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Molecular Detection of Urease and Hemolysine Genes in Proteus Mirabilis Bacteria Isolated From Urine of Rheumatoid Arthritis Patients in Najaf Province

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Annotation: Proteus mirabilis is a known uropathogen with significant virulence factors implicated in urinary tract infections (UTIs), particularly among patients with rheumatoid arthritis (RA). Despite prior studies, the molecular relationship between P. mirabilis virulence genes and RA remains underexplored. This study aimed to isolate P. mirabilis from the urine of RA patients, assess its antibiotic resistance profile, and detect the presence of two key virulence genes: urease (ureC) and hemolysin (hpmA). Using standard microbiological techniques, biochemical tests, antibiotic susceptibility assays, and PCR analysis, the study found that P. mirabilis was the most prevalent pathogen (51%) among 50 RA patients. All isolates showed complete resistance to cefotaxime,

amoxicillin, and ampicillin, while being 100% sensitive to meropenem, levofloxacin, and ciprofloxacin. Molecular analysis revealed that all P. mirabilis isolates harbored both ureC and hpmA genes. These findings suggest a potential pathogenic link between P. mirabilis virulence and RA, underscoring the need for further molecular and immunological studies to understand microbial triggers in autoimmune disorders.

Keywords: Proteus mirabilis, rheumatoid arthritis, urinary tract infection, urease gene, hemolysin gene, PCR, antibiotic resistance, virulence factors.

1.1 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 ., 2020).

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 control (**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 manyaspects of their physiology to other members of the family Enterobacteriaceae. When transferred to a solid medium, Proteus bacilli undergo morphogenesis to swarmer 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 includ 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 acqusition systems and toxins:

hemolysins, Proteus toxin agglutinin (Pta), and lipopolysaccharide (LPS) endotoxin (Nielubowicz &Mobley2010).

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 proteas es 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 in the urinary tract (Jones et al., 2005).

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).

2. 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).

3. 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 precipitation that leads to stone formation (Alamuri et al.,2009).This pH modification is essential during colonization of the Proteus mirabilis catheter, facilitating bacterial adhesion and the development of biofilm incrustation (Carlini and Polacco,2008).

Stone forming is a characteristic of infection with Proteus mirabilis, which contains a variety of advantages like evade of the host immune system, ureter blockage, ammonia exposure to the host cells, and clear harm to tissues (Konieczna et al.,2012).

3. Quorum sensing Several types of bacteria use cell-cell contact to sense population density and organize gene expression (Stankowska et al.,2012). This quorum sensing system does not impact swimming or swarming motility, swarming cell differentiation, or the in vitro test of virulence (Wang et al.,2006).
4. Immune Evasion The presence of bacteria in the ill prevent innate and adaptive immune reactions (Norsworthy and Pearson,2017).Proteus mirabilis has multiple modes of evasion (Belas et al.,2004).Proteus mirabilis encodes a metalloproteinase (ZapA), that cleaves serum and secretory immuno-globulin A1 (IgA1), IgA2, and IgG (Liu et al.,2015).

✓ Siderophores

For a long time, it has been known that virtually all bacteria require soluble iron as an important nutritive compound. It is indispensable for growth and metabolism, mainly for most redox processes in all ecological systems: in the natural environment (soil, water), in artificial media, and in such niches as living organisms. In the presence of a deficiency of iron, bacteria produce iron chelators, named siderophores, which are excreted to the surroundings; they bind iron and transport it into the bacterial cells by using suitable receptor proteins and appropriate transport mechanisms. The synthesis of siderophores is under the control of chromosomal or plasmid genes. In all kinds of the host-bacterium relationships (commensals and conventional and opportunistic

pathogens), the bacteria are in competition with their host for iron. Eukaryotic proteins like transferrin and lactoferrin, with high iron affinity, render prokaryotic cells iron deficient.

