MOLECULAR DETECTION OF UREASE AND HEMOLYSINE GENES IN PROTEUS MIRABILIS BACTERIA ISOLATED FROM URINE OF RHEUMATOID ARTHRITIS PATIENTS IN NAJAF PROVINCE

Abbas Kareem Magdy  
Department of pathological analyzes, College of Science, University of Kufa

Fatima Ali Hamid  
Department of pathological analyzes, College of Science, Sumer University

Yasmine Khudair Nasser  
Department of pathological analyzes, College of Science, Sumer University

Khlas Kazim Awaid  
Department of pathological analyzes, College of Science, wasit University

Ayaat Abbas Jawad  
Department of pathological analyzes, College of Science, Al_Basra University

Received: Feb 22, 2024; Accepted: March 29, 2024; Published: Jun 07, 2024;

Abstract: 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. According to the suggestion that Proteus microbes could possibly be involved in the etiopathogenesis. Proteus mirabilis was the commonest bacteria isolated in this study 15 (51% ) followed by E. coli in 6 ( 20.4 ) , Klibsiella spp.5 (17%) and Staphylococcus saprophiticus 3 (10.3%). All isolates of P. mirabilis appeared highest rate of resistance (100%) in the case of Cefotaxime, Amoxicillin, Ampicillin. And moderately resistance to Netilmicin (71.4% ). All isolates presented high sensitivity (100%) to Meropenem, Levofloxacain, and Ciprofloxacin. All isolates15 (100%) appeared the presence of urease and hemolysine genes by using PCR technique.

Keywords: Molecular, Detection, Arthritis Patients

Introduction

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 (AL-Hamadani et al ., 202). 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 (Hussien, 2016). 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 (Stankowska et al., 2008), including toxins like hemolysin and its function of pore formation, biofilm and regulation of the pathogenicity (Schaffer & Pearson, 2015). Urease enzyme which causes kidney and bladder stones (Al-Duliami et al., 2011). 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) mainly caused by P. mirabilis bacterium and RA (Gibofsky et al., 2014).

Infectious agents have been suspected as potential triggers of RA for a long time, and the search is still in progress. The suggestion that Proteus microbes could possibly be involved in the etiopathogenesis of RA and large number of studies have shown that antibodies against P. mirabilis were significantly elevated among RA patients but not in healthy controls (Ebringer et al., 2003). Furthermore, in an earlier study increased antibodies to P. mirabilis only, but not to four viruses; influenza, adenovirus, rubella and parvo-virus, and autoantigens were observed in RA patients in comparison to healthy controls. (Deighton et al., 1992). The association of RFs with increased anti-Proteus antibody titres in patients with RA could be explained on the basis that these anti-IgG auto-antibodies are produced secondarily as the result of B cells stimulation by exogenous and microbial antigens. RFs, however, are more likely to be involved in immune complex-mediated damage in RA, especially in those patients with associated ‘vasculitis possibly through activation of the complement system. (Kato, 2000).

This study was aimed to detect of two important virulence genes (UreC& HpmA) from Proteus mirabilis isolated from urine of RA patients. The Objective of this study include:

1. Isolation and identification of Proteus mirabilis bacteria from urine of RA patients by using convention methods and vitek system.
2. Phenotypic detection of Proteus mirabilis some virulence factors.
3. Detection of antibiotic susceptibility of Proteus mirabilis isolates to the most 7 antibiotic used for treatment
4. Molecular detection of urease (UreC,) and hemolysin (HpmA) genes as important virulence genes in in Proteus mirabilis isolated from RA patients by using PCR technique.

2.1 General characteristic of Proteus mirabilis

Proteus mirabilis is a member of the Enterobacteriaceae family and is a highly motile bacterium. Unlike the other members of Enterobacteriaceae, new phylogenetic tree classification based on shared core proteins, ribosomal proteins, and four multilocus sequence analysis proteins placing Proteus within a new Morganellaceae family (Adeolu et al., 2016).

Proteus bacilli are dimorphic bacteria. when grown in aliquid medium, the cells display swimming behavior and have a distinct morphology; i.e., they are motile, peritrichous flagellated(6 to 10 flagella per cell) rods, 1.0 to 2.0 mm in length. These bacilli, referred to as
swimmer cells, are similar in many aspects of their physiology to other members of the family Enterobacteriaceae. When transferred to a solid medium, Proteus bacilli undergo morphogenesis to swimmer cells and swarm over the surface of solid medium. This kind of growth of Proteus rods on solidified nutrient medium is termed the swarming phenomenon (Eberl et al., 1999).

