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Molecular Identification Of Virulence Factors genes of *proteus mirabilis* Isolated From Urinary Tract Infection

A Thesis

Submitted to the Council of College of Science\ University of Diyala in Partial Fulfillment of Requirement for the Degree of

Master of Science in Biology

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Dedication

To my heart ... and my life ... who supported and encouraged me and carried with me the burden of study ... my dear husband ... Bashar

To the one who raised me and drowned me with his compassion and tenderness ... to the light of my eyes ... my beloved father.

To the candle of my life, to the light that illuminated my path and wove through it my strength and my determination ... my beloved mother .

To my children (Muhammad, Amir, Shahd, and Shadan)

To those I cherish ... my childhood friends ... my brothers and sisters ... dear (Ali, Omar, Ibrahim, Muhammad, Tho-Alfaqar and Abdullah) (Shaima and Sara).

To everyone who supported me to complete my scientific career To everyone who taught me a letter.. My teachers, may God protect them.

To the pure souls that left us ... the martyrs of Iraq.

He consecrated the fruits of this humble effort

Dina Nazar

Acknowledgment

Praise be to God, Lord of the worlds, and prayers and peace be upon our master, ***Prophet Muhammad****, may God's prayers and peace be upon him, his family and companions, and who has guided his guidance to this day.*

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Dina Nazar

Summary

This study was aimed to detect some virulence factors of *Proteus mirabilis* isolated from patients with urinary tract infection using molecular methods. 300 urine specimens were collected from patients who attended (Al-Khalis general hospital, Al- Batool Teaching Hospital and Baquba Teaching Hospital) for a period extended from the 5th of August 2020 to 25th of January 2021. The diagnosis was done by consultant urologist. The age range of patients was from 2 to 56 years old. The isolation and identification of *P. mirabilis* was achieved by conventional methods including (Bacteriology, biochemical and Vitek kit) and molecular methods including (single-plex PCR of *ZapA* gene, *Urec* gene, Mrp gene, *FlaA* gene and *Esp* gene, multi-plex PCR *ZapA* gene and *FlaA* gene of and gene sequencing of *ZapA*). The antimicrobial susceptibility to 18 antibacterial was done using Kirby-Bauer disk diffusion method. Furthermore, The MIC for Cifipime and Meropenem was determined by microtiterplate .

According to the conventional detection methods, 25 isolates were diagnosed with 8.3% *protues mirabilis* bacteria from 300 samples. The highest isolation rate was found to be among those (20-29) years old patients 36.0% . Female 64.0%, rural residence 60.0%, acidic urine 76.0%, urine samples with10 pus cells/HPF, and urine samples with 10 RBCs/HPF. Regarding the virulence factors; all isolates 100% were positive for hemolysin, urease, siderophore, colony-factor antigen I, II, and III. Additionally, 11(44.0%) are strong and 11(44.0%) are moderate biofilm former, while only 3(12.0%) were non-biofilm formers. According to the susceptibility of studied antibiotics, the results showed that 17 out of 25 (68.0%) of *P. mirabilis* isolates were multi-drug resistant (MDR), while the remaining 8 32.0% were extended drug-resistant (XDR) isolates.

Single-plex PCR found that all 100% isolates had the *ZapA* gene, *Urec* gene, *Mrp* gene, *FlaA* gene. Whereas, the isolates 40% had the *Esp* gene. The result of multiplex PCR technique of (*ZapA* and *FlaA*) genes revealed that 100%. Gene sequencing results of *ZapA* showed the detection of three nucleic acid substitutions of *P. mirabilis* in isolates (G159A, G349A, and G439T). The G439T variant was observed in the phylogenetic tree toward the Chinese *Proteus mirabilis* strain ZA25, while the G159A was observed toward the Sweden *Proteus mirabilis*, CCUG 70746 strain. In contrast, the G349A variants did not induce any deviation in the phylogenetic positioning of currently investigated *P. mirabilis* isolates.

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List of Abbreviations

Abbreviate	Definition
B.H.I.	Brain Heart Infusion
BURST	Based Upon Related Sequence Types
Bp	Base pair
CIT	CitrateClinical and Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
EDTA	Ethylene diamine tetra acetic acid
Esp	Enterococcal surface protein
GEL	Gelatinase
GLU	Glucose
Mrp	Multidrug resistance-associated protein
Mrp	Multidrug resistance-associated protein
Mrp	Multidrug resistance-associated protein
MDR	Multi Drug-resistant
MIC	Minimum inhibitory concentration
MR	Methyl Red
NCBI	National Center for Biotechnology information
O.D	Optical Density
Spp.	Species
S	Sensitive
STM	Signature –tagged mutagenesis screens

TSI	Triple Sugar Iron
TDA	Tryptophane deaminase
URE	Urease
UTI	Urinary Tract Infection
UV	Ultraviolet
VP	Vogas-Proskauer
XDR	Extensively drug-resistant
Zap	Z-ring associated protein

Chapter One (Introduction)

Introduction

Proteus belongs to the Enterobacteriaceae of Gram-negative bacteria that are facultatively anaerobic. Gustav Hauser was the first to discover it in 1885. The willingness of its species to undergo morphological changes inspired the name of this genus, *Proteus* species are motile and have four to ten peritrichous flagella. (Ryan and Ray, 2010).

Proteus is an opportunistic pathogen that can cause serious invasive diseases in people who are chronically ill, elderly, or pregnant. It is a major cause of infections acquired in hospitals (Hamilton *et al*., 2018). *Protues mirabilis* can cause urinary tract infections, respiratory tract and wound infections, burns, and digestive tract infections, among other things (Smelov *et al*., 2016).

Protues mirabilis has many virulence factors like flagellum, capsules, fimbria and enzymes like (deaminase amino acid, urease and proteases), toxins such as endotoxins ,hemolysins and proteus toxic agglutinin (Pta), that detected using molecular techniques which explains the difficulty in achieving clinical therapy effectiveness (Cestari *et al*., 2013).

Protues mirabilis secretes a variety of cell-associated factors, including swarming, fimbriae, urease, proteolytic activity, and the development of hemagglutinins and hemolysins, iron acquisition systems, protease, and Lipopolysaccharides (LPS). This pathogen has evolved some pathogenic factors that allow it to colonize, survive, and develop in its host.

Protues mirabilis is also noted for its ability to shape biofilms that colonize routes and thus avoid antibiotic treatment. *Proteus* species are

the third most common cause of urinary tract infections (Flores-Mireles *et al*., 2015b).

A urinary tract infection (UTI) occurs when a pathogen enters the urinary tract system and multiplies to over 10^5 colonies per milliliter of urine, UTI is the second most common cause of infectious disease, affecting over 150 million people worldwide (Mann *et al*., 2017).
Protues mirabilis has been showing an increase in resistance to many antimicrobial agents in recent years (Gajdács and Urbán, 2019).

Antibiotic resistance has resulted in not only improvements in antimicrobial therapies, but also poor prognoses and a rise in hospitalized patient mortality (Giamarellos-Bourboulis *et al*., 2006).

Protues mirabilis has gained resistance to many antibiotic types, making treatment more difficult. Resistance to -lactams (both penicillins and cephalosporins), fluoroquinolones, nitrofurantoin, fosfomycin, aminoglycosides, tetracyclines and sulfonamides, in addition to the previously mentioned resistance to sulfamethoxazole and trimethoprim, has been identified (Schaffer and Pearson, 2017).

Thus, there are many virulence genes that assist survival of *P. mirabilis* within the urinary system such as urease, hemolysin, fimbriae, and flagella. However, *P. mirabilis* strains differ in the range and expression levels of virulence genes that can affect growth of bacteria and persistence within the urinary tract Abbas et al.,(2015).

A number of studie s have investigated the virulence characteristics of *P. mirabilis* and mechanisms involved in pathogenesis of UTI to identify the range of *P. mirabilis* virulence genes and their prevalence among *P. mirabilis* isolates(Hussein *et al* 2020) .In the present study, *P. mirabilis* isolates involving in human UTI are characteriz to identify

virulence gene markers in an effort to explore strategies involved in *P. mirabilis* pathogenesis and antibiotics susceptibility.

The aim of study:

This study was aimed to detect some virulence factors of uropathogenic *P. mirabilis* using multiplex PCR method. To achieve this goal, the steps of this study are:

1- Isolation and identification of *P. mirabilis* from patients with urinary tract infections.

2- Study the susceptibility of *P. mirabilis* toward certain traditional antibiotics.

3- Phenotypic detection of some virulence factors of *P. mirabilis* using cultural methods.

4- Molecular detection of some virulence factors using singlplex, multiplex PCR and gene sequencing methods.

Chapter Two
(Literature Review)

Literature Review

2.1. Diseases of urinary tract (UTI):

When a pathogen can penetrate the urinary tract system and cross more than 10^5 cfu /ml in urine, it is known as a urinary tract infection (UTI) (Smelov *et al*., 2016). According to previous studies, urinary tract infections (UTI) account for about 40% of all acquired hospitalized infections and more than 45% of bacteremia, which explain the increasing in mortality rate and morbidity (Saint *et al*., 2008). In general, women are more expected to infected with UTI at least one during their lifetimes in comparison with males, with a fifth of those patients developing the recurrence type of this problem (Brumbaugh *et al*., 2013).

Members of this family are present in commensal form in the gastrointestinal tract of humans and animals, and may be saprophytic when present in the water and soil environment (Ryan *et al*., 2010), while they are opportunistic pathogens when they move to other places of the body causing many injuries such as urinary tract infections and lung infections, Contamination of wounds and burns, injuries of the nervous system and the bloodstream (Cabral, 2010). Intestinal bacteria have many virulence factors that help them to invade the host and cause infection, such as their production of internal and external enzymes and toxins as well as the formation of the biofilm. Also, the composition of the cell wall of the Gram-negative bacteria is an important factor in its virulence because it contains a thin layer of peptidoglycan and a layer of lipoproteins and many Liposaccharides and phospholipids, in addition to their outer shell, which is characterized by optional permeability, as it provides protection for bacteria from lysosomes and antibiotics (Melnyk *et al*., 2015).

It has the ability to move in concentric waves similar to sea waves, forming what is known as the swarming phenomena, which is one of the diagnostic characteristics that are unique to the sex of fluctuations from the rest of the races of the intestinal family (Carey *et al*., 2013).

It was named *Proteus* for its possession of the phenomenon of pleomorphism, as it has the ability to change its shape between the long, moving linear L-Form and the short non-moving spherical O-Form (Zunion *et al*., 2003). This bacterium is spread in the environment as it can be isolated from soil, water and decomposing organic matter, and it is found as a natural house in the intestinal tract of humans and animals, but it is pathogenic and causes autoinfection when it leaves its original place, which led to the use of Anti-inflammatory with high effectiveness against this type of bacteria (Brooks *et al*., 2007 and Hegazy, 2016).

2.2 Classification and Gentic Characterization of *Proteus mirabilis*

The genus *Proteus* belongs to the Enterobacteriaceae family, which includes Gram-negative Bacillus, and to the Tribe tribe known as Proteeae, which includes three genera (*Proteus*, *Morganella*, *Providencia)Rozalski et al*, (2012). The genus Proteobacteria was previously divided into the following species based on the biochemical differences, which are *P. mirabilis*, *P. vulgaris*, *P. inconstance*, *P. rettgeri*, and *P. morganii*, but after using the DNA-DNA hybridization technique and determining the proportion of Quanine and Cytosine Scientists have been able to place new species within the correct genera (O'Hara *et al*., 2000).

On this basis, the species *P. rettgeri* was transferred to the genus Providencia to become Providencia rettgeri, and the species *P. inconstance* was placed within the genus Providencia for the same

reasons, which was divided into two types Providencia alcalifaciens and rovidencia stuartii, and the type *P. morganii* was included in the genus Morganella to become *M. morganii*, as it was found that the content of (G + C) in the DNA of these species is higher than that of the genus *Proteus* According to the modern classification, the genus *Proteus* includes five species, which are as follows: *P. mirabilis, P. vulgaris, P. myxofaciens, P. hauseri*, and *P. penneri* with three non-named genome species, all of which are isolated from human disease cases except for the type *P. myxofaciens*, it infects insects, as it was isolated from the mouth of the live and dead larvae of the gypsy moth (Porteria dispar Janda) and Abbot ,(2007). Recently, the species *P. myxofaciens* was excluded and placed under a new genus called Cosenzaea, as the results of partial analysis of the *rpoB* gene encoding the RNA polymerase enzyme led to the conclusion of the level The genetics to differentiate this species from that of *Proteus*-Providencia (Giammanco *et al*., 2011). The most recent and currently approved classification as shown in Table 2-1 (Adeolu *et al*., 2016).

Scientific classification

Formal Rank	**Example**
Kingdom	Bacteria
Phylum	Proteobacteria
Class	GammaProteobacteria
Order	Enterobacteriales
Family	*Morganellaceae*
Genus	*Proteus*
Species	*Mirabilis*

The chromosome is characterized by the possession of pathogenicity islands consisting of motile genetic elements called Integrative and Conjugative Elements (ICEpm1) consisting of 94 kilobases with a high percentage of (G + C) 44.84% (Flannery *et al*., 2009).

These islands are present in the genome of pathogenic strains only and have the ability to transmit between bacterial cells as they contribute to increasing their virulence. They are also distinguished by their containment of genes responsible for virulence factors as they encode the proteins needed for iron acquisition proteins and the production of Proteus toxic agglutinin (Alamuri and Mobley, 2008). *Proteus mirabilis* HI4320 contains a single plasmid of 36.289 nucleotide size that has 55 coded sequences with a (G + C) ratio of 36.21% and is closely related to Circular-R-factor molecules (R6K) of *E.coli* that regulates the construction of the penicillinase enzyme. The genome of this bacterium contains the gene *tetAJ* encoding for Tetracycline resistance protein and the gene encoding for the enzyme Acetyltransferase which detoxifying chloramphenicol (Pearson *et al*., 2008).

2.3 Proteus species:

Proteus could change shape at will to avoid being interrogated, which is why his name was proteus (Williams *et al*., 1978). Urease output from urine is high in these patients. They have virulence factors (hemolysin, IgA protease, fimbriae, and cytotoxins) that are essential in the pathogenesis of UTIs (Manos and Belas, 2006). They are intrinsically resistant to many antibiotic classes such as nitrofurantoin, tetracyclines, and colistin which can be used as a presumptive marker for identification of *Proteus mirabilis* (Mazzariol *et al*., 2017).

Proteus mirabilis is widely thought to have the most beneficial resistance patterns among the Proteae (Mazzariol *et al*., 2017). *Proteus*

mirabilis is a highly motile bacterium that belongs to the Enterobacteriaceae family. *Proteus mirabilis* differs from other genera of Enterobacteriaceae, it is not causes UTI in healthy people (Chen *et al*., 2012). On the other hand, *P. mirabilis* is often isolated from patients with complicated UTI, especially patients who underwent physiological problems such as a chronic indwelling urinary catheter or urolithiasis (Fatima and Al Mussaed, 2018).

The capacity of *P. mirabilis* to produce urease, which produces ammonia and raises the pH of urine more than 7.2, (Ali, 2012). The presence of magnesium and Calcium crystals in alkaline urine clots the lumen of catheter and causing bacteriuria, acute urinary retention and other complicated infections such as pyelonephritis and shock (Chen *et al*., 2012).