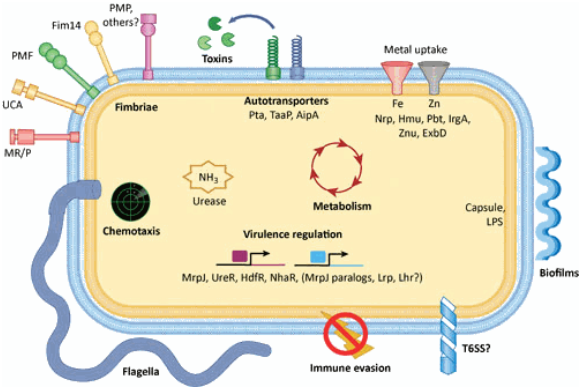

FIGURE (1) Concepts of *Proteus mirabilis* pathogenesis during urinary tract infection (UTI)

2.5 Antibiotic susceptibility

P. mirabilis is susceptible to b-lactams, aminoglycosides, fluoroquinolones, and trimethoprim/sulfamethoxazole, but is resistant to nitrofurantoin and tetracycline.7 In recent years, a trend has been observed where Enterobacteriaceae species, including P. mirabilis, show increased resistance to several antimicrobial agents.8,9 This increased resistance to antimicrobial agents has led not only to a changes in antimicrobial therapies, but also to poor prognoses and an increase in the mortality rate of hospitalized patients.10

The spread of multidrug-resistant (MDR) P. mirabilis isolates producing extended-spectrum β-lactamases (ESBLs) is constantly increasing worldwide. For example, to name a few, P. mirabilis strains harboring blaCMY2 were observed in Ireland (Mac Aogain et al.,2016) New Delhi metallo-beta-lactamase-5 (NDM-5)-producing Proteusisolate was analyzed in China [Sun et al., 2019], and NDM-1 positive strains were recently found in Italy [Bitar et al.,2020] and Tunisia [Kanzari et al., 2018]. P. mirabilis was also found to be the second most prevalent species, after Escherichia coli, among ESBL-producing Enterobacteriaceaefrom chicken meat in Singapore [. Lim et al., 2016], and ESBL production was significantly associated with mortality in patients with bacteremia caused by P. mirabilis . Several nosocomial infection outbreaks and community-acquired infections in Ethiopia and Nigeria (Chukwu et al., 2011) caused by this bacterial species were also reported. Studies mentioned above demonstrated that most bla-resistance determinants have a plasmid localization, and plasmids play a key role in antimicrobial drug-resistance of P. mirabilis. Moreover, such plasmids may have a hybrid origin (cointegrate/mosaic), which is important for the spread of multiple antibiotic resistance genes among Enterobacterales [17]. Besides the acquired resistance to the β-lactams, P. mirabilis is intrinsically resistant to tetracyclines and polymyxins, including colistin [Schaffer&Pearson,2015], which may facilitate the emergence of multidrug-resistant, or even extensively drug-resistant strains complicating the clinical treatment of infections caused by them.

3. Materials & Methods

3.1.1: Equipments and Instruments

Equipments and Instruments	Manufacturing company
Autoclave	CertoClav/Austria
Burner	Amal/Turkey
Centrifuge	Memmert/Germany
Compound light microscope	Olympus/Japan
Deep freezer	GFL/Germany
Digital camera	Sony/Japan
Distillator	Gallerellin Ltd/England

Electric oven	**Memmert**
Electrophoresis apparatus min	**Bio-Rad/Italy**
Eppendorf tubes	**Sterellin Ltd/UK**
Gel documentation	**Biometer/Germany**
Incubator	**Selecta/Spain**
Micropipette set (1-1000 µl)	**Eppendorf/Germany**
Millipore filter (0.22µm)	**Difco/USA**
Microcentrifuge	**Hettich/Germany**
Microwave	**Memmert/Germany**
Oven	**Selecta/Spain**
PCR system	**Agilent/USA**
PH-meter	**Orient/USA**
Sensitive balance	**Sauter/Switezeland**
Spectrophotometer	**Orient research/USA**
UV Lamp	**Ultra Violete products institute/USA**
Vitek-2 system Compact Auto analyzer	**bioMérieux/ France**
Vortex mixer	**Thermo scientific/Singapore**
Water bath	**Memmert/Germany**

