Ministry of Higher Education and Scientific Research
Al-Hilla University College
Department of Medical Laboratories Techniques

Molecular Identification using PCR-Technique of Proteus mirabilis Associated with Urinary Tract Infection

A Project Research

Submitted to the Department of Medical Laboratories Techniques/ Al-Hilla University College in Partial Fulfillment of the Requirements for the Degree of Bachelor of Medical Laboratories Techniques

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(مثل الذين ينفقون أموالهم في سبيل الله كمثل حبة

أنبتت سبع سنابل في كل سنبلة مائة حبة والله

يضاعف لمن يشاء)

صدق الله العلي العظيم

سورة البقرة – الآية 261

Dedication

T0 the great person Who I missed and wished he is with me now;

My Father

To my First teacher who gives me encouragement, happiness and inspiring me with hope;

My Mother

To the Candles Which Light My Life;

My Sister & Brothers

To My Colleagues in various Knowledge Fields;

My Friends

Acknowledgment

At first of all, thanks to Allah the most gracious and the most merciful, who gave me the ability and desire to achieve this study.

I would like to express my deepest appreciation and very honest gratitude to my supervisor, (M. Sc. Mustafa Abd Al-Jabbar) for his guidance, support, interest and his encouragement.

I would like to thank dean of Al-Hilla University College and the head of Department of Medical Laboratories Techniques and all my professors for supporting me in this work.

My special and sincere thanks to patients who gave me the samples from and may Allah heal them and have mercy on those who died because of this epidemic.

My sincere thanks are also to my family, friends and to anyone who helped and supported me by his pray for success in my work.

Abstract

This study included the diagnosis of *Proteus mirabilis* isolated from patients attended different hospitals, laboratories and privet laboratories in the Hilla city/Iraq (Hilla Teaching hospital, Childhood and Gynecology Hospital).

Fourteen isolates of *P. mirabilis* were identification based on phenotypic, microscopic and biochemical tests.

Some bacterial virulence factors related to Urinary tract infection have been investigated. The results showed of isolates to the production of hemolysin, urease, and formation of swarming.

The sensitivity test for all isolates of *P. mirabilis* were performed against six antibiotics commonly used to treat *P. mirabilis*, including Amoxicillin, cefotaxime, Amikacin, ceftazidime, Imipenem, and Meropenem. All isolates showed resistance against Amoxicillin and Cefotaxime were 100%, while the other antibiotics Amikacin, Ceftazidime, Meropenem, and Imipenem were 64.28%, 50%, 28.57% and 14.28%, respectively.

In order to confirm the results of the identification based on the biochemical tests, the results were confirmed by molecular identification using *ureR* gene of *P. mirabilis*. The bacterial DNA was extracted from 14 isolates and using the QIAGEN kit.

Out of 14 *P. mirabilis* isolates 7 isolates were positive for *ureR*; the other 7 isolates were negative for this gene. This identification may be more accurate than the phenotypic characterization which entails biochemical features.

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1.1 Urinary Tract Infection (UTI)

Urinary Tract Infection is one of common diseases, occurring from neonate up to adult age groups. More than 80% of Urinary Tract Infection is due to the bacteria like Escherichia coli and Proteus mirabilis (Jonhnson and Stamm, 1989). Urinary Tract Infection defines a condition in which the urinary tract is infected with the pathogen causing inflammation (Kunin and White, 1993). The major cause of UTI is Gram negative bacteria which belong to the Enterobacteriaceae family (Mobley *et al*., 1994). Walker (1999) stated that most common bacteria causing UTI is Proteus mirabilis which is gram negative, motile, swarmer bacteria and this bacterium attached to the penetrated tissue, resists host defenses and induce change to the host tissue.

1.2 *Proteus mirabilis*

The Proteus genus belongs to the Protease tribe in the family of Enterobacteriaceae; this tribe contains three types: Proteus, Providencia and Morganella. These bacteria are Gram negative rod measuring (0.4–0.8) μm in diameter and (1–3) μm in length fig (1-1), motile by peritrichous flagella, non-spore forming, non-capsulated, facultative anaerobic, major isolates have fimbriae, The genus of Proteus contains of four spp: *P. mirabilis, P. vulgaris, P. myxofaciens,* and *P. penneri* (Murray *et al*., 1999). Proteus spp (*P. mirabilis*, *P. vulgaris,* and *P. penneri*) are essential pathogens of the urinary tract and principal infectious agent in patients with indwelling urinary catheters (Jacobsen et al., 2008). Individuals were suffering from urinary tract infections initiated by *P. mirabilis* frequently progress cystitis, bacteriuria, bladder and kidney stones, and catheter obstruction due to stone deposit, and acute pyelonephritis (Burall *et al*., 2004). *Proteus mirabilis* is a communal reason of upper urinary tract infections that can include invasion urothelial of host cells (Jen liaw *et al*., 2000).

Fig (1-1): Gram stain of *Proteus mirabilis*

1.3 Proteus mirabilis scientific classification, (Jawetz, 2004)

Kingdom:	Bacteria.
Phylum:	Proteobacteria.
Class:	Gamma Proteobacteria.
Order:	Enterobacteriales.
Family:	Enterobacteriaceae.
Genus:	*Proteus.*
Species:	*P. Mirabilis.*

1.4 Pathogenicity of *Proteus mirabilis*

Pathogenicity of Proteus species is accomplished by the subsequent two stages: first the microorganism requirements to colonize the site of infection, and second the microorganism requirements to effectively avoid host resistances. Tissues colonization through using two of the four kinds of fimbriae named mannose resistant fimbriae (MRF) and Proteus species can be used to avoid the host resistances. The first is the creation of an IgA degrading protease which roles to split the secretory IgA (Watanakunakorn and Perni .,1994).