Protues mirabilis was found to have improved resistance to several antimicrobial agents (Lockhart *et al*., 2007). Increased antimicrobial resistance has resulted in not only improvements in antimicrobial therapy, but also poor prognoses and a higher mortality rate among hospitalized admitted patients (Chen *et al*., 2012). *Proteus mirabilis* may causes either symptomatic urinary tract problems like pyelonephritis and cystitis or asymptomatic like bacteriuria especially in diabetic patients and elderly ages (Kakde *et al*., 2018).

2.4 Epidemiology of *protues mirabilis*:

The genus of *Proteus* can be found in a variety of places in nature, including contaminated water, dirt, and manure. Proteus may be contributed in the decomposition of animal organic their ability to hydrolyze urea to ammonia and carbon dioxide, as well as their proteolytic ability make a difference, and oxidative deamination of amino acids. They can be found in both human and animal intestines. *Proteus*

mirabilis was isolated most frequently from cows, birds and dogs, while *P. Vulgaris* was isolated most frequently from cold-blooded vertebrates and pigs(Singh *et al*., 2019).

Proteus species, along with *Escherichia coli* and Klebsiella species, are natural flora of the human intestinal tract, with *E. coli* being the most common resident (Wilson, 2016). *Proteus* spp. are effective causes UTIs in both of nosocomial and community-acquired patients; 4 to 6% of *Proteus* infections in Europe and North America are community-acquired, and 3 to 6% are nosocomial (Koksal *et al*., 2017). Young people are considered pathogenic, while the elderly are considered opportunistic pathogens. The elderly are at the highest risk of infection, particularly those who have indwelling catheters (long-term catheterization) or are on regular antibiotic therapy (Cohen-Nahum *et al*., 2010). Other focus groups of sexes, with uncircumcised males having a higher infection rate (Hindi, 2014). A study conducted in Europe and North America proved that it is the main cause of nosocomial injuries, at a rate of 3-6% and of community acquired, at a rate of 4-6% (Abbott, 2007).

Protues mirabilis bacteria are endemic to hospitals and are transmitted through contaminated water, food, soil, and solutions taken intravenously. Also, the hands of patients and health care personnel are the cause of its spread, and it is the third cause of complex urinary tract infections after *E.coli* and *Klebsiella pneumonia*, which accounts for 12% of the infection, while It is the second cause of catheter-related UTIs after the bacterium providancia stuartii, which accounts for 15% of the infection (Jacobsen *et al*., 2008).

Chlabicz *et al.* (2011) Confirmed a 3.4% presence in uncomplicated urinary tract infections in Poland. *Protues mirabilis*

bacteria are a source of diseases, especially for people who suffer from weak immunity or from malnutrition, which forces them to take antibiotics frequently and this leads to the emergence of bacterial strains resistant, especially to the third generation cephalosporins, resulting in recurrence of infection and an increase in the mortality rate.

2.5 Urinary tract infection of *Proteus mirabilis:*

The majority of uropathogenic *P. mirabilis* are thought to be caused by bacteria ascending from the digestive tract, In healthcare environments, person-to-person transmission is the source of the others (O'Hara *et al*., 2000). Data suggest that the strains which isolated from urine and stool specimens in patients with UTI were same, whereas others do not (Mathur *et al*., 2005).

In comparison with *E. coli*-caused UTIs, *Proteus* spp. caused UTIs are often more isolated from patients with pyelonephritis.The majority of *Proteus* spp. bloodstream infections are caused by urinary tract infections and are often associated with urinary catheters (Thakare, 2016).

One study found that *P. mirabilis* bacteremic UTIs is more likely to have hydronephrosis, band neutrophils accounting for >10 percent of the WBC count, hyperthermia or hypothermia, an extreme SIRS reaction, and a high C-reactive protein concentration. Furthermore, the findings of the study indicate that uropathogenic *P. mirabilis* are more isolated from contracted infections (Chen *et al*., 2012).

Encrustation further obstructs the flows and induces vesicoureteral reflux, resulting in an increasing problem that can progress to bacteremia, pyelonephritis and shock. This could demonstrate that why patients with community-acquired infections are more likely to develop bacteremic *P. mirabilis* UTIs (Flores-Mireles *et al*., 2015a).

2.6 Pathogenicity of *Proteus mirabilis*:

Proteus mirabilis is a biofilm-forming agent that fouls the surface of a newly inserted urinary catheter easily. Fimbriae and other adhesins, which are surface organelles, tend to perform a key function in this process. Urease, plays a significant role in the pathogenicity, the method of removing excess nitrogen, urea, is found in excess concentations in urine about of 400 mM, is a substrate for urease and is hydrolyzed to NH3and CO2, the releasing ammonia led to ascend the pH of urine, causing soluble of cations and anions in the urine to precipitate. Urolithiasis develops as a result, with struvite (MgNH3PO4) or apatite (CaPO4) stones forming (Chelsie, 2017).

These crystals may grow on and inside catheter lumens, obstructing urine flow and requiring replacement or removal of catheter. Stones are develop in the tubules or pelvis of renal, causing inflammation and necessitating surgery. This bacterium can infiltrate bladder epithelial cells and produce cytotoxins that harm the epithelium, resultingin severe histopathology (Armbruster *et al*., 2018b). These bacteria greatly affect immunodeficiency, which is the main cause of hospital infection (Filimon and Iacob, 2007).

The fluctuations bacteria cause urinary tract infections, and there are two ways to cause infection, the haematogenous route and the ascending route, and is the most common ascending route as it intervenes. The bacteria Urethra, then the bladder, the ureter, until the kidneys reach Kidneys (Ahmed., 2015). *Protues mirabilies* penetrate the lining of the intestine and begin to proliferate as diarrhea occurs as a result of its secretion of intestinal toxins (Tortora *et al*., 2004).

2.7 The virulence factors of *Proteus mirabilis*:

Proteus mirabilis has developed several virulence factors which as essential for causing UTI, these include flagella for increasing to the kidneys through the ureters and urease for hydrolyzing urea, these virulence factors are most likely spatially and temporally regulated in their expression. *Proteus mirabilis* must enter urinary tract by the urethra, and habitat the bladder, climb the ureters to the kidneys, colonize the kidneys, and keep the infection going (Armbruster *et al*., 2017). *Proteus mirabilis* may often gain access to capillaries and cause bacteremia, it requires these in equal amounts to express different adhesions and flagella in tandem with morphological turns from vegetative cell to hyper flagellated cell, express toxins, and escape the responsibility of host immune (Kadhim *et al*., 2019).

The virulence factors are described as molecules formed by pathogenic bacteria that affect the host's body in a way that makes the bacteria grow and become more effective (Brown, 2013). Some fall within the cellular structure and others are excreted outside the body. The virulence factors encode by specific genes that enable the pathogen to control and colonize the host's defense lines (Relman and Falkow, 2009). *Protues mirabilis* possesses many of these factors which give an ability to invade the host and cause pathogenicity, including production of enzymes such as calorase, lipase and protease, as well as hemolysin, fluctuation (anthyl), adhesion to epithelial cells, bacteriocin production, biofilm formation (Jacobsen *et al*., 2008 and Armbruster and Mobley, 2012) show figure (2-1).

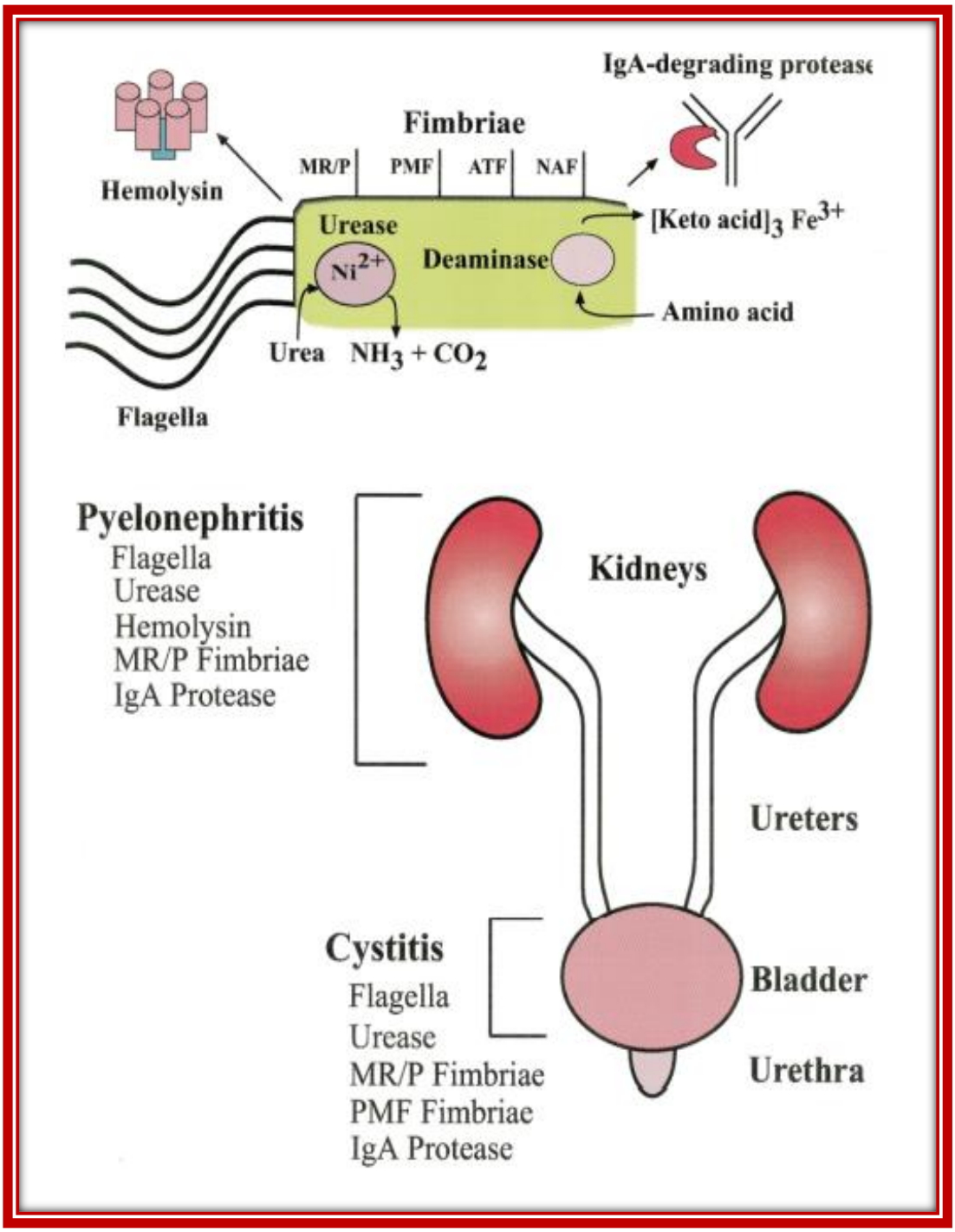


Figure (2-1): The sites of action of virulence factors for *Proteus mirabilis* (Ambester *et al*.,2018)

2.7.1 Urease:

Urea is hydrolyzed into ammonia and carbon dioxide by the cytoplasmic nickel metalloenzyme Urease, many bacteria prefer ammonia as a nitrogen source, and it can be predigested into biomolecules using glutamate dehydrogenase (GdhA) or glutamine synthetase (GlnA) (Carlini and Ligabue-Braun, 2016). The rise in local pH is a direct consequence of Urease activity and ammonia production. The alkalinity of urinary tract causes precipitation of magnesium and calcium ions which led to build urinary tract stones that made up of calcium phosphate and magnesium ammonium phosphate (Mora and Arioli, 2014).

Urease causes virulence by causing urinary stones to form and can obstruct urinary flow and cause tissue harm (Chew *et al*., 2012). *Proteus mirabilis* adherent to a urinary catheter can mix with the precipitated minerals, forming a crystalline biofilm with blocking of urine flow through the catheter (Stickler, 2008). These crystals can be seen in cultured epithelial cells of urinary tract that infected with invasive *P. mirabilis* in an experiment (Torzewska *et al*., 2014). Pathogens can be protected from drugs or the immune defence if *P. mirabilis* becomes embedded in these stones (Armbruster *et al*., 2017). Urinary stones may also serve as a focal point for other species of *p.mirabilis* to produce UTI (Róalski *et al*., 2012).

2.7.2 Flagella:

Protues mirabilis like many bacteria, swims across liquids and against chemical gradients using flagella, in liquid culture, *P. mirabilis* shows as a short rod, and usually has peritrichous flagella (Minamino and Imada, 2015). On the other hand, *P. mirabilis* differentiates into non-septate polyploid cells with many of flagella on rich solid media, typically (20-80) µm, while cells longer than 100 m have been observe

these swarmer cells migrate around surfaces as a group (Schaffer and Pearson, 2015). Hauser (1885) was the first to notice the swarming in *P. mirabilis* when he cultured it on solid surfaces. The bacteria re-differentiate into swarmer cells after some time, in a repetitive phase of swarming and consolidation, these bacteria migrate through several media surfaces, forming a distinctive bullseye pattern. Swarmer cells are morphologically recognize from vegetative or swimmer cells, with long lengths (typically 20 to 80 micrometers) and hyper-flagellation, as shown in Figure (2-2). The composition differs in peptidoglycan, lipopolysaccharide (LPS) and membrane fatty acid accompany the transition to the swarmer type (Strating *et al*., 2012). Swarm cells form rafts of parallel cells as they travel together as a group (Morgenstein *etal*.,2010).

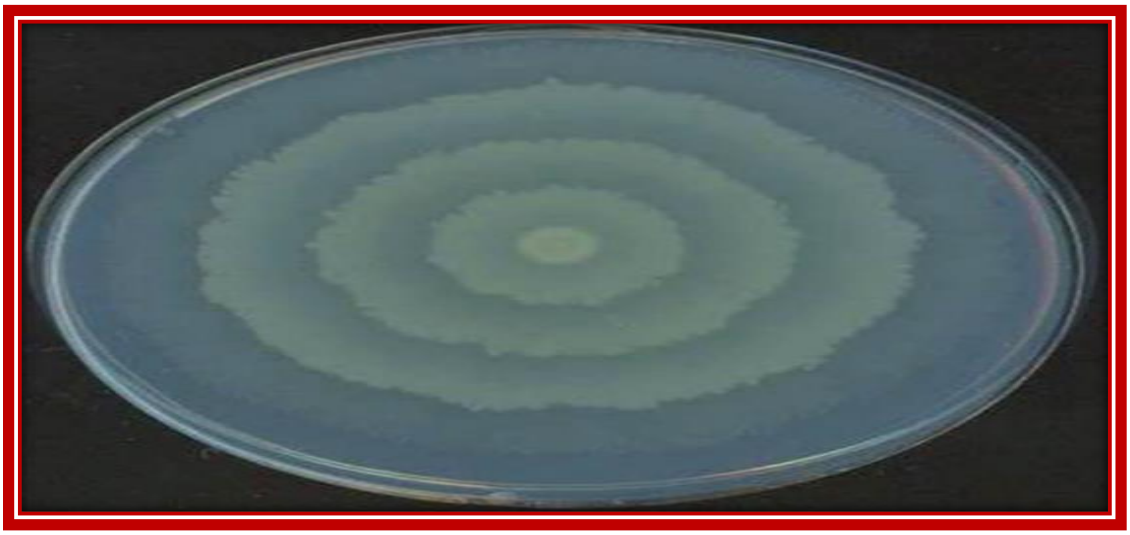


Figure (2-2): Swarming colony of *Proteus mirabilis* (Ayati, 2019)

When compared to older cultured bacteria or broth culture in the interior of a swarm colony, virulence factors such as urease , hemolysin and protease are stimulated at the time a swarm (Lazm *et al*., 2018). *Proteus mirabilis* swarming capacity is particularly important in since this organism will swarm, catheterized patients are at risk. through silicone or latex catheters (Jacobsen and Shirtliff,2011). Along with the

autotransportertoxin(Pta), *P. mirabilis* produces a Serratia-type calcium-independent hemolysin that acts as lyses of nucleated cells as well as RBCs (Schaffer and Pearson, 2015). The capability of the bacterium to invade cultured kidney cells is correlated with its hemolysin levels (Jung *et al*., 2019).

