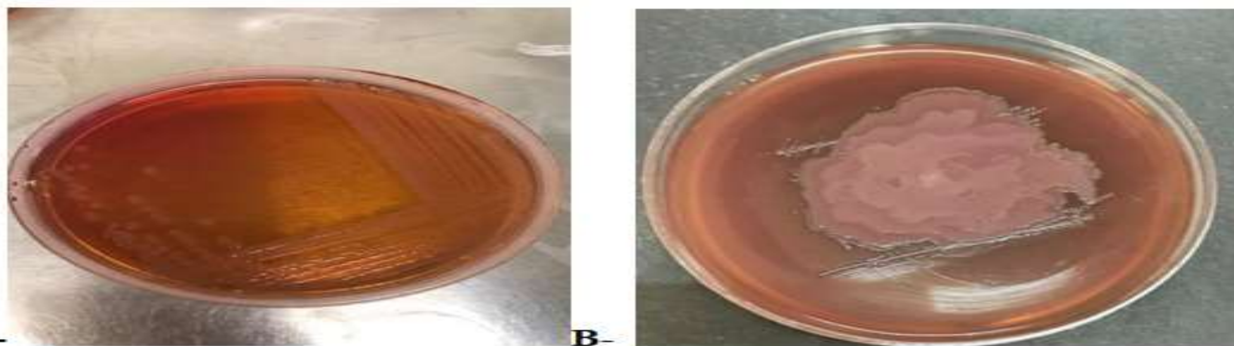


**Figure1 A*:* *Proteus* colonies are non-lactose ferment or on MaCconkey agar after incubation at 37° C for 24 hrs. B: swarming phenomena on blood agar base**

It was found that 103(64.3%) out of the total 160 samples collected gave positive results on MacConkey agar and Blood agar. These results were agreed with those reported by [12] who found that the percentage of positive cultures of urine samples were 64.6%. But such results were disagreed with those of [13] when found that percentage of positive culture of urine samples was (28.9%). The reason of the differences in percentage may be owed to differences in either size of samples or hospital locations as well as to the season and medications before sampling.

Result showed that 54 isolates belong to genus *Proteus* spp from the 103 positive cultures and, so the isolation percentage of *Proteus* from other bacteria of the UTI cases was (52.4%). This result was agreed with that of [14] who found that *Proteus* isolates were representing (60%) of the UTI cases tested. But this result disagreed with [15] who found that isolation percentage of *Proteus* occurrence (17.8%).From the previous results, *P. mirabilis* represent 74% (40 isolates) while *P. vulgaris* appeared only in 25.9% (14 isolates). The present results with previous study done in by [16].Who mentioned that *P. mirabilis* performed 77% while *p. vulgaris* performed 23%.

### Determination of Urease activity

It has been noted that all 40 isolates under study were capable of producing urease. However, ten isolates of *Proteus mirabilis* have ability to produce urease as shown in Table (4). Maximum specific activity of enzyme was found to be 3.7 units/ml and the lowest specific activity of was found to be 0.9 units/ml. These results that obtained by [17] who detected highest specific activity of urease as 3.9 unit/ml.