The second Immune system avoidance device is by three distinctive flagellin genes which must been presented to recombine and form flagella able of misleading the defense of host's. The third is by appearance of the MR/P fimbriae. The fourth machinery is the urease mediate formation of stone (Johnson *et al*., 2003). Urinary tract infection with *Proteus mirabilis* may due to serious complications, containing cystitis, acute pyelonephritis, bacteremia, fever, and death. In count to the production of hemolysin and the enzyme urease, fimbriae and flagellum-mediated motility have been proposed as virulence factors for this species. Proteus mirabilis responsible around 25% of bacteremia at a large community teaching hospital (Watanakunakorn and Perni .,1994).

1.5 Virulence Factors of *Proteus mirabilis:*

Proteus mirabilis have many virulence factors which help in causing the infections these are:

1.5.1 Hemolysin Production of *Proteus mirabilis:*

Two distinct hemolysins have been found among Proteus isolates which are HpmA and HlyA (Koronakis *et al*., 1987). Swihart and Welch, (1990) found that HpmA which is calicumin dependent hemolytic activities produced by all strains of *Proteus mirabilis* and most strain of *Proteus vulgaris*, while HlyA which is calcium dependent hemolysin activity is not found in *Proteus mirabilis* but it is found in some strains of *Proteus vulgaris*. In addition, hemolytic strains of *Proteus mirabilis* are more virulence than the non-hemolytic strains, when injected intravenously into mice.

1.5.2 Urease Production by *Proteus mirabilis:*

Urease is an important agent responsible for the pathogenesis of *Proteus* in the kidney, it is a cytoplasmic multimeric, nicked metallo enzyme which catalyzes the hydrolysis of urease to carbon dioxide and ammonia, elevated the pH and result in precipitation of magnesium ammonium phosphate and carbonate-apattite which form stones of kidney and bladder (Mobley and Chippendle, 1990). Larrson (1978) found that the increase in pH due to hydrolysis of urea by urease results in decrease of the biological activity of antibodies and distraction of leukocytes. The presence of ammonia has been implicated as directly toxic to epithelium of kidney (Mobley and Chippendale, 1990).

1.5.3 Swarming of *Proteus*:

Swarming was also considered as an important phenomenon during life cycle of *Proteus*, it is a cyclical differentiation process in which typical vegetative rods (2-4 µm in length) differentiate into long (up to 8 µm), a septate filaments that possess up to 50 fold more flagella per unit cell surface area (Liaw *et al*., 2000). Mobley and Belas (1995) stated that the swarming cell differentiation is a result of at least three separate phenomena including the production of elongated swarmer cells, the synthesis of vastly increased amount of flagellin and the coordinate multicellular interaction that result in a cyclic waves of cellular differentiation. There are many anti-swarming agents which can inhibit the swarming phenomena of *Proteus mirabilis* such as alcohol (6%), sodium azide, boric acid (Gupta, 1988) and poly nitrophenylglycerol (Liaw et al., 2000).

1.5.4 Antibiotic Sensitivity of *Proteus mirabilis:*

Many properties should be considered to choose drug for UTI treatment, these are; the drug activity against the infecting organisms, toxicity, the tissue concentration obtained, the effect of pH and possess no or little effect on normal flora of intestine and other regions (Glauser, 1986). There are different mechanisms by which microorganisms might exhibit resistance to drug, such as; production of enzymes that destroy the active drug, changing their permeability to the drug, developing an altered structural targets for the drug, and finally metabolic pathway which microorganisms produce an altered enzyme that can still perform its metabolic function but is much less affected by the drug (Jawetz *et al*., 1998).

1.5.4.1 β-Lactam Antibiotics:

They affect the cell wall by inhibiting enzymes that involve in the formation of peptidoglycan layer. β-lactam includes two groups which are penicillin and cephalosporin (Jawetz *et al*., 1998). Ampicillin and amoxicillin are active against *Proteus mirabilis* (Cercenado *et al*., 1990). Pagani *et al* (1998) studied the activity of pipracillin / tozobactam against Enterobacteriaceae and found it highly active against *Proteus mirabilis*, while Prescott et al (1990) studied the activity of carbencillin and cephalexin which were active antibiotics against *Proteus mirabilis*.

1.5.4.2 Aminoglycosides Antibiotics:

They inhibit protein synthesis by attaching to and inhibiting the function of 30S subunit of the bacterial ribosome (Jawetz *et al*., 1998). Amikacin is the important drug of choice in treatment infection caused by *Proteus mirabilis* (AlTalib and Habib, 1986). Gentamycin used widely in treatment of UTI (Merlin *et al*., 1988). Streptomycin is considered as the oldest aminoglycoside drug used to treatment UTI (Mingeot-Lectero *et al*., 1999).