3.1.2: The Biological and Chemical Materials

Materials	**Manufacturing company**
Agar-agar	**Himedia (India)**
Agarose	**BioBasic (Canada)**
Blood	**Blood bank (Najaf)**
Ethanol	**Teba (Iraq)**
Glycerol ($C_3H_8O_3$)	**Fluka (Switzerland)**
Gram stain	**Himedia (India)**
Hydrogen peroxide (H_2O_2)	**SDA (Iraq)**
Immersion oil	**SYRBIO/S.A.R.**
Normal saline	**Local (Iraq)**

3.1.3: Culture Media

Medium	**Manufacturer(Origin)**
Blood Agar Base	Oxoid(Paris,France)
Brain heart infusion broth	Himedia(India)
Brain heart infusion agar	Himedia(India)
Muller-Hinton agar	Himedia(India)
MacConkey agar	Himedia(India)
Kligler iron agar	Himedia(India)
Urea agar base	Himedia(India)
Methy red–Vogus Proskaur (MR-VP) medium	Himedia(India)

3.1.6: Antibiotic Disk

Table (3.4): Antibiotic disks used in this study

Antibiotic	**Code**	**Potency (µg/ disk**	**Origin**
Meropenem	MEM	10 mcg	Bioanalye / Turkey
Levofloxacin	LEV	5 mcg	Bioanalye / Turkey
Ciprofloxacin	CIP	10 mcg	Bioanalye / Turkey
Netilmicin	NET	30 mcg	Bioanalye / Turkey

Cefotaxime	CTX	30 mcg	Bioanalye / Turkey
Amoxicillin	AX	25 mcg	Bioanalye / Turkey
Ampicillin	Am	10 mcg	Bioanalye / Turkey

- PCR Materials

3.1.7.1 Genomic DNA Extraction Kit

Genomic DNA Extraction kit component	Manufacturing company / origin
Solution DS (30ml) solution MS (40ml) Proteinase K 20mg/ml (2ml) Wash Buffer PS (36ml) Wash Buffer PE (30ml) Elution Buffer TE (10ml) Spin Columns (100)	Dongsheng Biotech / China

3.1.7.2 Materials Used in Electrophoresis

Materials	Manufacturing company / origin
Loading dye Nuclease free water Ethidium bromide TBE (Tris-Borate EDTA) buffer TE (Tris- EDTA) buffer Agraose 1Kb DNA ladder (DNA marker)	Dongsheng Biotech / China

3.1.7.3 Primers

The sequences of the primers used in the laboratory work were as in reference and they were illustrated in Table (3-9):

Table (3-9): Sequences of the primers used.

Name	Oligo Nucleotide	Sequence (5′ to 3′.)	Size (bp)	Reference
urec	F	GGGGGCAATTTCGGTGATGT	319	((D'Orazio SE, et al., 1996)
	R	TGGCGCATAAGCGACCATAC		
hpmA	F	GCGGTGTAACGAAGCCAGTT	210	(Cestari et al., 2013
	R	CGGCTTGGCTATCGGTTTGT		

3.2: Methods:

3.2.1 Sample collection

A total of 50 urine samples were collected from patients with RA. Samples were collected from patients during the period from October 2021to 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 macckongy agar) , biochemical tests including; catalase test, oxidase reaction and urease test were used for more identification.

3.2.1: Preparation of Buffer and Chemical solutions:

3.2.1.1: McFarland's Standard Solution No.0.5 (1.5 X 10^8) cell/ml

The 0.5 McFarland standard tube was prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2.2H_2O$) solution to 99.5 ml of 1% (vol/vol) H_2SO_4. 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. A ccuracy of the density of a prepared 0.5 McFarland standard was checked by using a spectrophotometer. The absorbance of the wavelength of 625 nm should be between 0.08 and 0.1 (CLSI, 2020).

