MOLECULAR DETECTION OF UREC, HPMA, RSBA AND MRPA GENES OF PROTEUS MIRABILIS URINARY TRACT INFECTION IN PATIENT WITH RHEUMATOID ARTHRITIS

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ABSTRACT
This study was aimed to detect of four important virulence genes (UreC, HpmA, rsbA and mrpA) from Proteus mirabilis bacteria isolated and do the important four blood test (ACPA, RF, ESR, CRP) for patient with urinary tract infection and rheumatoid arthritis in Baghdad/Iraq. Fifty (50) clinical urine samples were collected and cultured on blood agar and maccckongy agar. Ten of the samples were identified as P.mirabilis and the automated system VITEK 2 used to complete the identification. Molecular methods using PCR technique targeting UreC, HpmA, rsbA and mrpA genes, using PCR technique and the results were UreC found in 90% of the samples, HpmA 90%, rsbA 100% and mrpA 80%. The P. mirabilis samples were also characterized for antibiotic resistance and the result showed that the majority of samples were show high resistant to Cefotaxime 90%, while the highest susceptibility was against Impenem antibiotic 70%.

Key words: automated system, targeting, PCR, blood culture bacteria infection,
INTRODUCTION
Rheumatoid arthritis RA is one of the more common autoimmune diseases, affecting approximately 1% of the population worldwide. The exact cause of RA is not known; however, initiation of disease seems to cause by an interaction among genetic susceptibility, environmental triggers, and chance. Different immunological and microbiological studies results support that there could be a link between urinary tract infections (UTI) and RA, this has mainly caused by P. mirabilis bacterium (10). Proteus mirabilis is a Gram-negative, facultative anaerobic rod shaped bacterium. It may found as part of the normal flora in intestine. This organism is not usually a pathogen, but does become a problematic when it comes into contact with urea in the urinary tract. So that, the infection can spread to the other parts of the body. This bacterium is well-known for its urease enzyme production and distinctive ability to differentiate into elongated swarm cells. P. mirabilis bacteria have a significant role in causing urinary tract infection which can be defined as the inflammatory response of urothelium to bacterial invasion, which is usually associated with bacteriuria and pyuria. A UTI can occur anywhere in the Urinary tract. It includes (urethritis, cystitis, pyelonephritis, epidydimitis, prostatitis, perinephritis), and it is considered as one among the most common infectious diseases that are widely seen among all age groups of individuals (9). Since Proteus mirabilis had many virulence factors that were important for inflicting UTIs, these factors had an importance role to make an infection in different areas of the urinary tract (17), including toxins like hemolysin and its function of pore formation, biofilm and regulation of the pathogenicity (14). Urease enzyme which causes kidney and bladder stones (2). The MR/P fimbriae are the most well-studied fimbriae of P. mirabilis, which are potentially involved in adhesion to the uroepithelium, contributes to the ability of an organism to establish infection in the urinary tract (15). The rsbA gene was regulator of swarming behavior that encodes a sensory, RsbA function may as a protein sensor of environmental conditions. It stimulated bio film formation and Extracellular polysaccharide formation (11, 12). This study was aimed to detect of four important virulence genes (UreC, HpmA, rsbA and mrpA) from Proteus mirabilis

MATERIALS AND METHODS
A total of 50 urine samples were collected from patients with UTI and RA. Samples were collected from patients during the period from October 2018 the end of August 2019 From the Medicine City/Baghdad Teaching Hospital, Medicine City/ Teaching Laboratory, private laboratories in Baghdad/Iraq. The urine of these 10 patients were identified by methods of routine tests: colony morphology (blood and macckongy agar), biochemical tests including; catalase test, oxidase reaction, urease test and also VITEK 2 system used for more identification.

Blood tests
Five millilitres of venous blood has been collected and divided into parts, some putted into Erythrocyte sedimentation rate (ESR) tube and the remained putted in a clean plain tube and allowable to clot at 37 C˚ for 10 minutes (for ESR test). Then centrifuging at 2500 rpm for 5 mine. until serum separaeted. Serum is storeed at 20 C˚ until used for the other three tests (RF, CRP, and ACPA).