1.6 Regulatory Gene (*UreR* gene)

Urease operon transcription is positively activated by *ureR* a dimer of identical 293 amino acid polypeptides that bind urea resulting in the binding of protein avidly to both *ureR* and *ureD* promoters, and then RNA polymerase is activated by *ureR* and thus will initiate transcription (Coker et al., 2000). Because of the important role of urease in the pathogenicity of *P. mirabilis*, this study was aimed at investigating to detect the correlation between *ureR* and *ureC* in urease production.

Aim of this study:

The study was aimed to use PCR-technique to confirm identification of *Proteus mirabilis* associated with urinary tract infection.

2.1 Materials

2.1.1 Laboratory Instrument

Table (2-1): The equipment and Instrument were used in this study:

No.	Instruments	Company	Country of Origin
1	Autoclave	Tripod	**UK**
2	Bench centrifuge	Memmert	**Germany**
3	Cooling centrifuge	Hittich	**Germany**
4	DNA extraction tubes 100 µl.	Eppendorf	**Germany**
5	Eppendrof tubes	Eppendorf	**Germany**
6	Hood	Bio LAB	**Korea**
7	Horizontal gel electrophoresis	Bio- Rad	**Italy**
8	Incubator	Selecta	**Spain**
9	Light microscope	Olympus	**Japan**
10	Oven	Olympus	**Japan**
11	PCR system	Clever	**Germany**
12	PCR tubes	Eppendorf	**Germany**
13	Petridish	Sterilin	**England**
14	Plain tubes	DMD-DISPO	**Syria**
15	Refrigerator	Kiriazi	**Egypt**
16	UV-transilluminator	UtraViolete products institute	**USA**
17	Vortex mixer	Griffin	**Germany**
18	Water bath	Gallen Kamp	**Germany**

2.1.2 Antibiotic disks

Table (2.2): The antibiotics disks were used in the present study

No	Antibiotics	Symbol	µg \ disk	Manufacturing company
1.	Amikacin	(AK)	**30**	**Bioanalyse(Turkey)**
2.	Cefotaxime	(CX)	**30**	
3.	Ceftazidime	CAZ	**30**	
4.	Imipenem	IMP	**10**	
5.	Meropenem	(MEM)	**10**	
6.	Amoxicillin	AX	**30**	

2.1.3 Commercial Kits

Table (2-3): Commercial Kits were used in the present study

No.	Types of Kit	Company	Country
1	Gentra®Puregene®(Genomic DNA Isolation)	QIAGEN	**U.S.A**
2	DNA leader	Bioner	**Korea**
3	Green master mix	Gene aid	**Taiwan**
4	**Primers**	**Promega**	**U.S.A**

2.1.4 Culture Media

Table (2-4): Culture media were used in the present study

No	Type of media	Manufacturing company	rigin
1.	Agar-Agar base	Himedia	**India**
2.	Blood agar base	Himedia	**India**
3.	Brain heart infusion agar	Himedia	**India**
4.	Brain heart infusion broth	Himedia	**India**
5.	Kligler Iron agar	Diffco-Michigan	**USA**
6.	Luria broth	Himedia	India
7.	MacConkey agar	Himedia	**India**
8.	MR–VP broth	Himedia	**India**
9.	Muller –Hinton agar	Himedia	**India**
10.	Nutrient agar	Himedia	**India**
11.	Nutrient broth	Himedia	**India**
12.	Peptone water	Himedia	**India**
13.	Simmone citrate agar	Diffco-Michigan	**USA**
14.	Urea agar base	Diffco-Michigan	**USA**

2.2 Methods 2.2. 1 Reagents and Solutions

2.2.1.1 The (Catalase, Oxidase, Methyl Red, Kovac´s**,)** reagents were prepared as described in MacFaddin (2000):

2.2.1.2 Gram Stain Solution

Gram Stain solution was supplied from Syrbio company. The solution was used to study bacterial cells morphology and their arrangement (Forbes *et al.*, 2007).

2.2.1.3 Urea Solution

It was prepared by dissolving 20 g of urea in small volume of distilled water, completed up to 100 ml D.W. and then sterilized by millipore filter paper. The solution used in urease test for the detection of urease positive bacteria (MacFaddin, 2000).

2.2.1.4 McFarland's Standard Solution No. 0.5 (Biomerieux, France).

2.2.1.5 Ethidium Bromide (10 mg/ml)

A quantity of 1g of Ethidium bromide dye was dissolved in 100 ml of distilled water, stirred on a magnetic stirrer for several hours until the dye completely dissolved. The solution was kept in dark bottle at room temperature (Sambrook and Russell, 2001).

2.2.1.6 Tris-Borate-EDTA (TBE) Buffer

Tris-borate-EDTA buffer was used at concentration of 1X (1: 10 dilution of the concentration stock). The stock solution was diluted by D.W. and stored at room temperature. Also (TBE) buffer is used and prepared by Promega company/USA.

2.2.2 Preparation of Culture Media

2.2.1 All culture media were prepared according to the instructions of the manufactures, and then sterilized by autoclaving at 121^{o}C at 15 pound per square inch for 15 min

2.2.2 Blood Agar Medium

Blood agar medium was prepared according to manufacturer Instructions by dissolving 40 g of blood agar base in 1000 ml D.W. The medium was autoclaved at 121 ºC for 15 min and pressure 15 pounds per square (psi), cooled to 50 ºC and 5% of fresh human blood was added. This medium was used as enrichment medium for the cultivation of the bacterial isolates and to determine their ability of blood hemolysis (Forbes et al., 2007).