2.7.3 Metalloprotease

Antibodies and antimicrobial peptides are among the host defenses that *P. mirabilis* must resolve to live in the urinary tract. *Proteus mirabilis* does this byencoding a variety of proteases (Armbruster *et al.*, 2018a). The gene *ZapA* is a metalloprotease that can degrade a wide range of host proteins *in vitro, Zap*A degrades mouse immunoglobulins *in vitro*, however, new research indicates that a degradation of the human antimicrobial proteins -defensin-1 and LL37 is more efficient than *Zap*A-mediated degradation of native immunoglobulins (Carson *et al*., 2011 and Schaffer and Pearson, 2017).

In vivo, ZapA is expressed at low levels, a *Proteus mirabilis* Z*ap*A mutant colonizes the urine, bladder, and kidneys independent tests, wild type performs less well than wild type. challenge murine infections (Pearson *et al*., 2011). In the fiveopen reading frames, originated from *Zap*A, sequencing found four additional putative metalloproteases (Pearson *et al*., 2008).

The gene *zapE* was named after the gene immediately originated from Z*apA*,and the three metalloproteases assumed to be copies of *ZapE*. Since the mutagenesis used a transposon, which may influenced the development of putative metalloproteases, STM was used to classify one of the *ZapE* copies as a virulence gene. (Himpsl *et al*., 2008). In a separate STM review, a U32family peptidase ,(PMI3442) was reported an essential for establishment of infection (Schaffer and Pearson, 2015).

2.8 General characterization of *p.mirabilis*:

The most popular form of detection infection with *P. mirabilis* is a culture. *Proteus* species are rod-shaped, gram-negative bacteria that are facultatively anaerobic, the majority of strains are lactose intolerant and have swarming motility that will show up on agar plates, to make a correct diagnosis, one must always compare positive culture findings with the patient's clinical appearance (Jamil *et al*., 2017). Urine sample screening for pyuria and leukocyte esterase is one of the additional evaluations, since pyuria is usually present in bacterial urinary tract infections, the absence of pyuria may suggest a different cause of symptoms, while a leukocyte esterase dipstick is a good substitute for microscopy, it is less sensitive than microscopic inspection, although the absence of bacteriuria does not rule out infection, Gram staining of urine can help expose microscopic bacteriuria, which would confirm infection (O'Keefe *et al*., 2019). *Proteus* species are characterized on culture plates by their ability to swarm. On MacConkey agar, *Proteus* spp form 2-3 mm colorless, flat colonies, while on blood agar and luria broth agar plates, they swarm in waves (Chelsie E. Armbruster, 2017).

2.9 Antimicrobial susceptibility of *Proteus mirabilis*:

Despite advances in novel antibiotics over the last decade, treating urinary tract infections has become a more difficult task for clinicians (Calzi *et al*., 2016). Antibiotic resistance is on the rise, severely limiting the drugs choices for these diseases (Gajdács, 2019a). Nonetheless, with the advent of carbapenemase and extended spectrum Beta lactamase producing strains in uropathogenic bacteria, multidrug resistance (MDR) are becoming an increasing concern, as these bacteria may be immune to a wide range of treatments when combined with other acquired and inherent resistance mechanisms (Gajdács, 2019b).

In recent years, a pattern has emerged in which *P. mirabilis* has become more resistant to a variety of antimicrobial agents (Gajdács and Urbán, 2019). Antibiotic resistance has resulted in not only improvements in antibiotic treatments, but also poor prognoses and a higher mortality rate among hospitalized patients (Giamarellos-Bourboulis *et al*., 2006) .

Proteus mirabilis has gained resistance to many antibiotic groups around the world, complicating care. The resistance to cephalosporins, fosfomycin, penicillins, nitrofurantoin, sulfonamides, aminoglycosides, tetracyclines, and fluoroquinolones has been identified in addition to sulfamethoxazole – trimethoprim resistance (Schaffer and Pearson, 2017). Most isolates, in particular, are tetracycline-resistant (O'Hara *et al*., 2000).

This organism's proclivity for being encased in crystalline biofilms or urinary stones on catheters of urinary tract will serve as a barrier to bacteria, resulting in treatment failures. Antimicrobial peptides such as protegrin, defensin, polymyxin B and LL-37 are also highly resistant to *P. mirabilis* (Belas *et al*., 2004). LPS modifications and extracellular proteases like *ZapA* are responsible for this resistance (Schaffer and Pearson, 2017) .

2.10 Diagnosis based on molecular methods:

Some of them can only detect a single pathogen or only Gram-positive or negative bacteria (Pasticci *et al*., 2020). A molecular approach has the benefit of quickly detecting UTI causes or genital infections(McKechnie *et al*., 2011). Several molecular methods were used to detect virulence factors of *P. mirabilis* like flagellum, fimbria, many enzymes capsules and toxins such as proteus toxic agglutinin , hemolysins, and endotoxins that clarify the difficulty in achieving clinical treatments performance (Alamuri and Mobley, 2008).

The identification of *Protues mirabilis* genes or virulence factors is done using molecular methods (Cestari *et al*., 2013). Polymerase Chain Reaction (PCR), Dot Blot Test, Real Time-PCR and Gene Sequencing techniques are examples of molecular methods (Kim *et al*., 2015). This technique is a low-cost, easy-to-use, and effective target enrichment technique for both next-generation sequencing and large-scale Sanger confirmation sequencing (Lefever *et al*., 2017). PCR-based methods, particularly quantitative PCR, are primarily used to classify and quantify pathogens or beneficial populations using the 16S rRNA genes or their unique functional genes (Wehrle *et al*., 2010). Multiplex PCR methods for simultaneously detecting common pathogens, such as those developed to detect Enterobacteriaceae and clinically relevant bacteria (Clifford *et al*., 2012), have also been recommended.

2.11 Gene Sequencing:

The method for determining the sequence of nucleotide bases in a piece of gene which known as Gene sequencing. Sequencing a short piece of gene is now relatively simple with the right equipment and materials (Blauwkamp *et al*., 2019). Purified DNA contains fragments of studied gene from pathogens that cause disease in different sites of the body, increasing the likelihood of noninvasive identification of a wide range of diseases by sequencing the studied bacterial gene. Despite the promise of next-generation sequencing (NGS)-based approaches for clinical researches, quality controls and validation strategies that address the unique challenges of clinical metagenomics are required (Simner *et al*., 2018 and Hong *et al*., 2018).

The first complete sequence of *P. mirabilis* was published in (2008) when working on the strain HI4320 isolated from the diuretic of a patient suffering from urinary catheterization for long periods. The

numbers of coding protein sequences reached 3,685 with seven sites coding for rRNA, and the ratio of guanine bases of guanine and cytosine (G + C) was about 38.88%, and it was described as the smallest among the intestinal family chromosomes, including the pathogenic strains of *E. coli* (Pearson *et al*., 2008).

In the following years, several sequences of several strains of this bacterium were published, such as the strain BB2000, C05028 and PR03 in addition to three other strains, and they were close to each other as it was found that the genetic content of these strains consisted of a chromosome size of 3.82 - 4.6 million base pairs and the ratio (G + C) ranges between 38.5 - 39% (Schaffer and Pearson, 2015).

Chapter Three
(Materials and Methods)

3. Materials and Methods

3.1. Instruments and Equipment

Instruments and Equipment with their company and origin are listed in table (3-1)

Table (3-1)The equipmentand instruments that used in this study

Equipment & Instruments	Company/origin
Autoclave	Labtech / Korea
Centrifuge	Fisher Scientific/USA
Cylinders	Bomex / Germany
Conical flasks	Marienfeld/Germany
Cotton Swab	Afco/Jordan
Distiller	Nova /Turkey
Digital Camera	Sony/Japan
Disposable petri	Sun/China
Electric oven	Memmert/Germany
Electrophoresis	Thermo Scientific/USA
ELISA reader	HS-Human Reader/Germany
Eppendorf tubes	LP/Italy
Gel Imaging System	Major Science
Incubator	Binder/Germany
Light microscope	Nikon/Japan
Microwave oven	Bosonic/China
Micro Spin Centrifuge	My fugene /China
Millipore filters(22.0 and 0.45 μm)	Sartoius/Germany
OWL Electrophoresis system	Thermo/USA
pH meter	Ino lab/Germany
Polystyrene tissue culture	AFCO/China

Parafilm	Pechieneg/USA
Quantus fiuorometer	Promega/USA
Refrigerator	Samsung/Korea
Standard Wire loop(1μ)	Himedia/India
Sensitive balance	Sartorius/Germany
Slides	Superstar/India
Thermocycler	Bio-Rad/USA
Tips	Sterellin Ltd/UK
Test tubes	Superstar/India
Tubes (1.5,0.5 and 0.2)ml	JET BIOFIL/Singapore
UV-Transilluminater	Major Science/Taiwan
Vortex	Quality Lab System
Vitek-2 compact	BioMerieux/France
Water bath	Memmert/Germany

3.2.Culture media:

The culture media used throughout this study are listed in table (3-2)

Table (3-2): Culture media and their origin used in the study

Culture media	Company/Origin
Blood base medium agar	Himedia/India
Brain-heart infusion broth	Himedia/India
Cimoon citrate agar	Himedia/India
Kligers iron agar	Himedia/India
MacConkey medium agar	Himedia/India
Muller Hinton medium agar	Oxoide/England
Muller-Hinton broth	Oxoide/England
Nutrient medium agar	Himedia/India
Nutrient broth	Himedia/India
Phenyl alanine medium agar	Himedia/India
Skimmed milk medium agar	Himedia/India
Tryptone soya broth	Oxoide/England
Urea base medium agar	Himedia/India

3.3 Biological and chemical materials:

The biological and chemical materials used in this study are listed in table (3-3).

Table (3-3): Biological and chemical substances and their origin

Biological and chemical materials	Company/Origin
Absolut ethanol (99%)	Promega/USA
Agar-Agar	Oxoid/England
Agarose	Promega/USA
Antibiotic discs	BDH/England
Antibiotic powder	AstraZeneca,LDP/England
Barium chloride	AstraZeneca/England
Blood Human AB	Blood Bank/Dyala
Ethanol 70%	BDH/England
FeCl3	Oxoid/England
Glycerol	BDH/England
H_2O_2	Oxoid/England
H_2SO_4	Promega/USA
Methanol Solution	BDH/England
Nuclease free water	Promega/USA
Peptone	BDH/England
Potassium hydroxide	BDH/England
Para-dimethyl aminobenzaldehyed	Fluka/Switzerland
Sodium Chloride	BDH/England
Sodium hydroxide	BDH/England
Sucrose	BDH/England
Tetramethyl-para-phenylene-diamine dihydrochloride	Fluka/Switzerland
Tris Boric EDTA(TBE)	Promega/USA
Tannic acid	Promega/USA
Urea	Oxoid/England
α –Naphthol	BDH/England

3.4 Stains and pigments:

Stains that used in this study are listed in table (3-4)

Table(3-4): The stains that used in this study

Stains or Pigments	Company/Origin
Ethidium bromide dye stain	Promega/USA
Gram stain	Synbio/Syria
Loading dye	Promega/USA
Methyl red stain	BDH/England
Phenol red stain	BDH/England
QuantiFluor dsDNA	Promega/USA

3.5 Commercial laboratory kits:

Table (3-5) shows the commercial laboratory kits that used in the study

Table (3-5): The laboratory kits used in this study

Laboratory kits	Company/Origin
Absolute Ethanol (Isopropanol)	ROMIL pure chemistry/UK
Ladder marker(100)pb	Promega/USA
PCRGO Taq Green Master Mix	Promega /USA
Primers	Macrogen/Korea
Vitek2 Kit	Biomerieux/France
Wizard Genomic DNA Purification Kit	Promega/USA

3.6 Antibiotics:

The tables (3-6) and (3-7) show the antibiotic powders and discs, repectively that used in this study

Table (3-6):The antibiotic powders that used in this study

Antibiotics	Company \ Origin
Cefepime	LDP (Barcelona-Spain)
Meropenem	Astrazeneca(UK)

Table (3-7): The antibiotic discs that manufactured by bioanalysis\ Turkey

Class	Antibiotic	Sym.	Conc. µg/ml	Inhibition diameter (mm)		
				S	I	R
Penicillins	Ampicilline	AM	10	≥17	14-16	≤13
	Piperacillin	pI	100	≥ 21	18-16	≤17
B-lactamcombination agents	Amoxicilline-Clavalante	AuG	30	≥18	14-20	≤17
Cephems parenteral includince cephalosporins I,II,III,IV	Cefepime	Cpm	30	≥25	-	≤19
	Cephotaxime	CTX	30	≥25	-	≤18
	Ceftazidime	CAZ	30	≥21	18-20	≤17
	Cefoxitin	CX	30	≥18	15-17	≤14
CARPENEMSE	Meropenem	MRP	10	≥23	20-22	≤19
	Imipenem	IPM	10	≥23	20-22	≤19
Aminoglycosine	Gentamicin	GM	10	≥15	13-14	≤12
MACROLIDES	Azithromycin	ATH	15	≥13	-	≤12
Tetracycline	Tetracycline	T	30	≥15	12-14	≤11
Quinolones and Fiuoroqulnolones	Ciprofioxacin	CIP	5	≥26	22-25	≤21
	Levofioxacine	LEV	5	≥21	17-20	≤16
	Nalidixic acid	NA	30	≥19	14-18	≤13
Folatepathway Antiagonists	Trimethoprim-sulfamethaxozole	STX	25	≥16	11-15	≤10
Phenicol	Chlorophenicol	C	30	≥18	15-17	≤12
Nitrofurantion	Nitrofuranton	F	300	≥17	15-16	≤14

R= Resistant, I= Intermediate, S= Sensitive, Con. = concentration

3.7 Preparation of the reagents and solutions:

3.7.1 Reagents:

3.7.1.1Kovac's reagent:

Five grams of Dimethyl amino Benzaldehyde (DMAB) were dissolved in 75 milliliters of amylalcohol to create it then 25milliliters of concentrated HCL were in addition to the mixture progressively. It was used in the diagnostic indole procedure, which assessed the organism'sability to split indole from tryptophan. Under aconditions used,the indole produces a red complex with paradimethylaminobenz aldehyde (Baron *et al*.,1994).

3.7.1.2 Methyl redreagent:

Methyl red reagent was prepared by dissolving 0.1 grams of methyl red in 300 militters of (95%) ethanol. Then, the volume was completed to 500 milliliters by adding distilled water. This reagent was used to detect complete glucose hydrolysis (Holt, 1994).

3.7.1.3Vogues – Proskauer reagent (Barrett's reagent):

It was made up of the following two solutions:

A. Five grams of alphanephthol were dissolved in 100 milliliters of ethanol (99%) and placed away from light in a dark bottle.

B. Forty grams of potassium hydroxide were dissolved in 100 milliliters of pure water (MacFaddin, 2000).

3.7.1.4 Oxidase reagent:

The reagent was prepared instantaneously by dissolving 1 gm of Tetramethyl-para-phenylenediamine dihydrochloride in 90 ml of distilled water, then the volume was completed to 100 ml and used to

investigate the ability of the isolates to produce the oxidase enzyme(Forbes *et al*., 2007).