*P. mirabilis* is well known for its ability to produce urease, which generates ammonia and elevates the pH of the urine to > 7.2.5 Calcium and magnesium crystallization in the urine of alkaline pH blocks the catheter lumen and causes acute urinary retention and the development of bacteriuria and other ascending infections6

*P. mirabilis* can be found in a wide variety of environments, including soil, water sources, and sewage, but it is predominantly a commensal of the gastrointestinal tracts of humans and animals (2, 3). While the bacterium is capable of causing a variety of human infections, including those of wounds, the eye, the gastrointestinal tract, and the urinary tract, it is most noted for infections of the catheterized urinary tract, known as catheter-associated urinary tract infections (Sanmarti et al., 2009).

*P. mirabilis* is not a common pathogen that causes urinary tract infections (UTIs) in normal hosts (Stankowsks et al., 2008). In contrast, *P. mirabilis* is isolated relatively frequently in complicated UTIs, such as those that present in patients with functional or anatomical abnormalities, especially patients with urolithiasis or a chronic indwelling urinary catheter (Cestari et al., 2013).

2.3 Pathogenesis and virulence factors

The pathogenic virulence factors of Proteus has been included fimbriae; flagella; enzymes: urease, (hydrolyzing urea to CO2 and NH3); proteases degrading antibodies, deaminase amino acid; tissue matrix proteins and proteins of complement system; iron acquisition systems and toxins: hemolysins, Proteus toxin agglutinin (Pta), and lipopolysaccharide (LPS) endotoxin (Nielubowicz & Mobley 2010).

The hemolytic activity produced by *P. mirabilis* is associated to hemolysin HpmA. This hemolysin is associated to the cell, calcium-independent, former of pores, encoded by two genes, hpmA and hpmB, that regulate the HpmA (166 kDa) and HpmB (63 kDa) proteins, respectively. HpmA hemolysin is responsible for tissue damage and is activated when its N-terminal peptide is cleaved, resulting in active HpmA (140 kDa). HpmB is responsible for HpmA activation and transport.

Clearly, *P. mirabilis* possesses an impressive arsenal of virulence factors (Fig. 1). Urease is a critical feature of this species, but the bacterium also expresses a startling number of fimbriae and other adhesins. The most well-studied fimbria is the mannose-resistant *Proteus*-like (MR/P) fimbria, whose expression is phase variable. As well, a variety of potent toxins and proteases compound virulence. Similar to other members of the *Enterobacteriaceae*, *P. mirabilis* carries numerous secretion systems, including types I, III, IV, V, and VI.

The important virulence factor of *Proteus mirabilis* includes

1. Adhesions

Attachment to the target cell may be the first essential step in the establishment of any pathogen in the host. Fimbria-mediated adherence has been suggested to contribute to pathogenesis of Proteus spp. Proteus mirabilis’s bacterial adhesion is a key step in colonizing and developing infections, which is done by fimbriae (Hasan, 2020). This bacteria attaches to and swarms across the surface of urinary catheters to gain a foothold.
fimbriae are surface appendages sometimes called pili which are shorter and finer than flagella and they are composed of structural protein subunits termed pillins. There are two classes of pili: ordinary pili which play a role in the adherence to host cells and sex pili which are responsible for the attachment of bacterial cells to each other during conjugation (Brooks et al., 2007).

There are five common fimbriae type that implicated in infection as following:

A. **Mannose-resistant / Proteus-like (MR/P) fimbriae**: This kind of fimbria is the most appropriate form in Proteus mirabilis

B. **B. Uroepithelial cell adhesion (UCA/NAF) fimbriae**: This type of fimbriae is taking the form of long flexible rods, the UCA/NAF fimbriae have an essential adhesion role to the uroepithelial cells also it has an important role in the colonization of the urinary tract (Jiang et al., 2018).

C. **Ambient-temperature fimbriae (ATF)**: In the ambient way of Proteus mirabilis, the ATF fimbriae are important (Hasan, 2020b).

D. **Proteus mirabilis fimbriae (PMF)**: The role of PMF fimbriae that shown is in old studies is introducing in the bacterial cells colonizing of urinary tract lower part but not the kidneys, recently, the studies proved that the role of PMF fimbriae is vital in the colonization of bacterial cells to bladder and kidneys (Sarshar et al., 2020).