2.2.3 Urea Agar Medium

Urea agar medium was prepared by adding 10 ml of urea solution (20% sterilized by millipore filter paper 0.22 μm) in volume of autoclaved urea agar base and completed up to 100 ml D.W and cooled to 50 ºC, the pH was adjusted to 7.1. The medium was distributed into sterilized test tubes and allowed to solidify in a slant form. It was used to test the capability of bacteria to produce urease enzyme (MacFaddin, 2000).

2.3 Collection of Specimens

Eighty four clinical specimens from mid-stream urine were collected from patients attended different hospitals, laboratories and privet laboratories in the Hilla city/Iraq (Hilla Teaching hospital, Childhood and Gynecology Hospital). Mid-stream urine samples were collected in sterilized screw-cap containers. The number of samples from males were 54 (64.28%), while from females were 30 (35.71%), which were isolated during 2018, and their identification was confirmed by routine biochemical tests.

1.4 Laboratory Diagnosis

1.4.1 Morphological Characteristics (Collee *et al*., 1996)

Samples were cultured on the MacConkey agar and blood agar by streak plate method and incubated at 37ºC for 24 hrs to observe the colony morphology (shape, size, surface texture, edge elevation, colour, opacity and hemolysis). Then colonies were stained by Gram stain to observe a specific shape, type of reaction, aggregation and specific intracellular compounds.

1.4.2 Biochemical Tests

2.4.2.1. Diagnosis of the bacteria was carried out by biochemical methods (Oxidase test, catalyse test, swarming, unease test, and IMVC test) according to (McFaddin, 2000).

2.4.2.2 Urease Production

The bacteria were inoculated on the entire slop of urea agar and incubated at 37°C. The result was read after 24 hrs to 6 days. Urease test is positive if the indicator was changing the colour of medium to purple-pink.

2.4.2.3 Hemolysis test

The presence of hemolytic activity was detected as a clear surrounding positive colony on blood agar.

2.4.2.4 Swarming phenomena

The bacteria were inoculated on center of blood agar as a spot and incubated at 37°C for 48 hrs. Swarming phenomena is positive if the concentric rings of growth that are formed as cyclic events of swarmer cell differentiation.

2.4.3 Antibiotic Sensitivity Test

Disk agar diffusion according to Kirby Bauer standardized antimicrobial susceptibility single disk method was carried out (Bauer *et al*., 1966; Forbes *et al*., 2007):

- Preparation of culture media and plates; Muller-Hinton medium was employed and heated to 4550°C, then poured in Petri dishes on a level surface to a depth of 4 mm. when the media was solidified the petri dishes were placed in the incubator at 37°C for 15-30 min. to let the excess moisture evaporated.
- Bacterial inoculums; with the sterile wire loop, the tops of 4-5 isolated colonies of E. coli were picked from the original culture and introduced in to a test tube containing 2 ml of normal saline to produce a bacterial suspension of moderate turbidity. Its turbidity was compared to that of the recommended turbidity standard to 0.5 McFarland turbidity equal to 1.5×10^8 CFU/ml.
- Inoculation of the test plates; within 15 min. of adjusting the density of the inoculums, a sterile cotton swab was dipped in to the standardized bacterial suspension. The swabs then streaked on the upper most surface of Muller-Hinton in three different planes to obtain uneven distribution of the inoculums. The lids were replaced and the inoculated plates were allowed to remain on a flat and level surface undistributed for 3-5min. to allow absorption of excess moisture.
- Disks were listed in table (2-2) placed on the inoculated plate and pressed in to the agar with a sterile forceps. Within 15 min. the inoculated plates were incubated at 37°C for 18 hrs. in an inverted position.
- Reading of the results; after incubation the diameters of the complete zone of the inhibition were noted and measured using reflected light and ruler. The end point measured to the nearest millimeter, was taken as the area showing no visible growth.
- Interpretation of zone size; the diameter of inhibition zone for individual antimicrobial agent was translated in terms of sensitive and resistant, the appearance of the inhibition zone of each antibiotic disk was measured and the results were interpreted by CLSI recommendation according to CLSI, (2014).

2.4.4 Genotyping Assays

2.4.4.1 Chromosomal DNA Extraction

The DNA of *Proteus mirabilis* was isolated according to Gentra puregene Bact. /kit. Following the manufacturer's instructions as follows:

 LB broth (5ml) was inoculated with single colony of *Proteus mirabilis* and incubated overnight at 37°C.

- A volume of 500µl of the culture (containing 0.5-1.5x10^9 cells) was transferred to a 1.5 ml microcentrifuge tube on ice centrifuged for 5 sec.at 8000 rpm, the supernatant was discarded.
- An aliquot of 300µl cell lysis solution was added to the pellet, and mixed by pipetting up and down. The sample was incubated at 80°C for 5 min., 1.5µl RNase A solution was added, and mixed by inverting 25 times, the sample solution was incubated for 30 min. at 37°C., then the sample solution was incubated for 15 min. on ice to quickly cool the sample.
- Protein precipitation solution (100µl) was added to the sample solution and vortexed vigorously for 20 sec. at high speed. Centrifuged for 3 min. at 8000 rpm. The pellet was discarded.
- DNA was precipitated by adding 300µl of isopropanol to the supernatant from the previous step, which was mixed by inverting gently 50 times. Centrifuged for 1min. at 8000 rpm. The supernatant was discarded and drain the tube by inverting on a clean piece of absorbent paper.
- An aliquot of 300µl of 70% ethanol was added to the pellet and the content was inverted several times to wash the DNA pellet, and centrifuged for 1min. at 8000 rpm. The supernatant was discarded, and drain the tube on a clean piece of absorbent paper, a volume of 50µl DNA hydration solution was added and vortexed for 5 sec. at medium speed, it was stored at -20°C for longer time periods.