3: Preparation of Reagents for bacteriological tests

3.2.6 Identification of Bacteria

3.2.6.1 Morphological and Cultural Characteristics

A single colony was taken from each primary positive culture on blood agar, and repeat growth for gain pure culture and then it was identified depending on its morphological and cultural characteristics (blood hemolysis, lactose fermentation , colony shape, size, color, borders, and texture) and then it was examined under the microscope after making smear from pure colony on clean slid and stain with Gram's stain for observation arrangement and reaction bacteria with stain (MacFaddin, 2000).

3- Biochemical Tests

3.1 Oxidase Reagent

This test was prepared by dissolving 0.1g of tetra methyl-P-phenylene diamine dihydrochloride in 10ml of distilled water. It was used immediately after preparation.

3.2.4.2 Catalase Reagent

It was prepared in 3% concentration of H_2O_2 and it was used to identify bacterial ability to produce catalase enzyme.

3.2.4.3 Methyl Red Reagent

Methyl red reagent was prepared by dissolving 0.1gm of methyl red in 300 ml of 95% ethanol and then the volume was completed to 500ml by distilled water. This reagent was used to detect the complete glucose hydrolysis.

3.2.4.4 Vogeus-Proskaur Reagents (VP)

The reagents were prepared as follows:

Reagent (VP1): 40% KOH in D.W.

Reagent (VP2): 5% α-naphthol in 96% ethanol .

3.2.4.5 Kovac's Reagent

The agent was prepared by dissolving 0.5gms from dimethyl aminobenzaldehyde in 15ml ethyl alcohol and 5ml of concentrated HCL. This reagent was used for the detection of indole production.

3.2.4.6 Nitrate Reduction Reagents

The reagent was prepared as follow:

Reagent A: dissolving 0.8gm of Sulphanilic acid in 100ml of 5M acitic acid.

Reagent B: dissolving 0.5gm of α-naphthol in 100ml of 5M acitic acid.

Three drops of reagent **A** with an equal volume of a reagent **B** were added to the suspension of bacteria in broth.

3.2.5 Preparation of Culture and Diagnostic Media

All media (solid & broth) were prepared according to the manufacturer's instructions, after sterilized by autoclave (121°C for 15minutes) then incubation of culture media for 24 hours after pouring it in sterile plates. It was done to avoid the contamination, then it was stored at 4˚C until use.

3.2.5.1 Blood Agar

It was prepared by suspending 40gm of blood agar base in 1000ml of D.W, heated to boiling and sterilized in autoclave and 7-10% of fresh human blood was added after cooling to 45˚C. It was used to show the colonial morphology and heamolysin production (Collee *et al*., 1996).

- Muller-Hinton Agar

It has been prepared according to manufacturer's instructions, heated to boiling and sterilized in autoclave and 5% of fresh human blood was added after cooling to 45˚C. It was used to determination of antimicrobial susceptibility testing (AST) (CLSI, 2020).

3.2.5.3 Nutrient agar

This media was prepared according to the manufactureing company. It was used for general experiments.

3.2.5.4 Simmons Citrate agar

It was used as differential medium to detect the ability of bacteria to use citrate as a sole source of carbon**.**

3.2.6 Isolation and Identification of Bacterial isolates

3.2.6.1 Cultural Characteristics

All samples were cultured on MacConkey agar, blood agar, Pseudomonas Isolation Agar and nutrient agar media using standard loop method,The media were incubated at 37 ˚C for 24-48 hrs. Morphological colonies characteristics were recorded on the media that are used . The pure colonies were prepared for biochemical tests to differentiate *P. aerogenosa* from other bacteria.

3.2.6.2 Microscopic examination

Depending on microscopical examination, Gram's stain was used to examine the isolated bacteria for studying differentiate gram negative cells ,detecting the shape and arrangement of bacterial cells (Jawetz *et al*.,2007).