3.7.1.5 Catalase reagent:

It was prepared by dissolving 3 gm of hydrogen peroxide H2O2 in 100 ml of distilled water, to obtain a 3% solution kept in opaque bottles and used to detect the ability of bacteria to produce the enzyme catalase(Forbes *et al*., 2007)..

3.7.2 Solutions:

3.7.2.1 Normal saline-solution

It was prepared by dissolving 8.5 grams of NaCl in a small volume of distillied water, then it complete to 1000 milliliters. The pH was adjusted to7.2 and autoclaved at 121C̊,15 Ib /inch^2 for 15 min., this reagent stored at 4C̊ (Sambrook and Rusell, 2001).

3.7.2.2Phosphate buffer solution

Eighty grams each of NaCl,0.34 and KH2 PO4, and 1.12 grams of HPO4 K2 were desolved in 1000 milliliters of distilled water. The pH was adjusted to 7.3 and the solution was autoclaved at 121C̊,15 Ib /inch^2 for 15 min., this reagent stored at 4C̊ (Sambrook and Rusell, 2001).

3.7.2.3 TrisEDTA Buffer solution (TE buffer)

This buffer was prepared by dissolving 0.05 M Tris-OH and 0.001 M EDTA in 800 milliliters of distilled water.The pH was adjusted to 8 and autoclaved at 121C̊ , 15 Ib /inch^2 for 15 min., the buffer was stored at 4C̊ until it used (Stellwagen and Stellwagen, 2002).

3.7.2.4Tris Borate-EDTA-buffer solution(TBE)

This solution was prepared by dissolving 0.08M Tris-OH, 0.05M boric acid and 0.02M EDTA in 500 milliliters of distilled water. The pH was adjusted to 8 and autoclaved at 121Ċ, 15 Ib /inch^2 for 15 minutes and deposited at 4Ċ(Sambrook and Rusell, 2001).

3.7.2.5Ethidium Bromide solution

This buffer was prepared by dissolving 0.5 grams of Ethidium Bromide in 50 milliliters distilled water stored of a dark reagent bottle(Sabnis, 2010).

3.7.2.6Trichloroacidic acid (TCA) solution (5%)

Five grams of TCA crystals were dissolved in 100 milliliters of distilled water and kept in the dark. (Sambrook *et al*., 2001).

3.7.2.7 Macferland standard solution (0.5 ml)

The solution prepared from Biomérieux company was used to assay the number of bacterial cells, as it gives an approximate number of cells which is 1.5 x 10^8 cells/ml.

3.8Preparation of culture media

3.8.1 Ready-made media

According to the manufactor′s instructions, the culture media were autoclaved at 121^{o}C, 15 Ib/inch^2 for 15 minutes as follow:

3.8.1.1 MacConkey agar medium

MacConkey agar medium was prepared by dissolving 49.53 grams in 1000 mililiteres of distilled water. This medium was used to differentiate the lactose fermenters from non-lactose fermenters (Luis *et al*., 2004).

3.8.1.2 Nutrient agar medium

Twenty eight grams of nutrient agar were dissolved in 1000 mililiteres of distilled water. It used to activate the cultured isolates of *P. mirabilis* (MacFaddin, 2000).

3.8.1.3 Nutrient broth medium

Thirteen grams of nutrient broth were dissolved in 1000 mililiteres of distilled water. This medium was supplemented with 15% glycerol to preserve *P. mirabilis* isolates (MacFaddin,2000).

3.8.1.4 Brain heart infusion medium

Thirty seven grams of Brain heart infusion were dissolved in 1000 mililiteres of distilled water. This medium was supplemented with 15% glycerol to preserve *P. mirabilis* isolates (MacFaddin, 2000).

3.8.1.5 Blood agar medium

Forty grams of blood agar base were dissolved in 1000 milliliters of distilled water. New human blood was applied at a rate of 5%. This medium was used to test the capacity of *P. mirabilis* to hemolyse blood cells (Cheesbrough ,2012).

3.8.1.6 Trypticase Soy broth

Thirty grams of dehydrated medium were dissolved in 1000 milliliters of distilled water. This medium was used as enrichment medium for cultivation of *P. mirabilis* isolates (Hill *et al*., 1998).

3.8.1.7 Peptone water medium

Eight grams of peptone were dissolved in1000 milliliters of distilled water. It was used for the demonstration of the ability of *P. mirabilis* isolates to decompose tryptophan to indole (Colwell, 1996).

3.8.1.8 MR-VP agar medium

This medium was prepared and used to detect the partial and complete hydrolysisof glucose according to (MacFaddin,2000).

3.8.1.9 Simmons'citrate agar medium

Simmon's citrate medium was used for determining the ability of *P. mirabilis* isolates to utilize citrate as carbon source (MacFaddin,2000).

3.8.1.10 Kligler Iron agar medium:

Kligler Iron agar was used to detect the fermentation of glucose and lactose and production of hydrogen sulfide (H_2S) (MacFaddin, 2000).

3.8.1.11 Motility medium

Four grams of agar-agar were mixed with 100 milliliters of nutrient broth and dissolved in 1000 milliliters of distilled water. It was then sterilized by autoclaving at121C̊, 15 Ib/inch^2 for 15 minutes. The purpose of this medium was to detect the motility of *P. mirabilis* (MacFaddin, 2000).

3.8.1.12 Urea agar medium

Twenty nine grams of urea agar base were dissolved in 100 milliliters of distilled water and filter sterilize (0.45-mm pore size). Next, 15 grams of agar were dissolved in 900 milliliters of distilled water and autoclaved at at121Ċ, 15 Ib/inch^2 for 15 minutes. Then, it cooled at 50Ċ. Finally, The medium was distributed into sterilized test tubes and allowed to solidify in a slant form after the pH was adjusted to 7.1 . This medium was used for detection the ability of *P. mirabilis* to produce urease enzyme (Mahon and Manuselis, 2007).

3.8.1.13 Muller-Hinton agar medium

Thirty grams of muller hinton agar were dissolved in 1000 ml of distilled water. It used to detect the susceptibility of antimicrobial agents (Wood and Washington, 1995).

3.8.1.14. M9 medium

A six g of Na2HPO4, 3g of KH2PO4, 0.5g of NaCl, 1g of NH4CL were dissolved in 950 ml of distilled water with 2% agar and then sterilized in an autoclave at 121Ċ for 15 min. After cooled the mixture at 50 Ċ, 2 ml of 1M of MgSO4, 10 ml of 20% glucose and 0.1 ml of 1M of CaCl2 (all of which were separately sterilized by filtration) were added to the mixture. The volume was then completed to 1000 ml by adding distilled water. After the inoculation of this media with bacterial strain and incubation for 24 hr at 37 Ċ, 3ml of (5%) Trichloroacetic acid was added. The formation of transparent area around the colony indicated the positive result. This medium was used to detect the proteolytic activity of bacteria (Karen and Roger, 2002).

3.8.1.15 Skimmed milk agar medium

Fifty two grams of skimmed milk were suspended in one liter of distilled water. Then, mixed and dissolved completely. It was sterilized by autoclaving at at 121Ċ, 15 Ib/inch^2 for 15 minutes. afterthat, it cooled and distributed in Petri dishes and left to solidify. Finally two holes were done per each plate using the back end of the blue tip and the plates were kept in the refrigerator till use (Senior ,1999).

3.9 Collection of samples

This is a cross-sectional study was conducted in Al - Khalis General Hospital, Baquba Teaching Hospital, and Al-Batool Teaching Hospital for maternity and Children, during the period extended from the 5th of August 2020 to 25th of January 2021. A total of 300 urine specimens were collected from patients clinically complaining of signs and symptoms of UTI. According to the diagnosis of the urologist, each patient's samples were collected using sterile container of approximately 10 milliliters of clean midstream urine. Initially, all collected samples were streaked on blood agar and MacConkey agar media for primary isolation and identification of *P. mirabilis*. The streaked dishes were immediately incubated at 37 Ċ for 24 hrs. The questionnaire form that taken from each patient was listed in appendix (1).

3.10 Laboratory diagnosis

3.10.1 Bacterial isolation and identification:

The identification of uropathogenic *P. mirabilis* was detected by the morphological appearance, including colony size, shape, color, swarming, smell, the edge of translucency and the elevation of the texture, of one single colony from each suspected primary culture. Then,

Gram's stain was investigated in the cultivated colonies the final identification was achieved using specific biochemical tests such as Lactose fermentation, Oxidase, Indol, Methyl red, Vogues –Proskauer ,Citrate utilization and Phenylalanine deaminase Per MacFaddin's,(2000) diagnostic procedures and Benson,(2001).

3.10.2 Identification of bacteria using Vitek system:

Protues mirabilis was identified using the Vitek Kit system according to the following procedures:

Suspension preparation: In a 12 x 75 millimeters clear glass (polystyrene) test tube, the sterile swab, or the applicator stick, was used for the transmission of sufficient pure cultivated colonies of a sterile solution of sterility (aqueous 0.45 percent to 0.5 percent NaCl, pH 4.5 to 7.0).The suspension turbidity was adjusted for a negative Gram range (0,5-0,6 per McFarland turbidity range) and measured with the Densichek turbidity meter. Inoculation an identification card was inoculated with a turbidometrically controlled suspension of pure saline colonies. These cards contain various biochemical broths in reaction cells and a negative control cell to estimate suspension growth and viability. Microorganism suspensions with integrated vacuum devices inoculated the identification cards. A test tube was placed into a special cassette and the identification card in the neighboring slot was placed while the transfer tube was inserted into the corresponding suspension tube. A vacuum chamber station was filled with the cassette manually. The suspension of the organism was forced into micro nuts through the transfer tube after a vacuum was applied and the air was reintroduced into the station. Before cards were inoculated, traditional catalase, coagulase, and oxidase tests, as well as Gram stain results, where needed.Card sealing and incubation amechanism that cuts off the transfer tube and stitches the card before

loading it into the carousel incubator has transferred inoculated cards to the incubator on a carousel. Both cardforms were incubated at(35.5 + 1.0C̊) online.Each card was taken out of 15 minutes in the incubator The samples were taken to the optical system for reaction readings, then placed back in the incubator until the next read time.Throughout the incubation cycle, data was collected at15-minute intervals.Depending on the organism's growth rate, incubation periods range from two to fifteen hours. By using an optical scanner to measure light attenuation, the Vitek programmed machine decides whether each well was positive or negative.The reactions were automatically analyzed after the incubation period was finished, and the identification was printed.

3.11.Preservation of *Proteus mirabilis* isolates:

3.11.1 Short term preservation

One pure colony was inoculated into nutrient agar slant tubes and incubated for 24 hours at 37C̊ before being stored at 4C̊(Forbes *et al*., 2007)

3.11.2 Long term preservation:

Protues mirabilis were kept alive by culturing them in brain heart infusion broth with glycerol (15%) and storing them at -20 C̊(Collee *et al*.,1996).

3.12.Detection of virulence factors by cultural methods:

3.12.1. Hemolysin production:

Protues mirabilis isolates were streaked onto the surface of blood medium agar to detect Hemolysin production. After overnight incubation period at 37C̊, the cultured plates were examined for the presence zone of

hemolysis around bacterial growth indicat a positive result.(Al-Mayahi,2017).

3.12.2. Urease production:

A small amount of pure colony of *protues mirabilies* was transferred using a sterilized loop and streaked on to surface of slanted urea agar, then incubated at 37 Ċ for 24 hrs to detect color change from yellow to pink color indicats a positive result. (Al-Mayahi, 2017).

3.12.3. Colonization Factor Antigen (CFA):

A-Detection of CFA/I

Protues mirabilis isolates were cultured on Tryptic soy medium agar and incubated for 24 hours at 37°C to detect CFAI;In the presence of D-mannose,a RBC group agglutinates with bacteria on the slide (Al-Mayahi, 2017).

B- Detection of CFA/II

The method of CFAII was the same method as in CFA/I, with the exception that chicken blood was used instead of human blood (Al-Mayahi, 2017).

C- Detection of CFA/III

CFAII was detected in the same methodas as CFA/I, but instead of Dmannose, tannic acid was used (Al-Mayahi, 2017).

3.12.4.Biofilm formation

3.12.4.1.Microtiter method.

The biofilm formation test was carried out according to Ghellai's *et al*. (2014) process, which included the following steps:

1. Twenty- five microliters of overnight *P. mirabilis* were incubated in flat-bottom- microtiter wells containing 180 microliters of Brain Heart infusion broth with sucrose(2%). Just 200 microliters of Brain heart infusion broth with 2% sucrose were used in the control wells (0.5 gm sucrose for each 25 milliliters of brain-heart broth).
2. During the incubation at 37 Ċ for 24 hours, the covered microtiterplate was sealed with Parafilm.
3. The wells were washed three times with regular saline pH=7.2 to extract unattached bacterial cells.
4. After 15 minutes of drying at room temperature, 200 µl of crystal violet solution (0.1%) were applied to the wells for 15 minutes.
5. All wellswere washed three times with distal water to remove the unbounded dyeafter extracting a crystal violet solution, and allowed to dry at room temperature.
6. Extracted with 200 µl of 95 % ethanol.
7. Using an ELISA reader, the absorbance of each well was calculated at 630 nm. All of the test OD values were subtracted from the control well's OD value.
8. The results were divided into three groups based on the absorbance: strong, moderate, and weak; this classification is shown in the table below (Bose *et al*., 2009).

Table(3-8):Classification of *P.mirabilis* according to biofilm formation

"OD" ≤ "ODc"	biofilm-free
"ODc < OD ≤ 2 x ODc"	Biofilm producer, moderately
"2 x ODc < OD"	Biofilm producer who is strong

OD= optical density reader of isolate, ODc= optical density reader of control.

3.12.4.2.The tube method of *Proteus mirabilis*.

Firstly, five milliliters of trypticase soy broth containing 1% glucose were inoculated with overnight cultured isolates. Next, it incubated at 37Ċ for 24 hrs. Then, the cultures were decanted and tubes were washed with regular saline. After that, the dryed tubes were stained with crystal violet (1%) and the residual color was washed away with distilled water. Finally, the tubes were dried upside down and the wall was lined with a stained film. (Mathur *et al*., 2006).

3.12.5 Extracellular protease

The isolates of *P. mirabilis* were cultured in 1 milliliter of skimmed milk broth. The cultured plates were incubated at 37 Ċ for 24 or 48 hrs. The diameter of the zone hydrolysis was determined around developing colonies positivity of the extracellular protease was described as 22-25 mm in diameter (Senior, 1999).

3.12.6 Siderophores production

Protues mirabilis isolates were cultivated on medium M9 and incubated at 37 Ċ for 24 hrs to detect the production of siderophores (Sambrook and Russell, 2001).

3.13 Antibacterial Susceptibility Test (AST)

3.13.1 Disk diffusion method

The disk diffusion susceptibility method was carried out according to Kirby-Bauer method (CLSI, 2019). The test is performed by following steps

1. A bacterial broth culture was prepared by transferring a separated colony on 24 hours old MacConkey agar plate culture to a tube containing 5 milliliters of sterile normal physiological saline and its turbidity was compared the Micferland standard which gives (1.5 $\times 10^8$) CFU/ml. The method of susceptibility to disk diffusion was implemented by Kirby-Bauer method (CLSL, 2019).
2. A sterile swab was used to streak 0.1 milliliter of broth culture onto the Mueller-Hinton agar plate's surface. The plates have been rotated around 60 degrees every time to ensure that the inoculum is evenly distributed. The plates were kept dry at room temperature for 5 minutes.
3. Antibiotic discs were transferred to the Mullar-Hinton agar plate with sterile forceps at a rate of 5-6 discs per plate using sterile forceps. Overnight at 37Ċ, the plates were incubated.
4. The growth inhibition zones around each of the antibiotic disks are determined by nearest millimeter.
5. The zone diameters of each drug were interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards diffusion test were “qualitative,” in that a category of susceptibility (ie, susceptible, intermediate, or resistant).