2.4.4.2 Molecular Detection of *ureR* Gene by PCR (Polymerase Chain Reaction)

Primer and PCR conditions were used to detect gene of *ureR* are present in table (1).However, each 25µl of PCR consist of each upstream and downstream primer (2.5 µl), free nuclease water

(2.5 µl), DNA extraction in concentration 0.1µg/ml (5µl), and master mix (12.5 µl). The polymerase chain reaction amplicon was detected by gel electrophoresis on 2% agarose gels for 2hr at 70 V.

Table (2-5): Primer pairs sequence and amplicon size

Gene	Primer sequence (5′-3′)	Amplicon size(bp)	Reference
ureR (F)	GGTGAGATTTGTATTAATTGG	225 bp	Zhang *et al*., 2012
ureR (R)	ATAATCTGGAAGATGACGAG		

2.4.4.3 Amplification Conditions

Table (2-6): Program of PCR conditions to *ureR* gene

Steps	Temperature	Time	No. of cycles
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	40 sec	35
Annealing	58 ºC	1 min	
Extension	72 ºC	20 sec	
Final extension	72 ºC	10 min	1
Hold	4 ºC	10	

2.4.4.4 DNA Amplification (PCR)

A PCR (Polymerase Chain Reaction) is performed in order to make a large number of copies of a gene. Therefore, the quantity of DNA is insufficient and cannot be used for other methods of testing. A PCR is performed on an automated cycler, which heats and cools the tubes with the reaction mixture in a very short time. According to (Johnson, 2000), PCR is performed for 30-40 cycles, in three major steps:

1. .Denaturation
2. .Annealing
3. .Extension

2.4.4.5 DNA Detection (Agarose Gel Electrophoresis)

Agarose gel electrophoresis is one of several physical methods for determining the size of DNA. In this method, DNA is forced to migrate through a highly cross-linked agarose matrix in response to an electric current. In solution, the phosphates on the DNA are negatively charged, and the molecule will therefore migrate to the positive (red) pole. (Lodish *et al*., 2004).

3.1. Isolation and Identification of *Proteus mirabilis*

Fourteen *Proteus mirabilis* isolates (16.6%) were obtained from 84 clinical specimens of urine, collected from both sex with different ages were isolated from different hospitals, laboratories and privet laboratories in Babylon province, table (3-1). Identification of isolates was accomplished by routine biochemical tests.

Table (3-1): Distribution of age and sex of patients by sources of isolation

Age (year)	UTI No. (%)		Total No. (%)
	Male	Female	
0 – 10	4(4.7)	3(3.5)	7(8.3)
11 – 20	5(5.9)	4(4.7)	9(10.7)
21 – 30	15(17.8)	10(11.9)	25(29.7)
31 – 40	5(5.9)	3(3.5)	8(9.5)
41 – 50	10(11.9)	5(5.9)	15(17.8)
51 – 60	15(17.8)	5(5.9)	20 (23.8)
Total	**54(64.28)**	**30(35.72)**	**84(100)**

According to age groups, the clinical samples were divided into six groups. Difference in UTI clinical samples between males and females were found, table (3-1).

Based on morphological and cultural characteristics of Proteus mirabilis, the present results revealed that only 14 isolates (16.6 %) were Proteus mirabilis, and 39 (46.42%) isolates were identified as other bacterial spp , while no growth was obtained from 31 (36.90%) of samples figure (3.1).

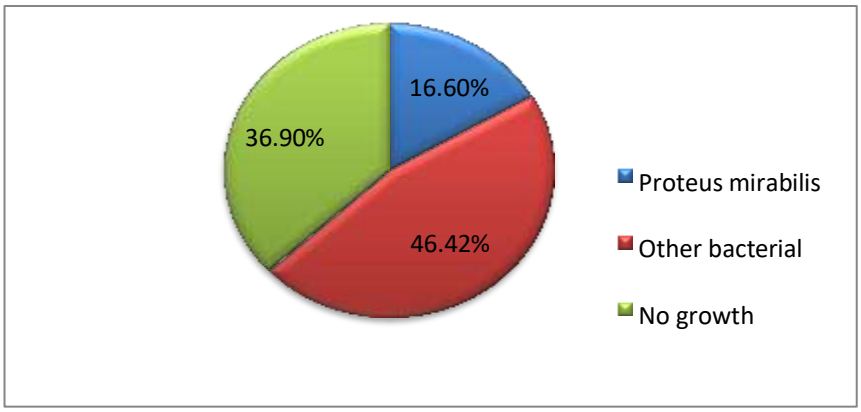

Figure (3.1): Percentages of bacterial isolates from UTI clinical specimens.

The present study revealed that the rate of infection in males (64.20%) was higher than females (35.70%), especially in the UTI, as shown in figure (3.2). However, the killer effect of vaginal fluid (which has low pH) may act naturally as a selection pressure against Proteus (Stamey, 1975).