3.2.7 Biochemical Tests

All biochemical tests were prepared according to MaccFadin,(2000).

3.2.7.1 Oxidase Test

Small part of fresh colony(18-24hr) was transferred with sterilized wooden stick to ready used oxidase reagent, appearance of deep violet color after (10-20 sec) indicates positive results**.**

3.2.7.2 Catalase Test (Hydrogen Peroxide 3%)

An amount of growth was placed on clean glass slide and drop of 3% Hydrogen Peroxide solution was added to it. Immediate release of oxygen bubbles was indicated as a positive result.

3.2.7.3 Indole Production Test

Peptone water was inoculated with a young bacterial colonies at 37°C for 24-48 hrs. A few drops of Kovacs reagent were added to each tube. Formation of pink ring indicates a positive test.

3.2.7.4 Methyl Red Test

MR-VP broth media was inoculated with a young bacterial isolates incubated at 37°C for 24 hrs. 5 drops of methyl red solution were added, mixed, and the result was read immediately. A positive

result incubated when a bright red was appeared.

3.2.7.5 Vogues-Proskauer Test

MR-VP broth was inoculated with a young bacterial isolates and incubated at 37°C for 24- 48 hrs. One ml of 40% KOH solution and 3 ml of 5% solution of α -naphthol were added to each tube. A positive reaction was indicated by the appearance of a pink color in 15-20 minutes.

3.2.7.6 Citrate utilizating test

The tube contained slant of simmon citrate agar was inoculated with tested bacteria by stabbing in the bottom and streaking on the slant, then incubated in at 37ºC for 24-48 hr. Change of the medium from green to blue indicates a positive result.

3.2.6: Preservation of Bacterial Isolates

3.2.6.1: Short Time Preservation

Preservation single pure colony of bacterial isolate was inoculated on trypticase soya agar slants in screw-capped tubes, incubate overnight at 35˚C, and then store in a refrigerator. Transfer every two weeks (Vandepitte *et al.*, 2003).

3.2.6.2: Long Time Preservation

Long-term preservation methods permit intervals of months or even years between subcultures (Vandepitte *et al*., 2003).

3.2.10: Antibiotics Susceptibility Testing

2.2.10.1: Disk Diffusion Method

It was performed according to Clinical Laboratory Standared Instittute (CLSI, 2020) by using a pure culture of previously identified bacterial organism. The suspension of 5 isolated colonies grown on blood agar plates to 5 ml of nutrient broth. This culture was then incubated for 2 hours to produce a bacterial suspension of moderate turbidity that compared with turbidity of ready-made 0.5 McFarland tube standard. A sterile swab was used to obtain an inoculum from the standardized culture, this inoculum was then swabbed on Muller–Hinton agar plate and the antibiotic discs were placed on the surface of this medium and incubated at 37ºC for 24 h. Antibiotics inhibition zones were measured and zone size was compared with standard zones from the CLSI (200), to determine the susceptibility of organism to each antibiotic.

-Genomic DNA Extraction:

Kit (Geneaid) was used for DNA extraction as the following:

Step1: Cell Harvesting/pre-lysis

1- A volume of 1 ml of bacterial suspension containing approximately (up to 1×10^9) cell/ml was transferred to 1.5 ml microcentrifuge tube, centrifugation for 1 minute at 14-16,000×g and discards the supernatant.

2- A volume of 200 µl of Gram+ Buffer(make sure lysozyme was added) was added to the tube and resuspend the cell pellet by shaking vigorously or pipetting, then left at room temperature for 10 minutes. During incubation, invert the tube every 2-3 minutes.

Step 2: Lysis

3- A volume of 200 µl of GB buffer was added to the sample and mix by shaking vigorously for 5 seconds, then incubated at 60ºC for at least 10minutes to ensure the sample lysate is clear. During incubation, the tube was inverted every 3 minutes. At this time, the required Elution Buffer (200 µl per sample) pre-heat at 60ºC (for step 5 DNA Elution).