6. The zone diameters was compared with standard curves of CLSI (2019) and CLSI (2020).

3.13.2Minimum Inhibitory Concentration

According to CLSI (2019), the MIC for all isolates was calculated as follows:

1- The isolates were grown at 37Ċ for 24 hrs on MacConky agar and approximately, 3-4 colonies were moved to normal saline before the turbidity reached 0.5 McFarland.
2. The cefepime and Meropenem antibiotic solution used in this analysis was prepared and diluted in Müeller Hinton Broth (stock 2). The top concentration in the test, 105 g/mL (stock 1), was diluted to 2048 g/mL after being prepared in advance according to paragraph (3.2.2.2.5). (stock 2).
3. Using a sterile micropipette and its tip, one hundred microliters of Mueller Hinton Broth was applied to all wells of the microtitre plate.
4. To get 1024 ml, a micropipette was used to transfer 100 microliters of (stock 2) antibiotic solution into the wells of column 1.
5. The antibiotics were combined with a micropipette by sucking up and down 5-8 times.
6. Hundred microliters of column 1 were removed and added to column 2. Transfer to column 3 and mix by sucking. Apply the same technique to the next column.
7. Nine-fold dilution (4, 8, 16, 32, 64, 128, 256, 512 and 1024). A hundred microliters of column 9 was discarded. As a positive control, five microliters of bacteria were poured into wells in columns 1 to 9 and column 12 that contained only broth. As a control negative, 100 microliters of stock 2 were applied to wells in column 11, and nothing

was added to column 10 (except broth), so that was also a control negative.

8. The plates were incubated at 37Ċ for 24 hrs.
9. The results were read manually using a black card and electronically with an ELISA reader at 630 nm wavelengths.

3.14 Genotyping assay

3.14.1 Extraction of *Proteus mirabilis* DNA

According to a result of antibiotic suscepility, (15 out of 25) isolates of *p.mirabilis* were chosen for Genomic DNA Extraction using Wizard Genomic DNA purification Kit, as following steps:

- To prepare pellet cells, 1ml of overnight culture for centrifuged 2min at 13000 rpm. Supernatant was then discarded.
- Five hundred microliter of nuclei lysis solution were added to cell pellet and pipetted gently for mixing.
- All mixes were incubated at 80Ċ for 5 minutes. Then, it cooled at room temperature.
- Three microliter of RNase solution were added to the mixed,and incubated at 37Ċ for 15 minutes.
- Two hundred microliter of protein precipitation solution were add to cell lysate. Next, it mixed well by vortexing and incubated in deep freeze (-30 Ċ). Then, it centrifuged at 13,000 rpm for 5 minutes.
- The diluted DNA was transferred to clean tubes containing 500 microlitiers of room temperature isopropanol. After mixing gently, centrifuge as in pellet cells above, and supernatant was decanted.
- Five hundred microlitirs of ethanol (70%) were added at room temperature. Then, it centrifuged at 13,000 rpm for 2 minutes.
- Ethanol aspirates the pellet and air-dries it.

- The DNA pellet was rehydrated at 65Ċ for 1 hr in 100µl of rehydration solution.

3.14.2 DNA quantification

The Quantus Fluorometer was used to detect the quantification of extracted DNA to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quantifluor Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

3.14.3 Agarose gel electrophoresis

After PCR amplification, the existence of amplification was confirmed using agarose gel electrophoresis.The extracted DNA parameters were entirely reliant on PCR.

1.Solutions

(1X TAE buffer,DNA ladder marker,loading dye, Ethidium bromide10 mg / ml).

2.Preparation of agarose

- A beaker was filled with 100 mL of 1X TAE.
- One and a half gm of agarose (1.5 %) was added to the buffer.
- The solution was heated using microwave for boiling until all of the gel particles were dissolved.
- One liter of Ethidium bromide (10 mg/ml) was added to the agarose.
- The mix was stirred to blend the agarose and reduce bubbles.
- The solution was allowed to cool at 50-60Ċ.

3.Casting of the horizontal agarose gel

After sealing both edges with cellophane tapes, the agarose solution was poured into the gel tray and allowed to solidify at room temperature for 30 min. The comb was removed carefully and the gel was put in the gel tray. 1X TAE-electrophoresis buffer was poured into the tray until it reached 3-5 mm above the gel's surface.

4.DNA loading and Gel electrophoresis

For PCR product, 5µl was directly loaded to well and electrical power was turned on at100v/mAmp for 1 hr. DNA moves from Cathode to Anode poles.The stained bands were visualized using Gel imaging system**.** Samples were loaded using a pre-prepared loading buffer by mixing 2 µl of buffer with 5 µl of extracted DNA to be carried over and placed in the gel pits. Next, it connected to the electrical current where it was fixed to a voltage of 100 volts for 1 hr. Then, the gel was examined using an ultraviolet source at a wavelength 336 nm.

3.14.4 Primers Preparation

The primers (forward and reverse) were dissolved with distilled, Nuclease freewater to prepare a stock solution, at a concentration of (100 pmol / µl) which was kept at a temperature of -20 Ċ until use, after which the working solution was prepared by taking 10 µl of the solution. Storage and add it to 90 µl of distilled water devoid of cutting enzymes to obtain a final concentration in10 pmol / µl.

3.14.4.1 The Primers and sequences genes of *Proteus mirabilis.*

The specific primers that used in this study were listed in table (3-9).

Table (3-9):The primer sets that used for PCR assay in this study.

Gene name	Oligo sequence (5'-3')	Product size (bp)	Anne. Temp (°C)	Reference
Esp	**F** TTGCTAATGCTAGTCCACGACC **R** GCGTCAACACTTGCATTGCCGAA	955	61	(Shankar *et al*., 1999)
FlaA	**F** AGGATAAATGGCCACATTG **R** CGGCATTGTTAATCGCTTTT	417	54.2	(Ali and Yousif, 2015)
Mrp	**F**ACACCTGCCCATATGGAAGATAC TGGTACA **R**AAGTGATGAAGCTTAGTGATGGT GATGGTGATGAGAGTAAGTCACC	550	40	(Zunino *et al.*, 2001)
UreC	**F**CCGGAACAGAAGTTGTCGCTGGA **R** GGGCTCTCCTACCGACTTGATC	533	63	(Takeuchi *et al.*, 1996)
ZapA	**F** ACCGCAGGAAAACATATAGCCC **R** GCGACTATCTTCCGCATAATCA	540	53	(Stankowska *et al.*, 2008)

3.14.5. Reaction Setup and Thermal Cycling Protocol

All DNA extracted from the fifteen of the twenty-five isolates in this study passed via polymerase chain reaction. Table (3-10) shows the procedure is to target the five genes under study. Each reaction was PCR Final volume is 20 µl.

Table (3-10): PCR Component Calculation for singleplex and multiplex PCR

Name of primer	Type of reaction	Reaction requirement					
		Master Mix (2X)	Nuclease free water	Primer		DNA (ng/µl)	Final volume (ml/µl)
				F (10µM)	R (10µM)		
Esp	**singleplex PCR**	**10**	**5**	**1**	**1**	**3**	**20**
FlaA	**Singleplex PCR**	**10**	**5**	**1**	**1**	**3**	**20**

Mrp	**Singleplex PCR**	**10**	**5**	**1**	**1**	**3**	**20**
UreC	**Singleplex PCR**	**10**	**5**	**1**	**1**	**3**	**20**
ZapA	**Singleplex PCR**	**10**	**5**	**1**	**1**	**3**	**20**
FlaA* and *ZapA	**Multiplex PCR**	**10**	**3**	**2**	**2**	**3**	**20**

3.14.6. Polymerase chain reaction:

The PCR is one of the molecular techniques which was designed for creating thousands to millions copies of demanding DNA fragment. DNA target (DNA template) that includes the region of DNA to be amplified, primers (forward and reverse primers) that are complementary to the DNA template, Thermus aquaticus DNA polymerase (Taq polymerase) enzyme, deoxy nucleotide triphosphates (dNTPs), and buffer solution that provide an appropriate chemical environment are all required for PCR. Initiation, Denaturation, Annealing, Extension / Elongation, and Final Extension are the basic PCR stages.

3.14.7 PCR Program

Programs of PCR thermocycling conditions for detection of genes(all the primers) PCR Program used in the study.

Table (3-11): PCR program used in the current study

Step		Tempreature (°C)	Time (minutes)	Cycle
Initial Denaturation		95	05:00	1
Denaturation		95	00:30	30
Annealing	*Esp*	61	00:30	
	FlaA	54		
	Mrp	40		
	UreC	63		
	ZapA	53		
Extension		72	00:30	
Final extension		72	07:00	1
Hold		10	10:00	

3.15 Gene Sequencing

3.15.1 DNA Sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from termini, forward, and reverse, according to instruction manuals of the sequencing firm (Macrogen Inc. Geumchen, Seoul, South Korea). Only transparent chromatographs from ABI (Applied Biosystems) sequence files were examined further, ensuring that the annotation and differences were not due to PCR or sequencing artifacts. The virtual positions and other information of the retrieved PCR fragments were established by comparing the observed DNA sequences of local samples with the retrieved DNA sequences of the bacterial database. BioEdit ver. 7.1 was used to compare DNA chromatograms with the deposited bacterial DNA sequences, demonstrating the nucleic acid variations (DNASTAR, Madison). SnapGene Viewerver.4.0.4 (https://www.snapg ene.com) was used to annotate each detected variant inside the *P.mirabilis* genes.

3.15.2 Comprehensive phylogenetic tree construction

A specificcomprehensive In this analysis, the tree was built using the neighbor-joining protocol defined by Sarhan *et al* (2020). Using the NCBI-BLASTn server, the observed variants were compared to their homologous, neighboring reference sequences (Zhang *et al*. 2000). Then, a complete inclusive tree, which includes the observed version, is constructed.was built by the neighbor-joining method and annotated as a cladogram tree using iTOL suit (Letunic and Bork, 2019). The sequences of each classified phylogenetic species-group in the comprehensive tree were colored appropriately.

3.16 Statistical analysis

The available statistical software, SPSS-27, was used to analyze the data (Statistical Packages for Social Sciences- version 27). Simple measures of mean, standard deviation, frequency, percentage, and range (minimum-maximum values) were used to present the data. The Pearson Chi-square test (two-test) was used to determine the significance of different percentage differences (qualitative data). and performed using GraphPad Prism version 3.06.The correlation matrix between viralance genes and susceptibility agents was estimated by using spearmans correlation coefficient analysis. Correlation coefficients were considered significant at P-values ≤ 0.05.

Chapter Four
(Results and Discussion)

4. Results and Discussion

4.1 Isolation of *Proteus mirabilis*

A total of 300 patients who suffering from urinary tract infaction for a period extended from 5 th August 2020 to 25th of January 2021. The percentage of positive culture of *P. mirabilis* was 8.3% (25 out of 300), these isolates of *P. mirabilis* were diagnosed based on bacteriological, biochemical and ViteK2 system criteria. In spite of that all urine samples were from patients complaining signs and symptoms of UTIs, but the low number of the isolates was most probably related to the administration of antibiotics before doing the urine culture. Locally, most consultant urologist make the culture and sensitivity tests as their last option in the management strategy of UTIs. So, patients during this stages were already received more than one course of antibiotics. These undoubtedly inhibit bacterial growth and reduce the culture outcomes (Jones *et al*., 2014). Furthermore, UTIS may be due to infection by fastidious bacteria that are not technical faults in the collection of urine specimen or culturing technique (Richard *et al*., 2008; Dougherty *et al*., 2020).Also, it has been showed that in acute care and emergency departments, urine reflex culturing decreased the number of urine cultures (Lynch *et al*., 2020).

4.2 Identification of *Proteus mirabilis*

4.2.1 Morphological and Cultural Characteristics

Proteus mirabilis grows on the center of MacConkey agar medium, it forms a single, circular, medium in size, pale, non-fermented colonies of lactose, as well as a rotten fish-like odor. In the midst of the blood agar, no clear colonies can be observed due to their swarming

movement, which is one of the primary diagnostic characteristics of the wonderful *Proteus* (Al- Bassam and AL-Kazaz, 2013). Appendix 3.

4.2.2 Microscopic diagnosis

The results of the microscopic examination of *P.mirabilis* after performing the staining process with the Gram stain on a smear taken from pure colonies showed that their cells are negative for the Gram stain (Holt *et al*., 1994).

4.2.3 Biochemical Tests

the results of biochemical tests showed that all isolates of *P. mirabilis* included in the study were positive for catalase, Methyl red, urease and phenylalanine tests, whereas negative result for indole, oxidase and Voges –Proskauer tests, and they were different in their ability to consume citrate and have the ability to move. While it showed its ability to ferment glucose sugar and its inability to ferment lactose when grown on KIA medium, it produced hydrogen disulfide H2S gas in the form of a black precipitate at the bottom of the tube Table (4-1) appendix 4.

Table (4-1):The results of biochemical tests

NO.	Biochemical Tests	*Result*
1	Gram Stain	-
2	Lactose fermentation	-
3	Oxidase	-
4	Catalase	+
5	Urease	+
6	Indol	-
7	Methyl red	+
8	Voges –Proskauer	-
9	Citrate utilization	+/-
10	Motility	+
11	Phenylalanine deaminase	+
12	KIA (H_2S and CO_2 Production)	++A/K

(+)Positive, (-) Nagative and (A/K) Acide\ Alkaline

4.2.4 Identification by Vitek-2 Compact

The results of the confirmatory diagnosis using the GN Card negative for the Vitek-2 Compact showed that 25 isolates belong to *P. mirabilis*, and the diagnostic accuracy ranged from 99.99%. The result of the current study agreed with The current study's findings are consistent with those of a previous study the researcher Al- Dawah *et al*. (2015) in Babylon, as the percentage of their isolates diagnosed with the Vitek-2 device is 99.99%. With Gram-negative bacteria, it gives accuracy by performing 47 tests for a period betwee 5-8 hours, as well as unconventional tests that are not present in other diagnostic kits, which allows the diagnosis of isolates within Record time with high accuracy and with a low error rate, as the device shows the results of the isolates in the direction of a specific reaction and is denoted by the symbol (-)

evidence of negative interaction and the symbol (+) evidence It consists of 16 numbers, as the calculator linked to the Vitek 2 device compares it, giving a rapid diagnosis within a maximum period of eight hours. Appendix (2) shows a sample of the test result and the biological chemical reactions of one of the isolates of *P. mirabilis.*

4.3. Correlation between *protues mirabilis* isolates with age group:

Bacteria of *P. mirabilis* were isolated from different patients with ages ranging from 2 to 56 years as shown in Table (4-2). The highest percentage of *P. mirabilis* was in age group (20-29) years, which was 36% (9 out of 25), while the lowest rate was in age groups (<10, 20-19 and 40-49) years olds, which was 8% (2 out of 25). The mean age ± SD of patients was 31.4±14.3 years, while the range of the ages was (2-56) years. This result is similar to the result of Jawad and Al-Rumahi (2018) who revealed that the age group (21-30) years old had a high percentage of *P. mirabilis* isolates (34.7%). Furthermore, this result agrees with Pal *et al*. (2014) who reported the highest infection rate among those with (20-29) years old, with a percentage 21.78%. The plausible explanation of these results may be due to the stage of sexually active in age group (20-29) years old with its hormonal changes that hasten UTIs (Storme *et al* 2019).