This result agree with those reported by AL-Murjany (2002) who found that the isolation percentage of Proteus in male was (63.5%) and (36.5%) in female.

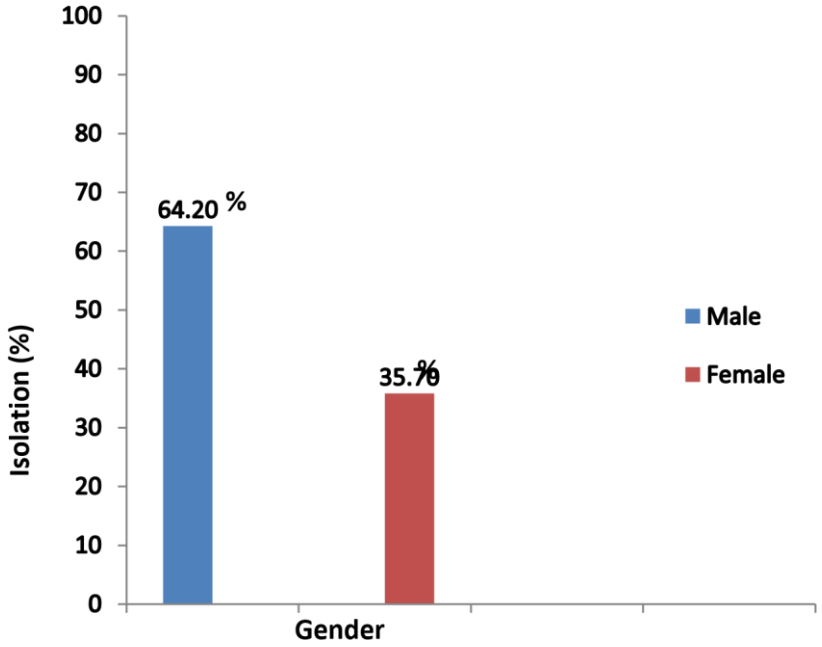

Figure (3.2): Prevalence of *Proteus mirabilis* by gender.

3.1.1 Cultural Characteristics and Biochemical Tests

Fourteen isolates were confirmed to be *Proteus mirabilis* based on traditional microscopic examination (Gram's stain), colony morphological features on MacConkey agar and blood agar, and standard biochemical tests. Table (3-2).

Table (3-2): Results of biochemical tests.

Test	Result
Gram staining	-
Indole Produce	-
Methyl Red	+
Voges-Proskauer	-
Citrate Utilization	-
Lactose Ferment	+
Oxidase Produce	-
Catalase Produce	+
Urease Produce	+
Motility Test	+
Hemolysin production	+

(-) negative, (+) positive

3.1.2 Virulence Factors of the Bacterial Isolates

3.1.2.1 Hemolysin production

Hemolysin production by Proteus was studied, and it was found that all isolates (100%) of *Proteus mirabilis* were capable to produce β-hemolytic on blood agar, the results are shown in figure (3-3)

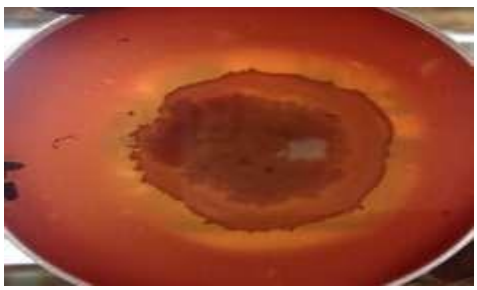

Figure (3-3):β-hemolysis on blood agar medium by isolated *P. mirabilis*

Production of hemolysin is generally associated with pathogenic bacteria, hence it is considered as important factors that contribute in their pathogenesis (Madigan *et al*., 1997).

Bahraini *et al*., (1991) and Sosa et al., (2006) who confirmed that all isolates (100%) of *Proteus* isolated from different clinical sources exhibit β- hemolysis on blood agar plates

3.1.2.2 Urease production

Urease production by Proteus spp. isolated was studied, and it was found that (100%) isolates of *Proteus mirabilis* were able to produce urease; these results were shown in figure (3-4).

Figure (3-4): Urease production by isolated *P. mirabilis*: pink color: positive and yellow color: negative

Urease enzyme was well-known to hydrolyze urea into ammonia and ultimately carbon dioxide (CO2). Therefor due to ammonia creation, arise in resident pH reasons sleet of normally soluble calcium and magnesium ions. These salts crystals can raise to significant mass to create kidney and bladder stones (Li etal., 2002; Stickler *et al*., 2005).

Mishara *et al*., (2001) who found that Proteus spp. produced high quantity of urease compared with other bacteria

3.1.2.3 Swarming Phenomenon

In this study, typical Proteus isolates were shown swarming motility by all isolates 100% when grown on blood agar plates. These results were shown in table (3-5).

Figure (3-5): swarming motility by isolated *P. mirabilis*

Proteus species have some unique features within the Enterobacteriaceae family, such as swarming motility, a coordinate multicellular phenomenon dependent on flagella that happens when cells grow on solid rich media or on sticky surfaces (McCarter and Silverman, 1998).

3.1.3 Antibiotic Susceptibility of Proteus mirabilis Isolates

Susceptibility test was done for all the 14 *Proteus mirabilis* isolates against 6 antibiotics, decision for considering an isolate resistant or sensitive was based on the comparison of the diameter of inhibition zone with that of standard value of CLSI, (2014). The results indicated that the various levels of susceptibilities to different antibiotics among isolates could be recorded. The results are summarized in figure (3.6).