E. **Proteus mirabilis P-like pili (PMP) fimbriae**: PMP fimbriae were first identified from a dog with urinary infection by Proteus mirabilis strain (Debnath et al., 2018).

1. **Motility**

   The motility is the most important virulence factor in Proteus mirabilis that influence in invading and spreading of infection in urinary tract parts (Kuan et al., 2014). The infection begins with the invasion of the periurethral region, then it travels through the urethra and progresses to the bladder, other areas of the urinary tract (Hickling et al., 2017).

   The presence of flagella on the surface of pathogenic and opportunistic bacteria has been thought to facilitate the colonization and dissemination from the initial site (Shirtliff and Leid, 2009).

   Swarming is a multicellular differentiation phenomenon that allows a population of bacteria to migrate on a solid surface in a coordinate manner. It is important in movement of Proteus species to new locations and most probably helps them in the colonization of microorganisms. (Verstraeten et al., 2008).

2. **Toxins and Enzymes**

   - **Hemolysin**: Hemolysin is a toxin that enters the target membrane of the eukaryotic cell and causes pores that trigger ion efflux and then cell disruption (Cabezas et al., 2017). Hemolysin promotes bacterial infection spread in the kidney and pyelonephritis develops in ascending UTIs (Los et al., 2013). Proteus mirabilis hemolysin genes are a two-part secretion (hpmA and hpmB) (Etxaniz et al., 2020).

   - **Proteus toxic agglutinin (Pta)**: Proteus toxic agglutinin is a protein that is designated as the outer membrane autotransporter that mediates aggregation of the cell and includes the catalytic α-domain that can lyse the kidney and bladder cells (Gupta et al., 2019). The Proteus mirabilis negative pta gene had reduced pathology, as well as a serious
colonization defect in urine, kidneys, and spleen (Engel et al., 2007).

B. Urease: Urease is significant in Proteus mirabilis pathogenesis, which catalyzes stones formation in the kidney and bladder or inhibits the urinary tract (Flannery et al., 2009). Urease is extremely important for Proteus mirabilis pathogenesis (Ranjbar et al., 2015). This enzyme catalyzes the formation of stones in the kidney and bladder or encrusts or obstructs the urinary tract (Armbruster et al., 2017). The cluster of urease genes (ureRDABCEFG) codifies the multimeric nickel metallo enzyme, which hydrolyzes urea into ammonia and dioxide, increases the PH, and induces multipurpose urinary ions.

Methods
2.1 Sample collection
A total of 50 urine samples were collected from patients with RA. Samples were collected from patients during the period from October 2021 to the end of March 2022 from the Medicine City/Al-Sadder Teaching Hospital in AL-Najaf/Iraq. The urine of all patients were cultured on different media colony morphology (blood and mackongy agar), biochemical tests including; catalase test, oxidase reaction and urease test were used for more identification.

2.2 Preparation of Buffer and Chemical solutions:
2.2.1 McFarland’s Standard Solution No.0.5 (1.5 X 10⁸) cell/ml The 0.5 McFarland standard tube was prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate (BaCl₂.2H₂O) solution to 99.5 ml of 1% (vol/vol) H₂SO₄. The turbidity standard was liquated into test tubes identical to those used to prepare the inoculum suspension. The McFarland standard tubes were sealed with parafilm to prevent evaporation and stored for up to 6 months in darkness at room temperature. Accuracy of the density of a prepared 0.5 McFarland standard was checked by using a spectrophotometer.

Results and Discussion
Out of 50 urine sample were collected from patient (39 female and 11 male) with UTI and RA that admitted to Medicine City/Al-Sadder Teaching Hospital in Najaf/Iraq during the period from October 2021 to the end of March 2022 from both gender with age ranged from 30-70 years old. Proteus mirabilis was the commonest bacteria isolated in this study 15 (51%) followed by E. coli in 6 (20.4%), Klubiella spp. in 5 (17%) and Staphylococcus saprophiticus in 3 (10.3%).

All P. mirabilis colonies gave biochemical result as in table (4.2). swarming motility on blood agar, and inability to metabolize lactose (on MacConkey agar plate) (Fig. 4.1). Also P. mirabilis produces a very distinct fishy odor. in another hand all 15 isolates gave negative result for oxidase, catalase and positive results for urease test.