Table (4-2): Presence of *P. mirabilis* distributed according the age groups.

Age (years)	Number of isolates	Percentage (%)
<10 years	2	8.0
10-19	2	8.0
20-29	9	36.0
30-39	5	20.0
40-49	2	8.0
≥ 50 years	5	20.0
Total	25	100%
Mean ± SD (Range)	**31.4 ± 14.3 (2-56)**	
P value	**0.01***	

***P-value <0.05 was considered statistically significant;SD:Standard deviation**

4.4. Correlation between *Proteus mirabilis* Isolates with the gender of Patients:

According to the gender of patients, females were more infected with *P. mirabilis* than males, with a percentage 64% (16 out of 25)(Figure 4-1). This result agrees with Ghofran *et al*, (2019) who found that the females were most infected with *P. mirabilis*, 61.53%, while disagrees with Hassan (2008) who found that the infection percentage of males is higher than females (59%). Females are more infected with UTIs than males because physiological, anatomical, hormonal and immunological discriminations (Lee *et al*., 2020; Mestrovic *et al* 2021).

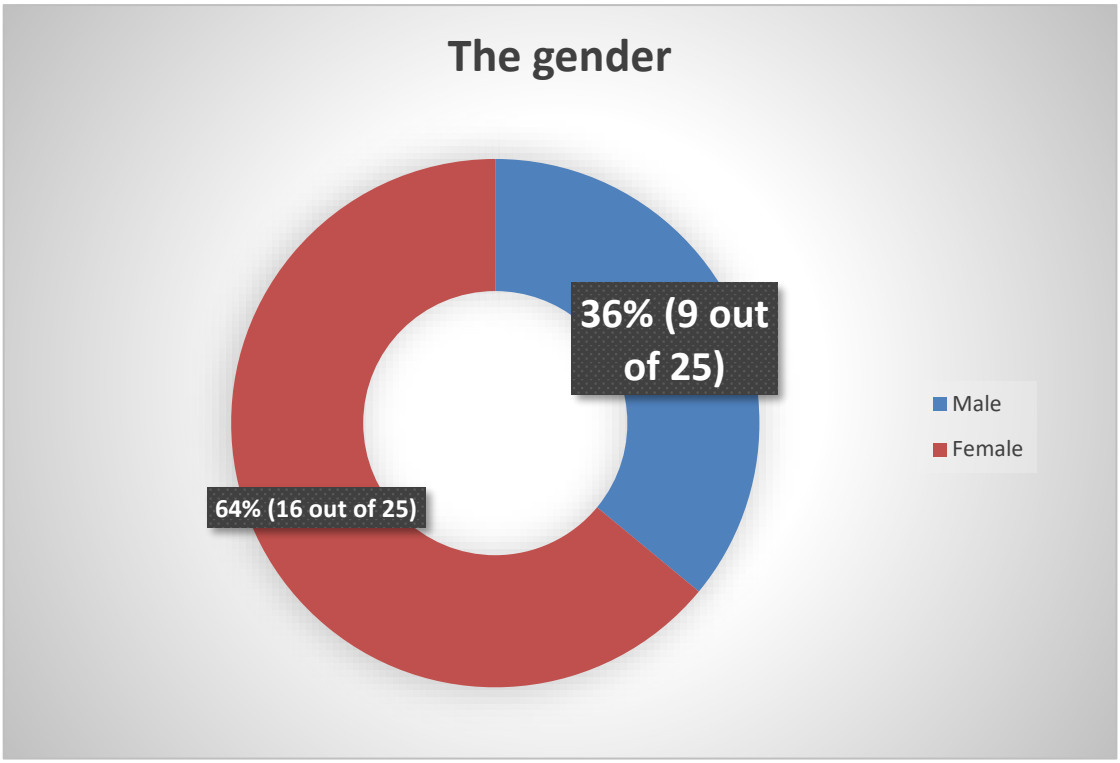


Figure (4-1): The Ratio of *Proteus mirabilis* isolates from male and female patients

4.5. The percentage of isolation *Protues mirabilis* according to aera of the study

Table (4-3) shows that the number of patients residing in the cities center areas (Urban) was 10 (40.0%), while the number of residents in the peripheral cities reached (Rural)15 (60.0%).

Table (4-3): Distribution of *P. mirabilis* isolates according to the aera of the study.

The area and No. of sample	Isolates No. (%)
(150) (Urban)	10 (40)
(150) (Rural)	15(60)
Total	25(100)
Chi-Square	1.3
P-value	0.4

With regard to housing, the results of the current study were similar to the results of Al-Obaidi (2006a) and Ghufran *et al*. (2019) who found that the rate of isolation of *P. mirabilis* from patients living in peri-urban areas was higher than that in urban areas. Low standard of living and low health education accompanied by neglect of health services in remote areas can be explained by these findings (WHO,2015; storm *et al*., 2019).

4.6.Correlation Between the Isolation of *Protues mirabilis* and Urine Reaction:

Table (4-4) shows that *P. mirabilis* was more isolated from alkaline urine samples, with a percentage (76.0%) than acidic urine samples (24%).

Table (4-4): Correlation between isolation of *Proteus mirabilis* and the reaction of urine.

Urine reaction	Isolates No. (%)
Alkaline	19 (76)
Acidic	6(24)
Total	25(100)
Chi-Square	25
p-value	**0.001***

***p -value≤0.05 was considered statistically significant**

The current results were partly in the agreement with (Ghofran *et al*, 2019) who found that the rate of alkaline urine among patients with UTIs was 11.9%. According to the urine reaction which was determined by measuring the pH, it was well documented that *P. mirabilis* is among the bacterial pathogens that able to produce urease enzyme which split

urea, the nitrogenous waste product in human urine, into ammonia and water leading to alter the urine reaction from acidic to alkaline and hasten bladder and renal urolithiasis (Armbruster *et al*., 2016). In reality, *Proteus* species have been found in 70% of bacteria-induced stone formation cases. (Prywer and Olszynski, 2017). It is important to remind that all (100%) of *P. mirabilis* isolates in the current study were urease producer.

4.7 The effect the presence of urine pus cells on the isolation of *Proteus mirabilis*

All urine samples included in the study were contain more than 10 pus cells/high power field. Accordingly 16 (64.0%) urine samples contain 10 pus cells/HPF (+), while 9(36.0%) of urine samples contain 20 pus cells/ HPF (++), as shown in table (4-5). Basically the presence of pus cells (blood neutrophils) in the urine is a primarily indication of infection, while the presence of red blood corpuscles (RBCs) is an indication of severe infection or probably renal damage (Waseem *et al*., 2014).

Table (4-5): Correlation between the presence of pus cells and the isolation of *Proteus mirabilis*

No. Urine pus cells	No. of the isolates	%
10 pus cells/HPF (+)	16	64.0
20 pus cells/HPF (++)	9	36.0
Total	25	100

HPF:High Power Field

4.8 Urine RBCs content and the presence of *Proteus mirabilis*

Table 4-6 shows that the number and ratio of the isolates which have been isolated from urine samples with RBCs content, basically, all urine samples included in the study contain ≥ 10 red blood corpuscles high power field. Urine samples contain 10 RBCs/HPF were 11(44.0%),

while those contain 20 RBCs/HPF were 9(36.0%), and the least were those contain 30 RBCs/HPF 5(30%).

The high rate of *P. mirabilis* recovered from urine samples with RBCs (≥ 10 corpuscles /HPF) may be due to the facts that in *P. mirabilis* UTIs, the pus cells tend to clumping, that easily be missed by microscopical urine examination (Schaffer and Pearson. 2015; Armbruster *et a*l., 2018). Furthermore, most of patients in our community were requested urine culture and sensitivity tests while they are in the convalescence stage when the number of pus cells and RBCs reduced. On the other hand, concomitant genital tract infection whether the bacteria or non-bacteria are characterized by profuse suppuration particularly in women (Bitew *et al*., 2017).

Table (4-6): The ratio of *Proteus mirabilis* according to urine RBCs content.

Urine RBCs	No. of the isolates	%
10 RBCs/HPF (+)	11	44.0
20 RBCs/HPF (++)	9	36.0
30 RBCs/HPF (+++)	5	20.0
Total	25	100

4.9. Detection of Virulence factors of *P. mirabilis* isolates:

Table (4-7) shows that all isolates of *P. mirabilis* were positive for hemolysin, urease, siderophor, Protease production and CFA (I, II and III), while the results of biofilm formation revealed that (44.0%) were strong biofilm former, (44.0%) were moderate biofilm former and (12.0%) were non-biofilm formers. All isolates were hemolysin releasing at the percentage of 100%, according to the current study. These results

disagree with (Salman,2008; Al-Hussaini *et al*., 2009; Al-Rajab, 2014; Al-Mayahi, 2017) who found that the isolates were produced of hemolysin at the percentage of 66.7%, 45%, 89.7% and 89.5%, respectively. Hemolysin may be a key virulence factor in the spread of *P. mirabilis* into the kidneys and the onset of pyelonephritis (Armbruster *et al*., 2018).

In terms of urease, these resuts agree with (Al- Dulaimi *et al*., 2011; Ali, 2015; Al-Mayahi., 2017) who found that all isolates of *P. mirabilis* were urease produced with a percentage 100% and disagree with (Salman, 2008; Al-Dawah *et al*., 2015) who found that 45.8% and 40%, respectively of their isolates expressed this enzyme. *Proteus mirabilis* has to produce significant urease activity in UTIs (Zalski *et al*., 1997).

The current results found that all *P. mirabilis* isolates are 100% positive for siderophore producing. The present result agree with (Zalski *et al*., 1997; Hagan and Mobley, 2009; Armbruster *et al*., 2018) who found that all their isolates were produced of siderphore. Whereas, disagree with (Al-Atabi., 2013) who found that 11.11% of *P. mirabilis* were siderophore positive. It has been found that the *P. mirabilis* genome At least two gene clusters linked to siderophore biosynthesis and ABC transport were discovered. One is a novel nonribosomal peptide synthetase-independent siderophore system, termed proteobactin, The nrp operon, which has been up-regulated in *P. mirabilis* during iron deficiency, is found in the other gene cluster (Pearson *et al*., 2008). The nrp operon was discovered to be encoded within the *P. mirabilis*, which has a high pathogenicity (Flannery *et al*., 2009).

The metabolite, -hydroxyisovaleric acid, has been identified as a potential siderophore, in addition to the possibility that nrp has a role similar to iron limitation (Himpsl *et al*., 2010). Amino acid deaminases' -keto acids and hydroxycarboxylic acids have also been proposed as potential siderophores in *Proteus*. The urinary tract is iron-limited, according to studies on uropathogenic bacteria, and iron acquisition by outer membrane receptors is essential during UTIs (Hagan and Mobley, 2009). Multiple outer membrane proteins of *P. mirabilis* are up-regulated in both human urine and iron-limiting medium, and three outer membrane proteins caused by iron-starvation are involved in heme absorption in *P. mirabilis*; one of these, act as a heme receptor, contributes to *P. mirabilis* uropathogenesis (Lima *et al*., 2007).

According to the production of CFA (I,II and III),the results of the current study are agree with (Al-Mayahi., 2017) who found that all of his isolates produced a colonization factor antigen, at a percentage of 100%. Many studies have reported that the presence of mannose-resistant Proteus-like (MR/P) fimbriae of *P. mirabilis* is important in UTIs through facilitating colonization of the urinary tract (Zunino *et al*., 2001; Jansen *et al*., 2004). Indeed, *Protues mirabilis* is thought to be the main species responsible for crystalline biofilm formation on catheters resulting in increased incidence of urolithiasis , bacteremia and blockage of urinary catheter particularly during polymicrobial infections, these complications are urease-dependent (Stickler and Feneley, 2010; Armbruster *et al*., 2014).

Several types of fimbriae are potentially involved in adhesion to the uroepithelium, can be expressed simultaneously by *P. mirabilis*; mannose-resistant/Proteus-like (MR/P) fimbriae, *P. mirabilis* fimbriae (PMF), uroepithelial cell adhesin (UCA), or nonagglutinating fimbriae

(NAF), and ambient-temperature fimbriae (ATF). The MR/P fimbriae are involved in biofilm formation on catheter walls during UTIs (Rocha *et al*., 2007). Accordingly, the expression of MR/P fimbriae is essential for the uropathogenesis of *P. mirabilis* to initiate UTI, and increased expression of MR/P fimbriae correlates with higher levels of colonization and the severity of the infection (Liu *et al*., 2015). Furthermore, other trimeric autotransporters as fimbrial surface adhesins and autoagglutinins were characterized in *P. mirabilis* (Alamuri *et al*., 2010).

This explanation totally agrees with the current results as all *P. mirabilis* isolates were recovered from patients with clinically evident UTIs. A further agreement with the studies reported that *P.mirabilis* has an extraordinary potential for adherence to host tissues, as its genome contains 17 operons encoding fimbriae, the highest number of fimbriae that any bacterial species sequenced (Pearson *et al*., 2008; Pellegrino *et al*., 2013).

According to the production of protease (Figure 4.2), these results were inconsistent with the findings of (El-Baghdady,2009; AL- Rajab, 2014; AL-Dulaimi.,2019) who found that all isolates of *P. mirabilis* were produced of protease. Whereas disagree with (Salman., 2008; Al-Dawah *et al* ., 2015) who found that the percentage of *P. mirabilis* isolates produced for this enzyme was 45.8% and 40%, respectively, and it was agree by the researcher Jabur *et al*., (2013) and Al-Mayahi *et al* .,(2017) as their isolates were 100% productive.

Table (4-7):Distribution of virulence factors among *Proteus mirabilis* isolates.

Virulence factors	Results	*Proteus mirabilis* isolates	
		No	%
Hemolysin	Positive	25	100
	Negative	0	0
Urease	Positive	25	100
	Negative	0	0
Siderophore	Positive	25	100
	Negative	0	0
CFA (I, II and III)	Positive	25	100
	Negative	0	0
Biofilm formation	Strong	11	44
	Moderate	11	44
	Non-biofilm	3	12
Extracellular Protease production	Positive	25	100
	Negative	0	0
Chi-Square	**21**		
p-value	**0.02***		

***: significant (p <0.05)**

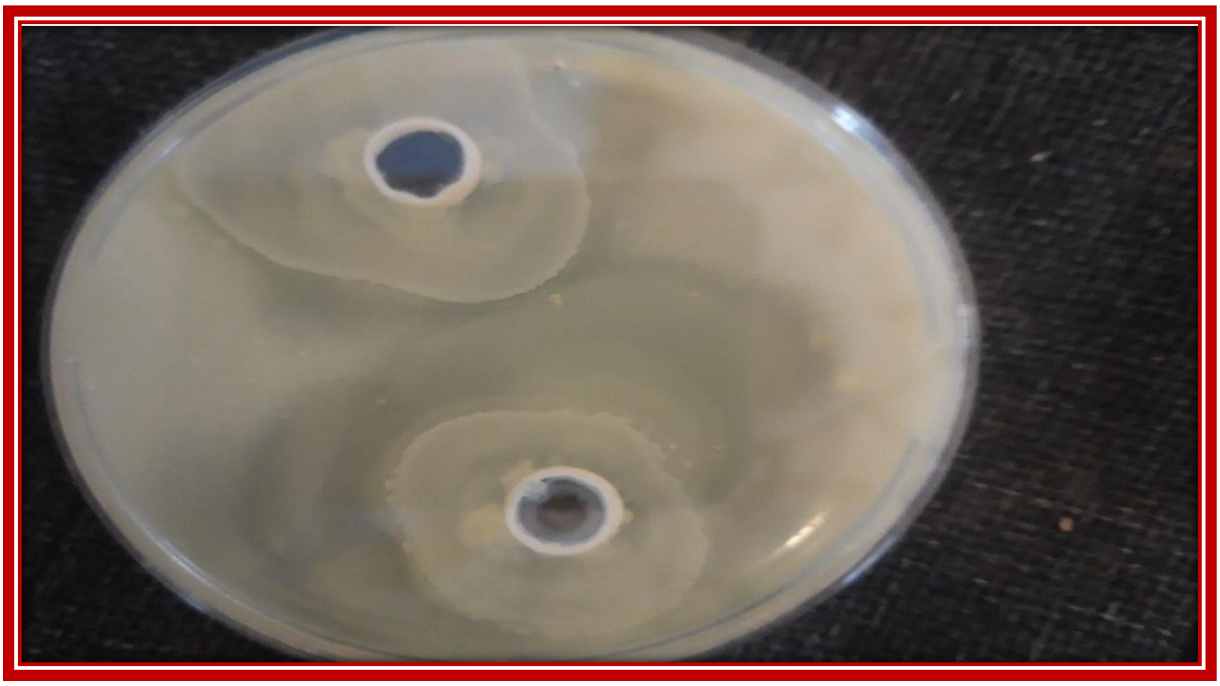


Figure (4-2) Production of Protease by *Proteus mirabilis* that curtured on skimmed milk agar medium.