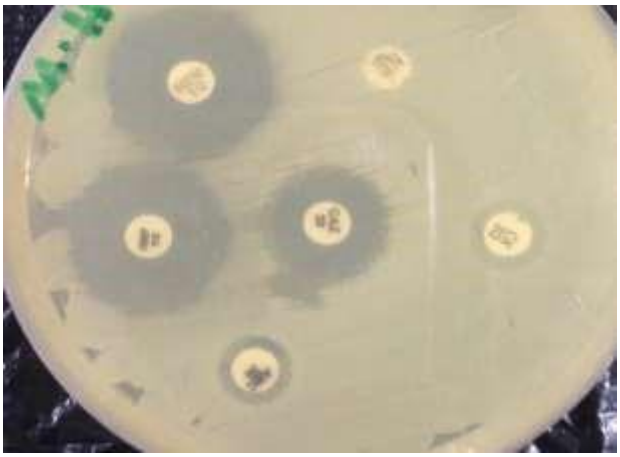

Figure (3.6): Antibiotics susceptibility of *Proteus mirabilis* isolates on Muller Hinton Agar. **CX**: Cefotaxime, **AK**: Amikacin, **CAZ**: Ceftazidime, **AX**: Amoxicillin, **IMP**: Imipenem, **MEM**: Meropenem **.**

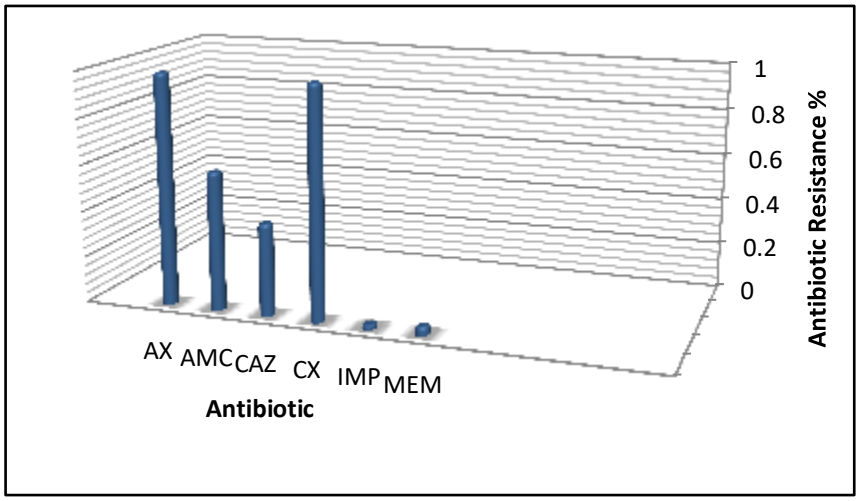

Figure (3.7): Antibiotics susceptibility of 14 *Proteus mirabilis* isolates. **CX**: Cefotaxime, **AMC**: Amikacin, **CAZ**: Ceftazidime, **AX**: Amoxicillin, **IMP**: Imipenem, **MEM**: Meropenem **.**

Figure (3.7) shows that Proteus mirabilis isolates were resistant 100% to Amoxicillin and Cefotaxime, Amikacin, (64.28%), Ceftazidime (50%), Meropenem (28.57%), and Imipenem (14.28%).

Molecular Study Extraction of DNA

The DNA was extracted from *Proteus mirabilis* by using the Gentra puregen cell kit figure (3.8).

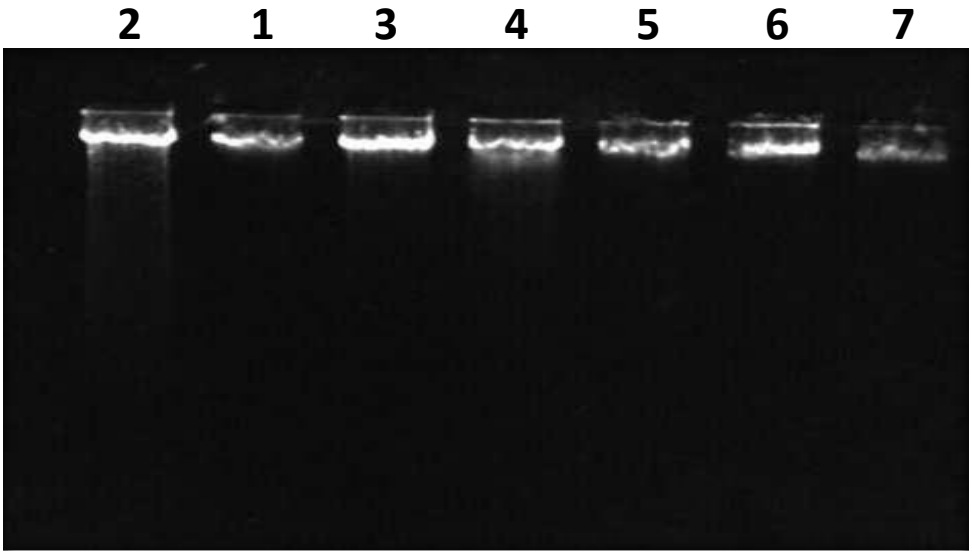

Figure (3.8): Gel electrophoresis of *P.mirabili* isolates DNA on 1% agarose gel at 60 volt for 1:30 hour.