Antimicrobial susceptibility
The antibiotic susceptibility tests was carried out for all the P. mirabilis isolates using Mueller-Hinton method to measure the zones of inhibition in accordance with the recommendations of clinical and laboratory standards institute. The antibiotics used were: (F): Nitrofuranton (100mcg/disc), (AK): Amikacin (10mcg/disc), (GM): Gentamicin (10mcg/disc), (APX): Ampicillin/cloxacillin (30mcg/disc), (C): CeftaCloramphenicol (30mcg/disc), (CTX): Cefotaxime (30mcg/disc), (IMP): Imipenem (10 µg/disc), (RP): Rafampicin (2µg/disc).

Molecular detection
DNA was extracted from activated pure culture of P. mirabilis bacteria using genomic DNA Bacteria Kit (geneaid). Detection of DNA bands using Agarose gelelectrophoresis (1%). Then conventional PCR were used in this study to detect the presence of the important virulence genes that related with causing UTI and RA (UreC, HpmA, rsbA and
mrpA) of the *P. mirabilis* bacteria using specific primers as shown in Table 1.

### Table 1. The Optimum Condition of Detection Gene

| Genes Name | primer Sequences | Size (bp) | Conditions | Ref. |
|------------|------------------|-----------|------------|------|
| Urec       | F: CCG GAA CAG AAG TTG TCG CTG GA R: GGG CTC TCC TAC CGA CTT GAT C | 533 | 94°C 3min <br> 94°C 1min <br> 63°C 30sec40x <br> 72°C 1min <br> 72°C 7min | (D’Orazio SE, et al., 1996) |
| rsbA       | F: TTG AAG GAC GCG ATC AGA CC R: ACT CTG CTG TCC TGT GGG TA | 467 | 94°C 5min <br> 94°C 60sec <br> 58°C 45sec35x <br> 72°C 1min <br> 72°C 7min | (Badi, S. et al., 2014) |
| hpmA       | F: GTT GAG GGG CTT TAT CAA GAG TC R: GAT AAC TGT TTT GCC CTT TGG TGC | 709 | 95°C 5min 1x <br> 95°C 30sec <br> 62°C 30sec 30x <br> 72°C 20 sec <br> 72°C 5min 1x | (Cestari et al., 2013) |
| 16sRNA     | F: GTG TAG CGG TGA AAT GCG R: ACG GGC GGT GTG TAC AA | 700 | 95°C 5min 1x <br> 95°C 30sec <br> 62°C 30sec 30x <br> 72°C 20 sec <br> 72°C 5min 1x | (Brause, 2003) |
| mrpA       | F: TTC TTA CTG ATG AGA CAT TG R: ATT TCA GGA AAC AAA AGA TG | 565 | 94°C 3min <br> 94°C 30sec <br> 40°C 30sec40x <br> 72°C 30sec <br> 72°C 7min | (Sosa, et al., 2006) |

**Protocol**

The PCR amplification mixture which used for detection of each gene includes FIREPol® Master Mix 5X (4 μl), 5 μl of DNA template, 1 μl (1 mM) of each forwarded and reversed primers and 9 μl of nuclease free water to complete the amplification mixture to 20μl, (Table 2).

### Table 2. PCR mixture components for each gene

| Component                  | Volume | Final Concentration |
|----------------------------|--------|---------------------|
| FIREPol® Master Mix , 5X   | 4 μl   | 1X                  |
| Forward primer             | 1 μl   | 1 μM                |
| Reverse primer             | 1 μl   | 1 Mm                |
| DNA template               | 5 μl   | 25 ng               |
| Nuclease free dh2O         | 14 μl  |                     |
| Final volume               | 25 μl  |                     |

After preparing the reaction volume in PCR tube the mixture was spin down and then PCR tube placed in the PCR thermo cycler and the amplification reactions was started according to the program described in Table 1.

**RESULTS AND DISCUSSION**

A total of 50 urine sample of patient with UTI and RA that admitted to different hospitals in Baghdad/Iraq during the period, from October 2018 to August 2019, from both gender with age ranged from 30-85 years old. All colonies were diagnosed in the lab as *P. mirabilis* due to characteristic swarming motility on blood agar, and inability to metabolize lactose (on MacConkey agar plate) (Fig. 1). Also *P. mirabilis* produces a very distinct fishy odor. in another hand all 10 isolates gave negative result for oxidase, catalase and positive results for urease test.
Fig. 1. *Proteus mirabilis* on Blood agar (A) and Macconkey agar (B).

The resistance pattern to nine antimicrobials disks was shown in Fig. (2). It shows high resistance reach to 70% against Gentamycin & Nitrofurantoin, Cefotaxime 90%, while the highest susceptibility was to the Imipenem antibiotic which was 70%.