4.10 Antibiotics susceptibility of the *Proteus mirabilis* isolates

The percentages of *P. mirabilis* resistant towards studied antibiotics are listed in table (4.8), the resistant percentage toward ampicillin and piperacillin were 96% and 72%, respectively. These result agree with the (Jawad *et al*, 2018; Ismail and Kazem, 2017) who found that the resistance to piperacillin were 72.0% and 78%, respectively. Whereas, the disagree with (Al-Bassam and Al-Kazaz, 2013; Zain Al-Abidin and Ahmad, 2015) who reported a resistant rate 20% and 40%, respectively. The ratio of the anti-augmentin (amoxicillin-clavulanic acid) that belongs to the beta-lactam group, the resistance rate obtained in this study was 64%, the present result was consistent with that of (Al-Atabi, 2020) who found a resistant rate at 61.2%. Whereas, disagree with (AL-Moussoy, 2013; Al-Rajab, 2014; Gupta *et al*., 2014; Perween *et al*., 2016) who found the resistant rates were 83.78%, 94.2%, 94.3% and 100% respectively. The origin of resistance is due to the ability of *P. mirabilis*

to produce beta-lactase enzymes, broad-spectrum beta-lactase enzymes ESβLs, that encodes a chromosomal or plasmid if it analyzes penicillins and sporins, and resulted the multiple resistance to antibiotics or due to the alteration of the target site and reduced permeability of the outer wall (Guilfoile, 2007). Additionally, may be due to the flow systems that expel the antagonists inside the cell to the outside (khalil *et al*., 2012).

The susceptibility to Nitrofurantion; the current results recorded that 84.0% of the isolates were resistant. These results agree with (Sayal *et al* .,2018; Hussein *et al*.,2020) who found the percentage rates of resistance were 90% and 85%, respectively. Whereas, disagree with (Al-Mazoki., 2004) who found that the percentage of resistance was 61%. The resistant rate of the current study to Tetracyclin was 72.0%. This result was matched with that reported (Jawad and Alramahy, 2018) who reported a resistant rate of 66.66%. Also, *P.mirabilis* isolates showed a resistant rate of 72.0% to Chloramphenicol. This result was close to that reported by (Jawad and Alramahy, 2018) who found that the resistant rate was 75.36%. However, it was less than that reported by Jawad and Alramahy, (2017) who found a resistant rate of 82.60%. On the other side, it was higher than that reported by Zuhair and Al-Obaidi, (2016) who found a resistant rate of 62.5%. Regarding the Naldixic acid, the current results reported a resistant rate of 88.0%. This result was identical to that reported (AL-Bahashwan, 2013). Nevertheless, it was incorresponding with the result of ,(Jawad and Alramahy, 2018), who recorded a resistant rate of 75.36%, and with the result of (Saeed, 2017) who reported a resistant rate of 100%.

The resistant rate of the *P. mirabilis* isolates to Ciprofloxacin and Levofloxacin were 20% and 28%, respectively. These results was close to results of (Ismail and Kadhim, 2017) who reported that the resistant rate

to Ciprofloxacin and Levofloxacin were 3.9% and 13.7%, respectively. The current study found a high resistance to Trimethoprim-sulfamethoxazole (Co-trimoxazole), with a rate of 92.0%. This result agrees with (Hindi, 2014) who found the resistance rate was 92.8%.

The current isolates showed that the resistance rate to Gentamicin was 76.0%. This result was close to results of (AL-Rajab, 2014) who found a resistance rate of 81% and inconsistent with (AL-baytti, 2010; Bahashwan and Elshafey, 2013) who showed the resistance rates were 90.9% and 62.2%, respectively. The rate of resistant of isolates to Imipenem was 52.0%. This result was higher than the resistance rate of 25% which was reported by Kadhim *et al*., (2014). However, the current result was inconsistent with the result of AL-Bassam and AL-Kazaz, (2013) who reported a resistant rate of 15%. As well as the study of AL - Rajab, (2014), who recorded a resistant rate of 16.2%. As for Meropenem, the rate of resistant was 32.0%, and the sensitivity rate of 56.0%. This result is closer to the resistant rate of 41.7% found in Dhe Qar by (Abbas and Kazem, 2014).

The isolates showed a resistance rate against the third generation Cephalosporins, as the rate resistance of *P. mirabilis* isolates to Cefotaxime was 64.0%. The current resistant rate is higher than that of (AL-Bassam and AL-Kazaz, 2013) who recorded a resistance rate of 35%. As well as higher than that reported by (Ismail and Kadhim, 2017), who found a resistant rate of 15.7%. Regarding the Ceftriaxone, the isolates showed resistant rate 76.0%. This result was lower than the resistant rate of Al-Mousawi, (2013) who found a resistant rate of 88.3%. Additionally, it was higher than the resistant rate reported in Karbala province by Shabeeb and Alghanimi (2018) they recorded a resistant rate of 47.3%. The current results found that the resistant rate against

Cephotoxime was 64.0%. This result was lower than the resistant rate 70% reported by (Feglo *et al*.,2010)

Antibiotic resistance in clinical isolates of *P. mirabilis* is a major public health concern. It produces AmpC-lactamases and extended spectrum-lactamases, which make infection control difficult (Literacka *et al*., 2019; Mirzaei *et al*., 2019). It may also form complex biofilms with accumulated layers of polysaccharides in which sessile cells are trapped, escalating the infection's severity (Shaaban *et al*., 2020).For a wild-type phenotype, *P. mirabilis* produces no chromosomally encoded -lactamase, resulting in complete susceptibility to all -lactams. Fluoroquinolones are normally toxic to them. Antibiotic-resistant strains of *Proteus spp*. are becoming more common, complicating the treatment of infections caused by these bacteria.

Table (4-8): Pattern of Antibiotic susceptibility of *Proteus mirabilis* isolates.

Antibiotic	Sensitive		Resistant	
	No	%	No	%
Ampicillin	1	4.0	24	96.0
Pipracillin	7	28.0	18	72.0
Amoxacillin - Clavunate	8	32.0	17	68.0
Cefepime	2	8.0	23	92.0
Cephotoxime	6	24.0	19	76.0
Cefoxitin	6	24.0	19	76.0
Ceflazidime	4	16.0	21	84.0
Imipenem	10	40.0	15	60.0
Meropenem	14	56.0	11	44.0
Gentamicin	6	24.0	19	76.0
Azithromycin	6	24.0	19	76.0

Tetracycline	7	28.0	18	72.0
Ciprofloxacin	20	80.0	5	20.0
Levofloxacin	16	64.0	9	36.0
Naldixic acid	3	12.0	22	88.0
Trimethoprim-sulfamethoxazole	1	4.0	24	96.0
Chloramphenicol	6	24.0	19	76.0
Nitrofurantion	2	8.0	23	92.0

4.11.Minimum Inhibitory Concentration of Cifipime and Meropenem:

According to (CLSI 2019), the minimum inhibitory concentration of Cifipime and Meropenem against 25 isolates of *P. mirabilis* was 131.20 ±233.11 mg/ml of the concentration (2-1024) mg/ml. Although the mean Meropenem concentration was 73.64±92.38 mg/ml, the MICs were (2-256) mg/ml, as shown in table (4-9).

Table(4-9): Minimum Inhibitory Concentration of Cifipime and Meropenem against *P.mirabilis* isolates.

MIC category	Mean ± SD concentration	Concentration mg/ml
Cifipime	131.20 ± 236.11	2-1024
Meropenem	73.64±92.38	2-256
p-value	0.001*	

4.12. Correlation between susceptibility of Antibiotic and Biofilms formed by *P.mirabilis*:

The results generally revealed that the strong biofilm former isolates had higher resistant rate to all tested antibiotics except the Meropenem, Ciprofloxacin and Levofloxacin. Similarly the modernly biofilm formers isolates had higher rate of antibiotic resistance except Meropenem, Ciprofloxacin and Levofloxacin. However, the differences were statistically insignificant as appeared in table (4-10).

Table (4-10): Correlation of biofilm formation with antibiotic susceptibility.

Antibiotics		Biofilm formation					
		Strong		Moderate		Non-biofilm	
		No	%	No	%	No	%
Ampicillin	Sensitive	1	9.1	-	-	-	-
	Resistant	10	90.9	11	100	3	100
	Intermediate	-	-	-	-	-	-
Pipracillin	Sensitive	1	9.1	4	36.4	2	66.7
	Resistant	10	90.9	7	63.6	1	33.3
	Intermediate	-	-	-	-	-	-
Amoxacillin-Clavunate	Sensitive	3	27.3	3	27.3	2	66.7
	Resistant	8	72.7	7	63.6	1	33.3
	Intermediate	-	-	1	9.1	-	-
Cefepime	Sensitive	1	9.1	1	9.1	-	-
	Resistant	9	81.8	10	90.9	3	100
	Intermediate	1	9.1	-	-	-	-
Cephotoxime	Sensitive	3	27.3	3	27.3	-	-

	Resistant	6	54.5	8	72.7	2	66.7
	Intermediate	2	18.2	-	-	1	33.3
Cefoxitin	Sensitive	3	27.3	2	18.2	1	33.3
	Resistant	8	72.7	9	81.8	2	66.7
	Intermediate	-	-	-	-	-	-
Ceflazidime	Sensitive	-	-	2	18.2	2	66.7
	Resistant	10	90.9	9	81.8	1	33.3
	Intermediate	1	9.1	-	-	-	-
Imipipenem	Sensitive	4	36.4	4	36.4	2	66.7
	Resistant	6	54.5	6	54.5	1	33.3
	Intermediate	1	9.1	1	9.1	-	-
Meropenem	Sensitive	5	45.5	7	63.6	2	66.7
	Resistant	4	36.4	3	27.3	1	33.3
	Intermediate	2	18.2	1	9.1	-	-
Gentamicin	Sensitive	2	18.2	4	36.4	-	-
	Resistant	9	81.8	7	63.6	3	100
	Intermediate	-	-	-	-	-	-
Azithromycin	Sensitive	2	18.2	3	27.3	1	33.3
	Resistant	9	81.8	8	72.7	2	66.7
	Intermediate	-	-	-	-	-	-
Tetracycline	Sensitive	3	27.3	3	27.3	1	33.3
	Resistant	8	72.7	8	72.7	2	66.7
	Intermediate	-	-	-	-	-	-
Ciprofloxacin	Sensitive	8	72.7	10	90.9	2	66.7
	Resistant	3	27.3	1	9.1	1	33.3
	Intermediate	-	-	-	-	-	-
Levofloxacin	Sensitive	7	63.6	7	63.6	2	66.7

	Resistant	3	27.3	3	27.3	1	33.3
	Intermediate	1	9.1	1	9.1	-	-
Naldixic acid	Sensitive	1	9.1	2	18.2	-	-
	Resistant	10	90.9	9	81.8	3	100
	Intermediate	-	-	-	-	-	-
Trimethoprim - sulfamethoxazole	Sensitive	-	-	1	9.1	-	-
	Resistant	10	90.9	10	90.9	3	100
	Intermediate	1	9.1	-	-	-	-
Chloramphenicol	Sensitive	4	36.4	2	18.2	-	-
	Resistant	6	54.5	9	81.8	3	100
	Intermediate	1	9.1	-	-	-	-
Nitrofurantion	Sensitive	-	-	2	18.2	-	-
	Resistant	10	90.9	8	72.7	3	100
	Intermediate	1	9.1	1	9.1	-	-

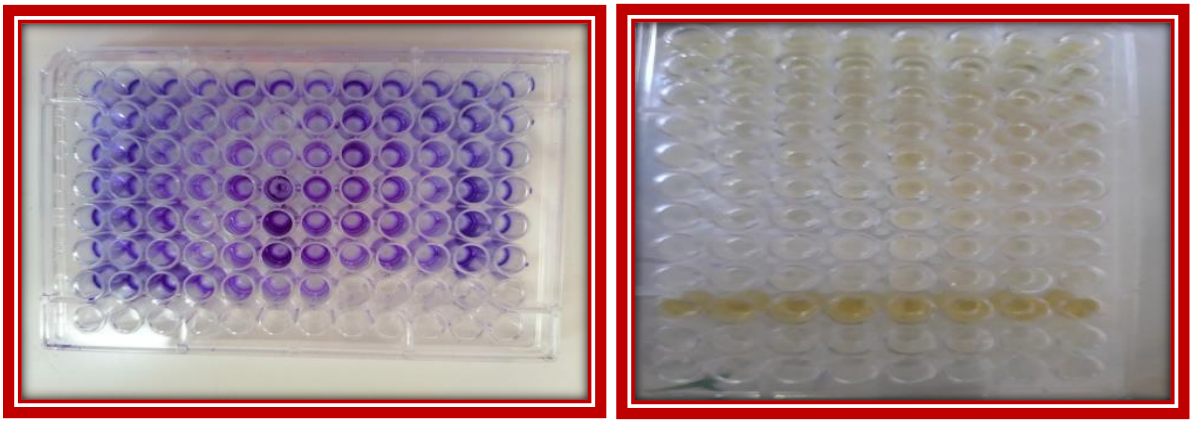
4.13. Minimum inhibitory concentration of Cifipime and Meropenem According to Biofilm Formation:

Table (4-11) shows that the strong and moderate biofilm former isolates had lower MIC then weak forms isolates of both Cifipime and Meropenem**.** The statistical differences were insignificant (p= 0.253 and p= 0.072), respectively . Shwos Figure (4-3) (A) shows the formation of biofilm isolates and (B) shows a microplate method for minimum inhibitor concentration

Table (4-11): Association of Biofilm Formation with MIC of Cifipime and Meropenem

	result	No.	MIC Cifipime	MIC Meropenem
Biofilm Formation	Strong	11	39.6±75.5	82.7±97.1
	Moderate	11	200.2±312.2	37.9±48.9
	Weak	3	214.0±265.7	171.3±146.6
	p-value		**0.235***	**0.072***

***Significant difference between percentages using Pearson Chi-square test (χ^2-test) at 0.05 level.**



(A) **(B)**

Figure (4.3): (A) Biofilm formation by *Proteus mirabilis*, (B) The microplate method for minimum inhibitor concentration

Locally, the present results are in agreement with the results of (Al-Duliami *et al*., 2011; AL-Ataby., 2013) .They discovered that 91.7 % and 88.88 % of *P. mirabilis* isolates in Diyala had the ability to form biofilms, respectively. Furthermore, the current findings were completely consistent with those of Rocha *et al*., (2007), who observed biofilm formation in all *P. mirabilis* isolates, although they were only partly consistent with the finding that *P. mirabilis* isolates had a modest capacity (65.1%) to shape biofilms (AL-Salihi, 2012).