Detection of ureR gene

Gel electrophoresis of PCR amplified products from extracted DNA isolates from urine and amplified with *ureR* primers. Out of 14 isolates the presence genes encodes (*ureR*) was detected in 7 of isolates showing amplification product of 225 bp, whereas other isolates were negative figure (3.9) and figure (3.10) respectively.

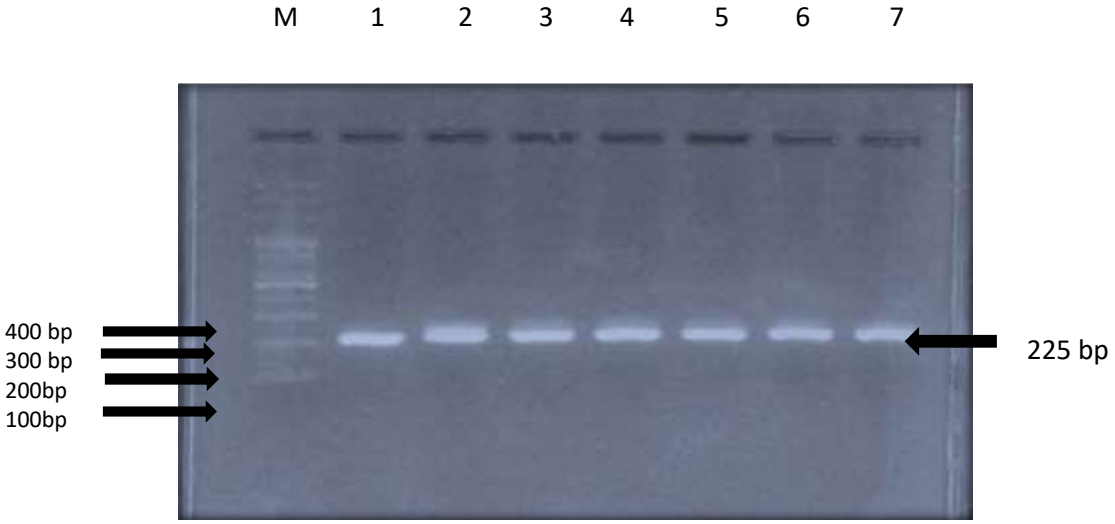

Figure (3.9): PCR amplification with specific primers for *ureR* gene in clinical isolates of *P. mirabilis.* Lane M, 100 - bp DNA marker. Lanes (1, 2, 3, 4, 5, 6, and 7) positive isolates on 2 % agarose at 70 volt for 2hrs.

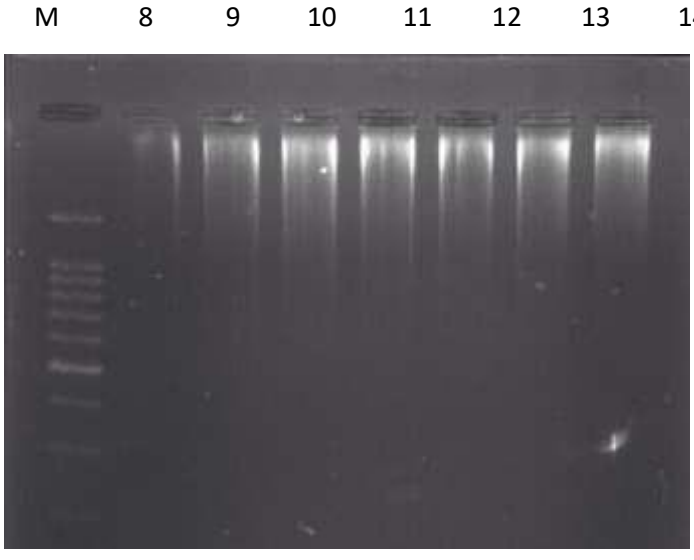

Figure (3.10): PCR amplification with specific primers for *ureR* gene in clinical isolates of P. mirabilis. Lane M, 100-bp DNA marker. All P. mirabilis (8, 9, 10,11,12,13, and 14) isolates show negative, on 2 % agarose at 70 volt for 2 hrs.

The current study shows that the presence of *ureR* gene approximately 225-bp DNA fragment appeared in PCR product, and thus the *ureR*-based PCR method can be used for specific detection of *P. mirabilis*.

PCR-based methods, in particular quantitative PCR, are used primarily to identify and quantify either pathogens or beneficial populations, including *Bacillus sp*., *Campylobacter sp*., *Legionella sp*., *Pseudomonas sp*., *Salmonella sp*., and *Vibrio sp*., etc., based on the 16S rRNA genes or their specific functional genes (Wehrle *et al*. 2010).

The *ureR* of *P. mirabilis* is the only one true regulatory gene that has been identified and is present only in those gene clusters that are inducible by urea (Mobley *et al*., 1995).

Naas *et al*., (2000),who reported that *P. mirabilis* was identification by the *ureR* gene of the most specialized and accurate gene in type diagnosis, and more specialized than the *ureC* gene in the diagnosis of *P. mirabilis*.

CONCLUSION

The study documented that all *P. mirabilis* isolated from urine clinical specimens have the ability to possess more than one virulence factors such as Urease, hemolysin, and swarming activity. The *ureR* - PCR technique is effective way to identification of *P. mirabilis*.

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