**Table 4: Urease activity produce by isolate of *P.mirablis***

| Isolates NO. | Absorbance at 588 nm | Ammonia conc. (u mole) | Specific activity (unit /ml) |
|---|---|---|---|
| 1 | 0.381 | 106 | 3.5 |
| 2 | 0.176 | 49 | 1.6 |
| 3 | 0.384 | 107 | 3.5 |
| 4 | 0.273 | 76 | 2.5 |
| 5 | 0.186 | 52 | 1.7 |
| 6 | 0.372 | 104 | 3.4 |
| 7 | 0.224 | 62 | 2.0 |
| 8 | 0.357 | 99 | 3.3 |
| 9 | 0.121 | 34 | 1.1 |
| 10 | 0.362 | 100 | 3.3 |
| 11 | 0.375 | 104 | 3.4 |
| 12 | 0.210 | 59 | 1.9 |
| 13 | 0.281 | 78 | 2.6 |
| 14 | 0.216 | 60 | 2.0 |
| 15 | 0.134 | 37 | 1.2 |
| 16 | 0.382 | 106 | 3.5 |
| 17 | 0.250 | 70 | 2.3 |
| 18 | 0.399 | 111 | 3.7 |
| 19 | 0.375 | 104 | 3.4 |
| 20 | 0.284 | 79 | 2.6 |
| 21 | 0.140 | 39 | 1.3 |
| 22 | 0.218 | 61 | 2.0 |
| 23 | 0.281 | 78 | 2.6 |
| 24 | 0.115 | 32 | 1.0 |
| 25 | 0.350 | 97 | 3.2 |
| 26 | 0.102 | 29 | 0.9 |
| 27 | 0.112 | 31 | 1.0 |

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| | | | |
|---|---|---|---|
| 28 | 0.213 | 59 | 1.9 |
| 29 | 0.236 | 66 | 2.2 |
| 30 | 0.221 | 62 | 2.0 |
| 31 | 0.211 | 59 | 1.9 |
| 32 | 0.218 | 61 | 2.0 |
| 33 | 0.152 | 42 | 1.4 |
| 34 | 0.169 | 47 | 1.5 |
| 35 | 0.371 | 103 | 3.4 |
| 36 | 0.150 | 42 | 1.4 |
| 37 | 0.098 | 27 | 0.9 |
| 38 | 0.110 | 31 | 1.0 |
| 39 | 0.315 | 88 | 2.9 |
| 40 | 0.099 | 28 | 0.9 |

## Detection of Adhesion in *P. mirabilis*

Adhesive ability of *p.mirablis* to epithelial cells isolated from female urine was compared by the frequency of distribution of bacteria on epithelial cells and by the mean number of bacteria adhering to (20) epithelial cells and considered as criteria for adhesive capability of cells, as long as adhering bacterial cells visible under light microscope and easy to count, all the isolates displayed differences in their adherence to human epithelial cells and showed a mean number of adhering bacteria ranging from(10.75 ± 0.63) to (18.00 ± 1.05) bacteria /epithelial cell as indicated in Table(5) It was noticed that isolates no. 1, 3, 6, 8 , 10, 11, 16 , 18 , 19 and 35 displayed high adhesive capability with mean (18.00 ± 1.05), (16.50 ± 0.89), (17.00 ± 1.26), (15.75 ± 0.86), (17.50 ±1.16), (17.25 ± 0.97), (17.75 ± 1.38), (16.00 ± 1.07) and (16.50 ± 1.15) bacteria /epithelial cell respectively, while the isolate 24 displayed a lowest adhesive capability with mean (10.75 ± 0.63) bacteria /epithelial cell. These result [18].Who found that forty bacteria/ uroepithelial cell of adhesion averagedof *P. mirabilis* on uroepithelial cells.