Table (4.1): Distribution of bacteria isolated from urine of RA patients

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteus mirabilis</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Catalse</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td></td>
</tr>
<tr>
<td>Methyl red</td>
<td></td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td></td>
</tr>
<tr>
<td>Simmons Citrate</td>
<td>+</td>
</tr>
<tr>
<td>H2S production</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Kligler agar</td>
<td></td>
</tr>
</tbody>
</table>

Table(4.2) Biochemical result of *Proteus mirabilis* isolated in this study

Figure(4.1): A- *P. mirabilis* on blood agar B-urease production

4.3 Antibiotic susceptibility of *P. mirabilis*

Table (4-3) show the resistance of *Proteus mirabilis* to 7 antimicrobial agents by using Kirby-Bauer disk diffusion method (Bauer et al., 1966). The results were interpreted according to the
diameter of inhibition zones and compared with inhibition zones determined by CLSI (2021), and to decide the susceptibility of bacteria to antimicrobial agent whether being resistant. In this study *P. mirabilis* showed a different susceptibility towards antibiotics. All isolates of *P. mirabilis* appeared highest rate of resistance (100%) in the case of Cefotaxime, Amoxicillin, Ampicillin. And moderately resistance to Netilmicin (71.4%). All isolates presented high sensitivity (100%) to Meropenem, Levofloxacin, and Ciprofloxacin as in figure (4.1) and table (4.3)

![Image](image_url)

**Figure (4.1):** Antibiotic susceptibility of *P. mirabilis* to 7 different antibiotic.

**Table (4.3):** Antibiotic susceptibility of *P. mirabilis* to 7 different antibiotic.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Symbol</th>
<th>Resistance NO.(%)</th>
<th>Sensitivity NO.(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meropenem</td>
<td>ME</td>
<td>0</td>
<td>15(100%)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>LE</td>
<td>0</td>
<td>15(100%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>CI</td>
<td>0</td>
<td>15(100%)</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>NE</td>
<td>4(26.6%)</td>
<td>11(71.4%)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>CT</td>
<td>15(100%)</td>
<td>0</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>AX</td>
<td>15(100%)</td>
<td>0</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Am</td>
<td>15(100%)</td>
<td>0</td>
</tr>
</tbody>
</table>

4.3 PCR detection of hemolysine and urease genes

Figure (4.3): PCR amplified products of urease gene of the *P. mirabilis* using the primers with expected size 319bp. 
Lane (L), DNA marker (100bp ladder). 
Lane (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15) No. of amplify of urease gene

Figure (4.4): PCR amplified products of hemolysin gene of the *P. mirabilis* using the primers with expected size 210bp.
Lane (L), DNA marker (100bp ladder).
Lane (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15) No. of amplify of hemolysin gene

Figure (4.3) and (4.4) shown that all isolates (100%) appeared the presence of urease and hemolysine genes. α-hemolysin is responsible for damaging tissue and activating when its N-terminal peptide has cleaved. Based on the results of this study carried out in relation to Proteus microbes (figure 4.3, 4.4).

Molecular similarity was observed between the "EQKIRRAA" amino acid sequences present in the RA-associated HLA-DR molecules and the "ESRRAL" amino acid motif present in hemolysins of *P. mirabilis*. (Gibofsky et al., 2014) Subsequently, another molecular homology was discovered between the "IRRET" pentapeptide present in Proteus urease and the "LRREI" amino acid sequences found in type XI collagen, which is a component of hyaline cartilage and present predominantly in the small joints. Reciprocal immunological cross-reactivity has been demonstrated between Proteus and HLA-DR4 peptides, (Wilson et al., 1995) and elevated antibody levels against the synthetic peptides from these cross-reactive molecules were found among RA patients from England, Japan and Norway (Tiwana et al., 1999). 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 were 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 (Schaffer et al, 2015).

Conclusion
In this study concluded that P. mirabilis was the commonest bacteria isolated from urine of RA patients. All P. mirabilis isolates have higher sensitive to Meropenem, Levofloxacin, and Ciprofloxacin and higher resistant to Cefotaxime, Amoxicillin, mpicillin. And moderately resistance to Netilmicin. All isolates presented urease and hemolysine genes.

References


[16] Sosa, V.; G. Schlapp, and P. Zunino, 2006. Proteus mirabilis isolates of different origins do not show correlation with virulence attributes and can colonize the urinary tract of mice. Microbiology. 152, 2149–2157