Step 3: DNA Binding

4- A volume of 200 µl of absolute ethanol was added to the sample lysate and mix immediately by shaking vigorously. If precipitate appears, breaked it up by pipetting.

6- GD column was placed in a 2ml Collection Tube.

7- All of the mixture (including any insoluble precipitate) was transferred to the GD Column and centrifuged at 14-16,000×g for 2 minutes.

8- The 2 ml Collection Tube containing the flow-through was discarded and placed the GD Column in new 2 ml Collection Tube.

Step 4: Washing

9- A volume of 400 µl W1 Buffer was added to the GD Column.

10- Then, centrifugation at 14-16000×g for 30 second

11- The flow-through was discarded and placed the GD Column back in the 2 ml Collection Tube.

12- A volume of 600 µl Wash Buffer (ethanol added) was added to the GD Column.

13- Centrifugation at 14-16,000×g for 30 second.

14-The flow-through was discarded and placed the GD Column back in the 2 ml Collection Tube.

15- Centrifugation again for 3 minutes at 14-16,000×g to dry the column matrix.

Step 5: DNA Elution

16- The dried GD Column was transferred to a clean 1.5 microcentrifuge tube. And 100 µl of preheated Elution Buffer was added to the center of the column matrix and let stand for 3-5 minutes or until the Elution Buffer is absorbed by the matrix.

18- Centrifugation at14-16,000×g for 30 seconds to elute the purified DNA.

19- Store eluted DNA at 4˚C or -20˚C.

PCR Reaction Mix Preparation

The DNA extract of P.mirabilis isolate were subjected to different gene by PCR . The protocols used depending on manufactures instruction .all PCR component were assembled in PCR tube and mixed on ice bag under sterile condition as in table (3-10)

Table (3-10): PCR reaction mix preparation

Mixture Contents	Volume (µl)
Master Mix	**12.5**
Primer	**3**
Template DNA	**2**
Distilled water	**7.5**
Total	**25**

3.2.12 PCR Thermo Cycling Conditions

Polymerase chain reaction assays were performed at a reaction volume of 25 µl. Depending on their reference procedure .The Rapid PCR amplification conditions performed with a thermal cycler were specific to each single primer set.

Table (3-11): PCR thermos cycling conditions:

Gene	Initial Denaturation	denaturation	Annealing	extension	Final extension	Cycles
urec	**94C 3min**	**94C 1min**	**63C 30sec**	**72 C 1 min**	**72 C 7min**	**35**
hpmA	**94C 5min**	**94C 60 sec**	**58C 45sec**	**72 C 1 min**	**72 C 7min**	**30**

3.2.13 Agarose Gel Preparation and DNA Loading

This method was carried out according to Bartlett and Stirling (1998) as following :

1-Agarose (1.5 gram) was added to 100 ml of Tris-borate-EDTA (TBE) with final concentration 10x (90 ml distilled water + 10 ml TBE).

2-The prepared Tris-borate-EDTA was boiled, then allowed to cool to 45°C, and 0.05 mg/ml of ethidium bromide was added.

3-The agarose poured in equilibrated gel tray, and left until cooled and became more hardened.

4-Five microliters of PCR product were loaded to the agarose gel wells followed by DNA marker to one of the wells. The gel try was fixed in electrophoresis chamber and TBE buffer was added to the chamber. The electric current was performed at 70 volt for 80 Minutes. The PCR products were detected in a 1.5% agarose gel.