**Table 5: Adhesion average of *P. mirabilis* to uroepithelial cell**

| No. of *P.mirablis* isolates | No. of adherent *P. mirabilis* to No. of epithelial cells | | | | Mean No. of adherent *P. mirablis* /cells ± SD |
|---|---|---|---|---|---|
| | 0 | 1-5 | 6-20 | >20 | |
| 1 | 0 | 1 | 6 | 13 | 18.00 ± 1.05 |
| 2 | 2 | 10 | 3 | 5 | 12.75 ± 0.63 |
| 3 | 0 | 4 | 6 | 10 | 16.50 ± 0.89 |
| 4 | 0 | 6 | 14 | 0 | 13.50 ± 0.52 |
| 5 | 1 | 12 | 5 | 2 | 12.00 ± 0.48 |
| 6 | 0 | 2 | 8 | 10 | 17.00 ± 1.26 |
| 7 | 2 | 8 | 7 | 3 | 12.75 ± 0.73 |
| 8 | 2 | 3 | 5 | 10 | 15.75 ± 0.86 |
| 9 | 2 | 13 | 5 | 0 | 10.75 ± 0.69 |
| 10 | 0 | 2 | 6 | 12 | 17.50 ± 1.16 |
| 11 | 0 | 2 | 7 | 11 | 17.25 ± 0.97 |
| 12 | 0 | 6 | 10 | 4 | 14.50 ± 0.61 |
| 13 | 0 | 12 | 6 | 2 | 12.50 ± 0.74 |
| 14 | 2 | 9 | 4 | 5 | 13.00 ± 0.68 |
| 15 | 2 | 9 | 6 | 3 | 12.50 ± 0.71 |
| 16 | 0 | 2 | 5 | 13 | 17.75 ± 1.38 |
| 17 | 0 | 7 | 13 | 0 | 13.25 ± 0.74 |
| 18 | 2 | 2 | 6 | 10 | 16.00 ± 1.07 |
| 19 | 2 | 3 | 5 | 10 | 15.75 ± 0.86 |
| 20 | 0 | 8 | 8 | 4 | 14.00 ± 0.82 |
| 21 | 1 | 9 | 6 | 4 | 13.25 ± 0.77 |
| 22 | 1 | 8 | 7 | 4 | 13.50 ± 0.82 |
| 23 | 0 | 10 | 7 | 3 | 13.25 ± 0.75 |

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| 24 | 2 | 13 | 5 | 0 | 10.75 ± 0.63 |
|---|---|---|---|---|---|
| 25 | 0 | 6 | 14 | 0 | 13.50 ± 0.78 |
| 26 | 1 | 12 | 5 | 2 | 12.00 ± 0.61 |
| 27 | 0 | 6 | 10 | 4 | 14.50 ± 0.95 |
| 28 | 0 | 12 | 5 | 3 | 12.75 ± 0.72 |
| 29 | 3 | 10 | 7 | 0 | 11.00 ± 0.47 |
| 30 | 1 | 12 | 5 | 2 | 12.00 ± 0.51 |
| 31 | 1 | 6 | 8 | 5 | 14.25 ± 0.82 |
| 32 | 1 | 8 | 6 | 5 | 13.75 ± 0.76 |
| 33 | 2 | 6 | 7 | 5 | 13.75 ± 0.81 |
| 34 | 0 | 8 | 7 | 5 | 14.25 ± 0.79 |
| 35 | 0 | 4 | 6 | 10 | 16.50 ± 1.15 |
| 36 | 1 | 12 | 5 | 2 | 12.00 ± 0.49 |
| 37 | 1 | 10 | 6 | 3 | 12.75 ± 0.77 |
| 38 | 2 | 12 | 6 | 0 | 11.00 ± 0.46 |
| 39 | 0 | 12 | 6 | 2 | 12.50 ± 0.65 |
| 040 | 0 | 6 | 9 | 5 | 14.75 0.92 |

## Amplification of *ure C* by Convential PCR Techniques

Amplification of urease gene was done using specific PCR primer. Results shown in figure indicate successful amplification of the gene for all isolates as indicated by the presence of band with molecular weight 317 bp. Regarding Ure C gene, these results are with a previous study by[19] who indicating the presence of Ure C about 96.6% isolates obtained from UTI patients.