![Fig 2. Show the resistance of bacteria to a variety of the antibiotic](image)

The extraction of chromosomal DNA was done, from all 10 isolates that diagnosed by VITEK 2 system. The results of gel electrophoresis showed the presence of DNA bands in the same level for all isolates in agarose gel (Fig. 3). Then, the concentration and purity of extracted DNA was measured by QuantiFluor® (Promega, USA).

![Fig 3. Genomic DNA extraction electrophorasis (1% agarose gel at 75 volt for 30min)](image)
Further molecular diagnosis for *P. mirabilis* bacteria by using 16sRNA gene specific primer, and the results were detected by electrophoresis on 2% agarose and exposed to U.V light in which the results indicates that all (10) isolates give the same result for biochemical diagnosis. (Fig. 4).

Fig. 4. 16sRNA (of size 700bp) extraction electrophorasis on 2% agarose gel at 75 volt for 30min

Approximately nine of all the isolated (10) samples gave positive results for *UreC & HpmA* genes (except one sample). The results of *UreC* and *HpmA* gene with molecular size 533bp and 709bp respectively were shown in Fig. 5 and 6. The gene α-hemolysin is encodes by two genes, *hpmA* and *hpmB*, that regulate the HpmA (166 kDa) proteins. α-hemolysin is responsible for damaging tissue and activating when its N-terminal peptide has cleaved, so the results can be activating HpmA (140 kDa) (8, 12)

Fig. 5. Agarose gel electrophoresis (2% agarose, 75 V for 1:45 hour) of *UreC* gene PCR products (533bp) codify for urease enzyme of *P. mirabilis* isolates

Fig. 6. Agarose gel electrophoresis (2% agarose, 75 V for 1:45 hour) of *hpmA* gene PCR products (709bp) codify for hemolysin A of *P. mirabilis* isolates.

While the result of gel electrophoresis for amplification PCR product showed that the presence of bands of *rsbA* in all the samples, which means that the primer of *rsbA* gene bind specifically to complement sequence within DNA template (Fig. 7).
For *mrpA* genes detection, a primer of 565bp were used and out of the ten *P. mirabilis* isolates eight of them were carrying this gene (Fig. 8).

Based on the results of this study carried out in relation to Proteus microbes table (3), it could be said that compelling evidence exists linking this microbe to RA, starting with recurrent sub-clinical Proteus UTIs and ending in the full development of RA. To prove the scientific logic of this possibility, many blood tests was done including (ESR, CRP, RF and AcpA) all of these tests was high in the patients whom their urine are having *P. mirabilis* bacteria. A meta-analysis has shown that the pooled sensitivities of ACPA and RF are similar, but ACPA positivity is more specific for RA than IgM RF, IgG RF, or IgA RF positivity Furthermore, the “shared epitope” EQRRAA and type XI collagen sequence LRREI each contain an arginine doublet which could be acted upon by (PAD) enzymes during inflammatory episodes and to produce further quantities of CCPs. There is thus a clear link between Proteus bacteria and the presence of AcpA in the early stages of RA (13).

### Table 3. Represented the results of blood tests for the 10 samples isolates

| Samples no. | Gender | Age | Types of bacteria | ESR (mm/hr) | RF  | CRP (Mg/dl) | ACPA (U/ml) |
|-------------|--------|-----|-------------------|--------------|-----|-------------|-------------|
| 1           | Female | 76  | *P. mirabilis*    | 73           | +   | 60          | 67          |
| 2           | Male   | 72  | *P. mirabilis*    | 62           | +   | 94          | 68          |
| 3           | Female | 83  | *P. mirabilis*    | 81           | +   | 80          | 72          |
| 4           | Female | 73  | *P. mirabilis*    | 33           | +   | 52          | 45          |
| 5           | Female | 82  | *P. mirabilis*    | 39           | +   | 48          | 43          |
| 6           | Female | 67  | *P. mirabilis*    | 41           | +   | 63          | 47          |
| 7           | Male   | 78  | *P. mirabilis*    | 67           | +   | 80          | 33          |
| 8           | Female | 59  | *P. mirabilis*    | 52           | +   | 73          | 49          |
| 9           | Female | 66  | *P. mirabilis*    | 61           | +   | 62          | 70          |
| 10          | Female | 61  | *P. mirabilis*    | 70           | +   | 78          | 81          |
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