5-Finally, the electrophoresis result was detected using gel documentation system; the positive results were proved when the DNA band base pairs of sample equal to the target product size

- Statistical Analysis

Chapter four

Results & Discussion

Out of 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) , *Klibsiella spp*.5 (17%) and *Staphylococcus saprophiticus* 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

Bacterial isolates	No.	%
Proteus mirabilis	15	51
Escherichia coli	6	20.4
Klebsiella spp.	5	17
Staphylococcus Saprophiticus	3	10.3

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

Test	Result
Oxidase	-
Catalse	-
Urease	+

Indole production	
Methyl red	
Voges-Proskauer	
Simmons Citrate	+
H2S production	+
Motility	+
Kligler agar	

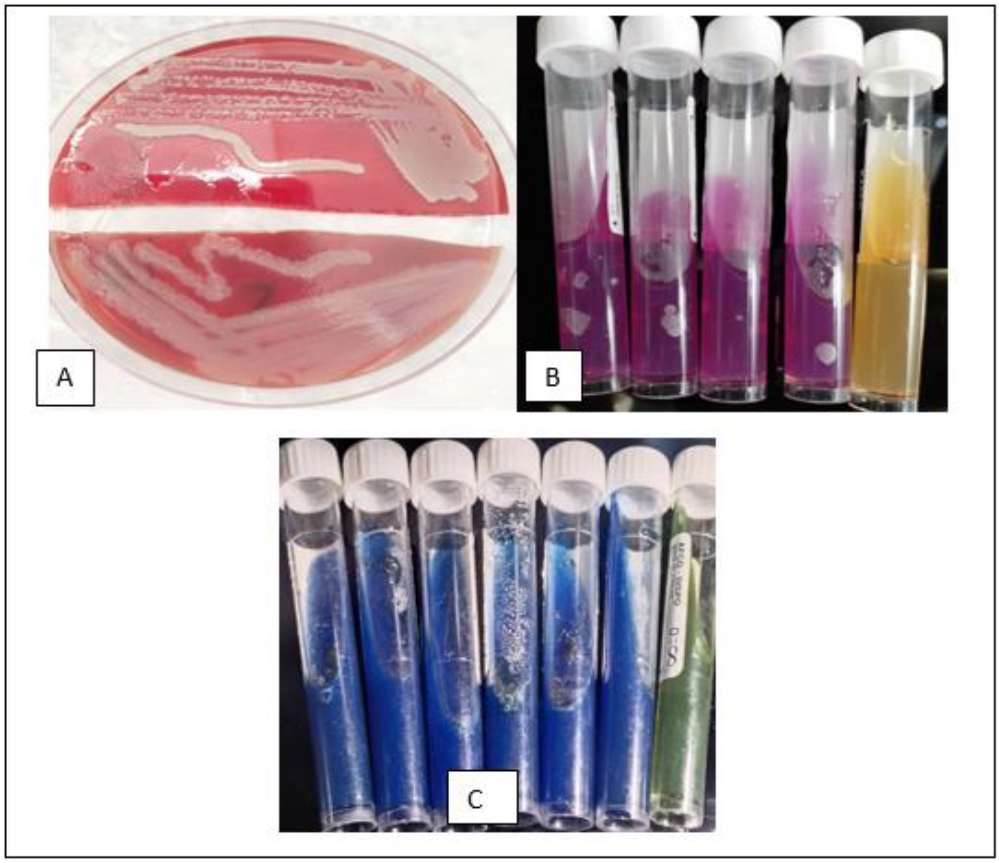

Figure (4.1): A- *P. mirabilis* on blood agar B-urease production

C- Simmons Citrate

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)

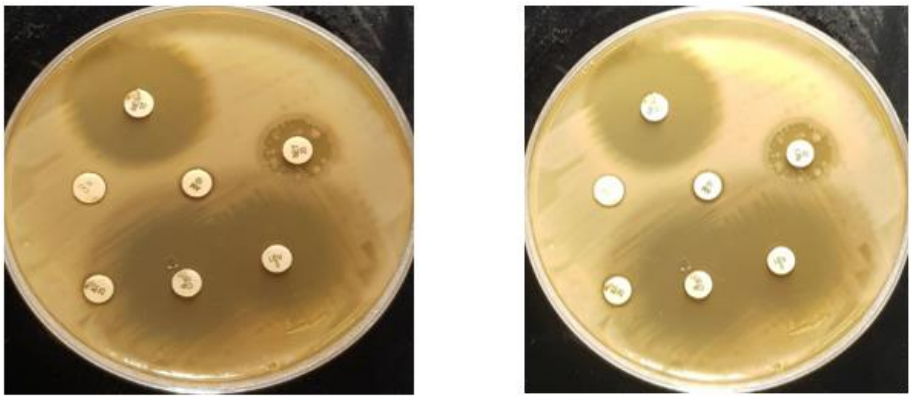

Figure (4.1): Antibiotic susceptibility of *P.mirabilis* to 7 different antibiotic .