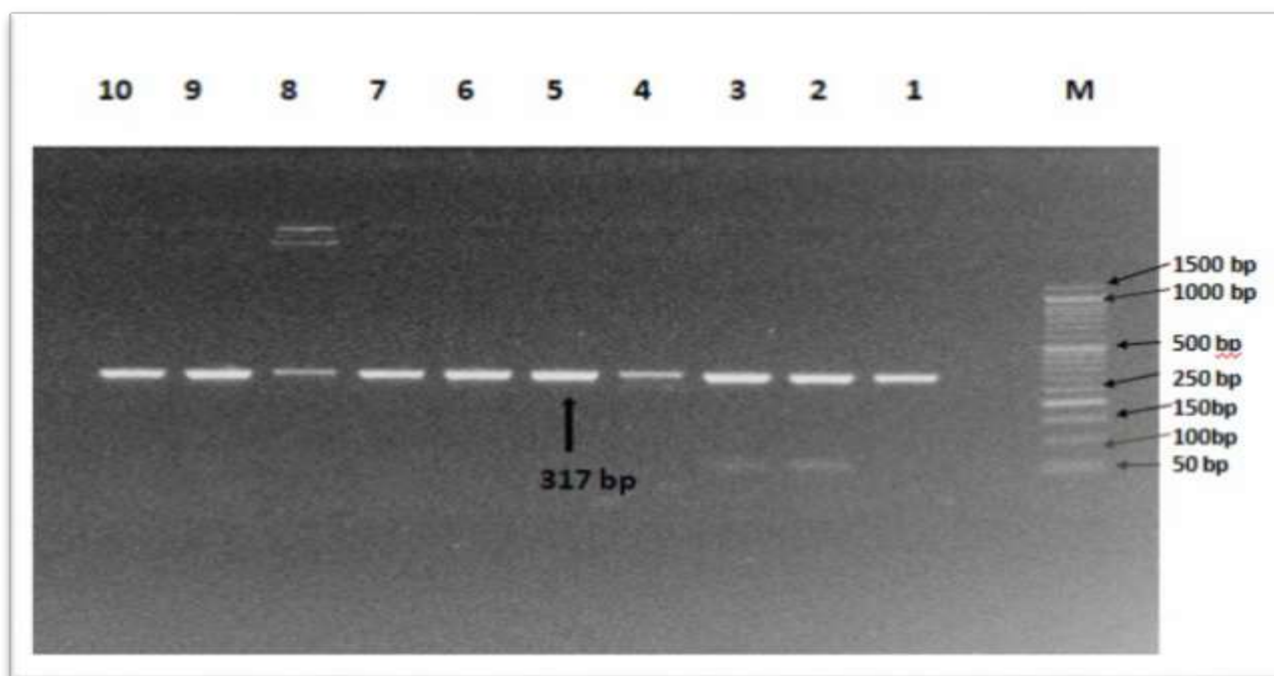


**Figure 2: Gel electrophoresis for amplified ure C gene of proteus mirabilis Lane M ladder (100 bp), Line: 1-10 represent isolates 1, 3, 6, 8, 10, 11, 16, 18, 19 and 35 respectively**

## Amplification of mrp A by Convential PCR Techniques

Amplification of *mrpA* gene was done using specific PCR primer. Results shown in Figure (3) indicate successful amplification of the gene for all isolates as indicated by the presence of band with molecular weight 648bp. This results [20] who found that *P. mirabilis mrpA* fimbrial gene was present in all isolates. This particular type of fimbriae is frequently related to *P. mirabilis* UTI pathogenesis.

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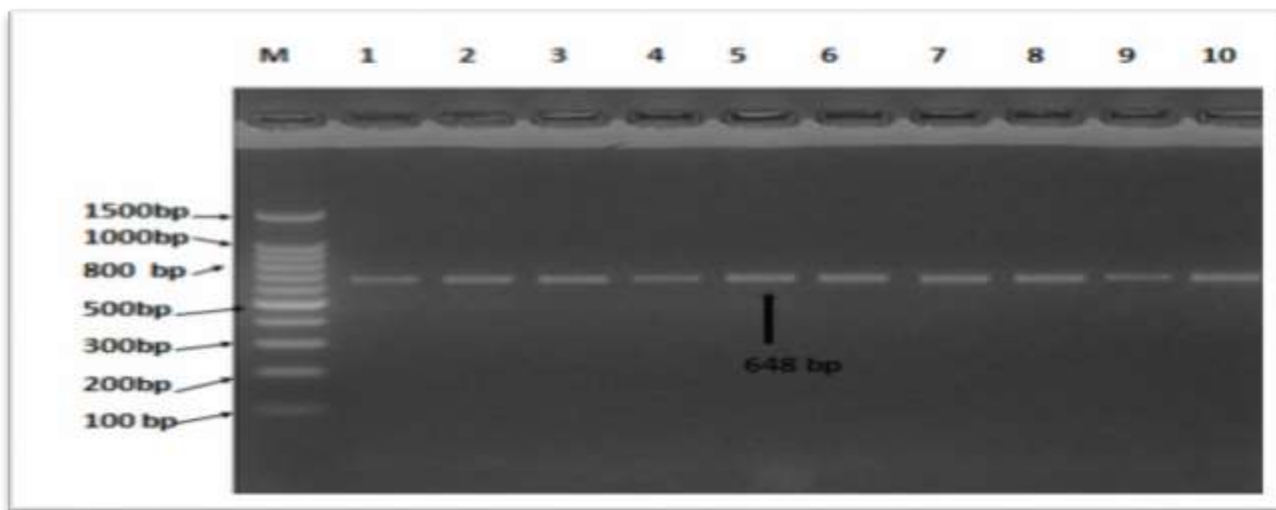


**Figure 3: Gel electrophoresis for amplified mrpA gene of proteus mirabilis Lane M ladder (100 bp), Line: 1-10 represent isolates 1, 3, 6, 8, 10, 11, 16, 18, 19 and 35 respectively**

## Sequence Analysis of *mrpA* gene

The sequence homology of *mrp*A gene was obtained by comparison with closest blast sequence in gene bank. Similarity was calculated using FASTA method. Data shown in table (6) indicate as much as 100% similarity of four isolates with that sequence of *P.mirablis* AR-0029.however, isolate 19 shows 100% homology with that of *P.mirablis* AR-0156.

**Table 6: Average similarity percentage of *mrpA* sequences of *P. mirablis* isolated from UTI patients in comparsion to sequence obtained from NCBI blast**

| No. of local isolate | NCBI Blast Alignment | *mrpA* Sequence Identity (%) |
|---|---|---|
| 1 | *P.mirablis* AR-0029 | 100% |
| 2 | *P.mirablis* AR-0029 | 100% |
| 3 | *P.mirablis* AR-0029 | 100% |
| 4 | *P.mirablis* AR-0029 | 100% |
| 5 | *P.mirablis* AR-0156 | 100% |

These results were in agreement with several other investigations namely; Pathirana, (2018) show that *mrpA* gene of *P. mirabilis* represents 96.40% of mean similarity and pet turtles to human respiratory isolates using the same gene of *P. mirabilis* represents 94.90% of mean similarity also [20] found similarity in *mrpA* amplicon sequences of chicken to human isolates.

## Sequence Analysis of *ure C* Gene

The sequence homology of ure c gene was obtained by comparison with closest blast sequence in gene bank. Similarity was calculated using FASTA method. Data shown in Table (7) indicate as much as 100% similarity of two isolated with that sequence of p.mirablisAR-0159. However, two isolate show 100% homology with that of P. mirablis AR-K1609 and other isolate show 100% homology with that of P.mirablisAR-379

**Table 7: Average similarity percentage of *ure C* sequence of *P.mirablis* isolated from UTI patients in comparsion to sequence obtained from NCBI blast**

| No. of Local Isolate | NCBI Blast Alignment | *Ure C* Sequence Identity (%) |
|---|---|---|
| 1 | *P.mirablis* AR-0159 | 100% |
| 2 | *P.mirablis* AR-379 | 100% |
| 3 | *P.mirablis* AR-0159 | 100% |
| 4 | *P.mirablis* AR-k1609 | 100% |
| 5 | *P.mirablis* AR-K1609 | 100% |

These results were several other investigation who [21] show *ure c* gene of *P. mirabilis* represents of obtained were sequenced and BLAST search, which revealed 100% identity.

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