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

Antibiotics	Symbole	Resistance NO.(%)	Sensitivity No.(%)
Meropenem	MEM	0	15(100%)
Levofloxacin	LEV	0	15(100%)
Ciprofloxacin	CIP	0	15(100%)
Netilmicin	NET	4(26.6%)	11(71.4%)
Cefotaxime	CTX	15(100%)	0
Amoxicillin	AX	15(100%)	0
Ampicillin	Am	15(100%)	0

4.4 PCR detection of hemolysine and urease genes

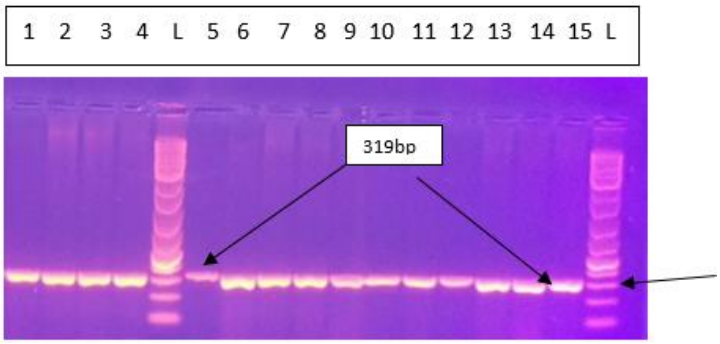

Figure (4.3): **PCR amplified products of *urease* gene of the *P. mirabilis* using the primers with expected size 319bp.**

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

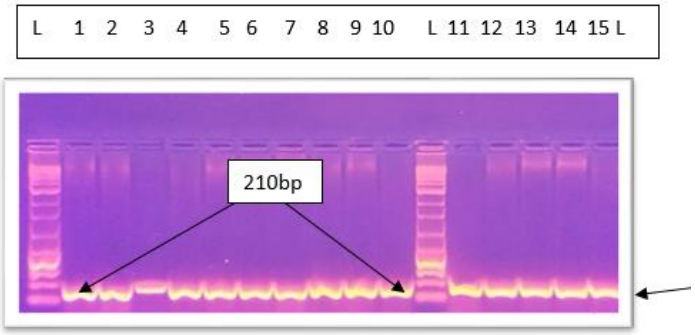

Figure (4.4): **PCR amplified products of *hemolysin* gene of the *P. mirabilis* using the primers with expected size 210bp.**

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 isolates15 (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 (figure4.3, 4.4) .

Molecular similarity was observed between the "EQKlRRAA" 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 car- tilage and present predominantly in the small joints. Reciprocal immunological cross-reactivity has been demonstrated between Proteus and HLA-DR4 pep- tides, (Wilson et al., 1995) and elevated antibody levels against the synthetic peptides from these cross-reactive mole- cules 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 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 (Schaffer et al, 2015).

Conclusion

1. *P. mirabilis* was the commonest bacteria isolated from urine of RA patients.
2. All *P. mirabilis* isolates have higher sensitive to Meropenem, Levofloxacin, and, Ciprofloxacin and higher resistant to Cefotaxime, Amoxicillin, Ampicillin. and moderately resistance to Netilmicin.
3. All isolates presented urease and hemolysine genes.

Recommendation

1. Make comparative study for the present some virulence genes among *P.mirabilis* isolated from RA and other infection .
2. Sequencings urease and hemolysine gene in *P.mirabilis* isolated from RA.
3. More immunological and molecular studies must be do for *P.mirabilis* in RA patients.

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