Bacteria form biofilms as part of their survival mechanisms, and biofilms are thus common in nature. There is evidence that accumulating biofilms play a role in protues mirabilis pathogenesis, especially in

chronic infections (Bjarnsholt, 2013). Bacteria can cause local tissue damage and then an acute infection by remaining dormant and hidden from the immune system. The bacteria within the biofilm respond to anoxia and nutrient limitation by changing their metabolism, gene expression, and protein production, which can result in a slower metabolic rate and slower cell division (Stoodley and Hall-Stoodley, 2009). By inactivating antimicrobial targets or lowering the criteria for cellular activity, these adaptations render bacteria more resistant to antimicrobial therapy (Fuqua *et al*., 2019; Uruen *et al*., 2021).

These are the plausible explanation of the increased antibiotic resistant of most *P. mirabilis* isolates of the current study against the majority of antibiotics used even these increment had insignificant difference statistically.

Protues mirabilis biofilms in the urinary tract, especially on the catheter's surface, have been the subject of the most research. Catheter incrustation and obstruction are caused by crystallized biofilms. They included two forms of crystals: struvite (magnesium ammonium phosphate) and apatite (hydroxyl calcium phosphate). It appears in biofilms in the urinary tract and obstructs urine flow (Jacobsen and Shirtliff, 2011). It can lead to bladder blockage, bacteriuria episodes, fever, sepsis, and shock (Jones *et al*., 2007). However, there were no statistically significant variations in biofilm formation capacity between *P. mirabilis* strains derived from catheterized and non-catheterized patients' urine. (Kwiecinska-Pirog *et al*., 2014). Furthermore, the current results are consistent with that reported by (Czerwonka *et al*., 2016).

4.14. Multiple drug resistant *P. mirabilis*:

The results of the current study found that 17 out of 25 (68.0%) of *P. mirabilis* isolates were MDR, while the remaining 8 (32.0%) were XDR isolates, as appeared in table (4-12).

Table (4-12): Multiple Drug and Extending Drug Resistant isolates.

Categories	No. of isolates	Percentage
Multi-drug resistant (MDR)	17	68.0%
Extended drug resistant (XDR)	8	32.0%

Actually these results are unexpected for the authors and alarming for the healthcare workers and the community. Because it implies that the treatment of this bacterium become very difficult. Therefore, it is matched the CDC 2019 report on antimicrobial resistance thread since the report classify the Carbapenem-resistant *Enterobacteriaceae* as urgent thread and the ESBL-producing *Enterobacteriaceae* as serious thread and that the fighting of these threats required a public health collaborative global approach (CDC, 2019).

Concerning the MDR, the current result is disagrees with than the 14.5% of the MDR *P. mirabilis* isolates recovered from patients with UTIs in Iran, all these isolates had the ability of biofilm and hemolysin formation, and all of them amplified *mrpH*, *mrpA*, *pmfA*, *ureG* and *hpmA* genes (Mirzaei *et al*., 2019).

Furthermore, the value of the current result is disagerrs than the result of (Boudjemaa *et al*., 2019) who reported 4.8% MDR rate of *P. mirabilis*.These isolates exhibited resistance to important antibiotics, including amoxicillin, amoxicillin/clavulanic acid, cefotaxime,

gentamicin and ciprofloxacin, 8.6% had an ESBL genotype and 71.4% had an AmpC/ESBL genotype . genes were detected in all isolates. Additionally, the current rate of MDR is disagree than that obtained in an Italian study, in which among *P. mirabilis* isolates recovered from patients with bloodstream infections, 33.3% of them were MDR (Tumbarello *et al*., 2012).

The current result is closer to those reported from Nigeria since 56% of *P. mirabilis* isolates recovered from 5 hospitals were MDR (Alabi *et al*., 2017).

Generally, MDR strains of *P. mirabilis* produce extended-spectrum β-lactamases (ESBLs) or the AmpC-type cephalosporinase and rarely carbapenemases, and their prevalence in some settings is relatively high (Cohen-Nahum *et al*., 2010; D'Andrea *et al*., 2011).

MDR infections had a significant impact on the prognosis and survival of hospitalized patients (Endimiani *et al*., 2005; Tumbarello *et al*., 2012).

In Korea, ESBL producing *P. mirabilis* was found to be significantly associated with mortality in patients with bacteremia (Ahn *et al*., 2017). Even an outbreak caused by *VEB-1* extended-spectrum β-lactamaseproducing MDR *Proteus mirabilis* sepsis in a neonatal intensive care unit was reported from India (Jain *et al*., 2016). Concerning the extended drug resistant *P. mirabilis*, It has been reported that an XDR *P. mirabilis* isolates were emerged which possess the metallo-β-lactamase 1 that is able to hydrolyze all β-lactams except monobactams, in *P. mirabilis* which is intrinsically resistant to tetracycline, tigecycline and colistin, that make clinical treatment extremely difficult, and represents a great threat for public health (Qin *et al*., 2015).

The current study believe that the emergence of MDR and XDR *P. mirabilis* in Diyala community, and probably whole Iraq, is not unusual since all medical, social, educational and commercial encouraging factors are actively cooperate for emergence of such isolates. In any case, these genetically altered strains necessitate further investigation on the country level.

4.15 Detection virulence factor of *proteus mirabilis* using singleplex PCR.

Fifteen isolates of *p.mirabilis* were molecular identified using single plex PCR techniques . The The results found that all isolates contained the *Zap*A gene, the *Urec* gene, the *MRp* gene, and the *FlaA* gene with apercentage (100%). While 6 isolates showed a percentage (40%) carrying the *Esp* gene table(4.13).

Table (4-13): Gene detection rate among the *Proteus mirabilis* isolates.

Gene detected	Detected		Not detected	
	No.	%	No.	%
Esp gene	6	40	9	60
FlaA gene	15	100	-	-
Mrp gene	15	100	-	-
Urec gene	15	100	-	-
Zap gene	15	100	-	-

The results of this study showed that all isolates of *P. mirabilis* are carriers *Urec* gene with apercentage (100%). The molecular weight of *Urec* gene wes 533bp of DNA ladder (100%) Figure (4-4), when the beams are compared with DNA ladde as shown in the figure 4.9. The

current result was consistent with that of Al - Janahi , (2013) and Al-Mayahi (2017) who found all isolates carriers *Ure*c gene with apercentage (100%) . The result was also agrees with that of Pathirana

et al., (2018) who reported that 100% of *p.mirabilis* isolates were positive. On the other hand, the current result was disagrees with Abbas *et al.,* (2015) ,Al- Oqaili *et al*., (2017) and Al-Duliami, (2019) who found that positive result of *p. mirabilis* we carried *Urec* gene were (18% ,60% and 85.7% respectively. Concerning the *UreC* gene**,** this gene is one of the basic structural genes for building the urease enzyme, and it is preserved and replicated greatly.

It is considered one of the important diagnostic characteristics of *P. mirabilis*, as it is the main cause of the rise of basophilic urea and the formation of urinary stones (Mohammed *et al*., 2014).*Proteus mirabilis* produces urea-inducible urease, which hydrolyzes urea into ammonia and carbon dioxide. Raising the local *pH* induces precipitation of normally soluble calcium and magnesium ions, which can expand to remarkable sizes to create bladder and kidney stones, which are a hallmark of *Proteus spp*. infections (Island and Mobley, 1995). Urease is therefore a significant virulence factor in *P. mirabilis* UTIs schaffer and Pearson,(2015).

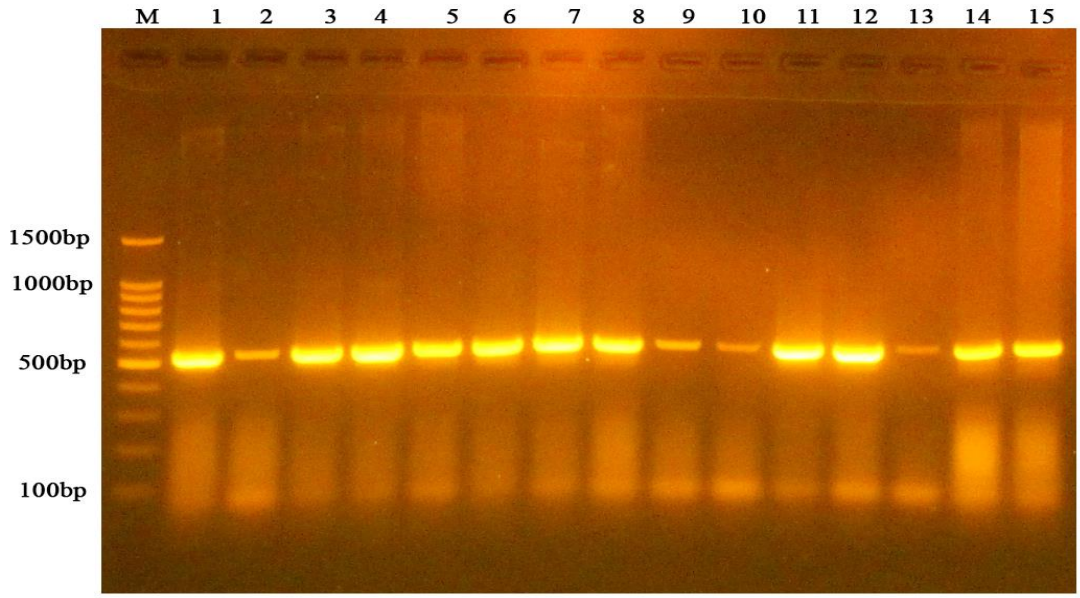


Figure (4-4): Amplification of *UreC* gene of *Proteus* samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes(1-15) represent *P.mirabilis* isolates.

The current results showed that all isolates had detectable *Mrp*A gene. The results of the polymerase chain reaction of the gene showed that the size of the bundle was 550bp, when the beams were compared with DNA ladder, as shown in the figure (4-5). The present result was in agreement with (Al- Mayahi, 2017) who also reported 100% detection rate for this gene, but disagrees with the findings of (Ghaima *et al*.,2017; AL-Oqaili *et al*.,2017; Ghaima *et al*., 2017) who obtained a detection rate of the *Mrp*A were 35%, 40% and 81.17%, respectively. In connection with *Mrp*A gene, (The most thoroughly studied *P. mirabilis* fimbriae were the Mannose-resistant *Proteus*-like fimbriae (MR/P). It was needed for biofilm formation and autoaggregation, and its expression is required for colonization of the bladder and kidneys (Schaffer *et al*., 2016; Norsworthy and Pearson, 2017). Furthermore, MR/P-mediated biofilm formation can be used to begin the quest for the physiological MR/P

fimbrial receptor. Jiang and colleagues (Jiang *et al*., 2020). The mrpABCDEFGHJ operon encodes the main structural subunit, the outer membrane usher, the periplasmic chaperone, the mrpB and is mrpE-G four minor subunits, the tip-located adhesin, and the transcriptional regulator. *Mrp*H is a two-domain adhesin (TDA) with an N-terminal receptor binding domain and a C-terminal pilin domain that binds the TDA to the fimbrial tip through a short linker 1B (Werneburg and Thanassi, 2018).

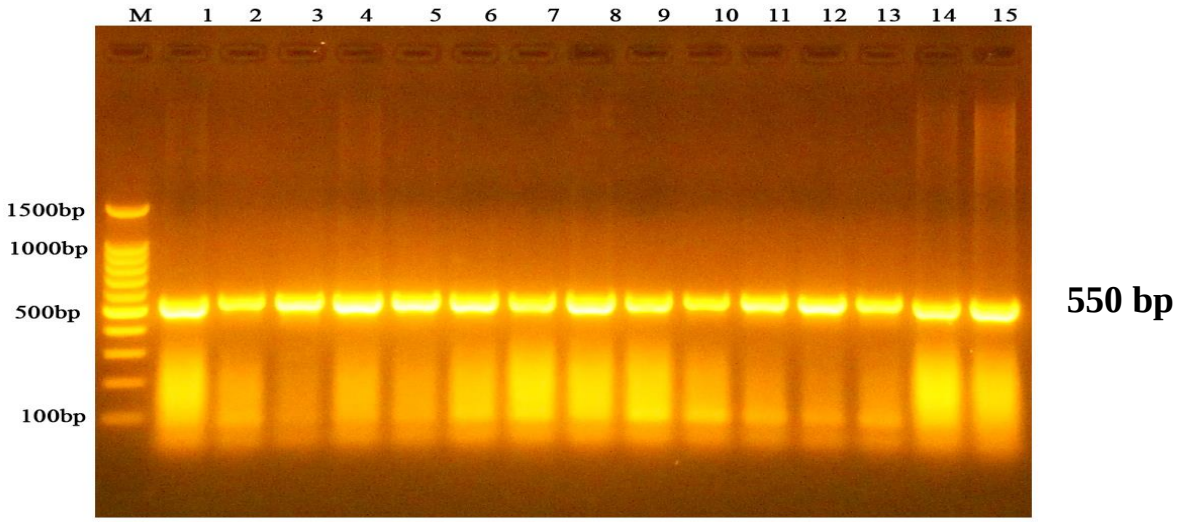


Figure (4.5); Amplification of *Mrp* gene of *Proteus* samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes (1-15) represent *P.mirabilis* isolates

About the *FlaA* gene, the results showed that all (100%) isolates of *P. mirabilis* had detectable *FlaA* gene as illustrated in figure (4.6).

The current results were in agreement with local studies which also obtained 100% detection rate (Mohammed *et al*, .2014; Ali and Yousif, 2015; Al-Duliami, 2019).

But are inconsistent with the finding of (Ali, 2015) who reported a detection rate of 86.88% *FlaA* gene among their isolates of *P. mirabilis*

was dimorphic, When cultured in nutrient broth, it produces short vegetative swimmer cells with a single nucleoid and peritrichous flagella. Swimmer cells, on the other hand, differentiate into nonseptated, elongated swarmer cells with multiple nucleoids and numerous flagella when cultured on nutrient agar (Rather, 2005). Flagellar rotation inhibition causes swarmer cell differentiation (Belas and Suvanasuthi, 2005). The expression of several virulence factors that help in the invasion of uroepithelial cells in human urinary tracts is linked to the differentiation of *Protues mirabilis* swarmer cells (Schaffer and Pearson, 2015). Swimming and swarming motility both necessitate functional flagella. The expression of the flagellar regulon, which allows flagellar genes and flagellum biosynthesis to be coordinated. (Chevance and Hughes, 2008).

Swarmer cell differentiation in *Proteus mirabilis* is distinguished by the presence of flagella. Flagellin synthesis, which is the flagellar filament protein, is coordinated as part of a larger regulon of genes whose expression is needed for urinary pathogenesis. The control of the *FlaA* locus, which consists of two tandemly repet and nearly identical copies of flagellin-encoding genes *FlaA* and *FlaB*. *FlaA* but not *FlaB*,is expressed by wild-type cells, and its transcription

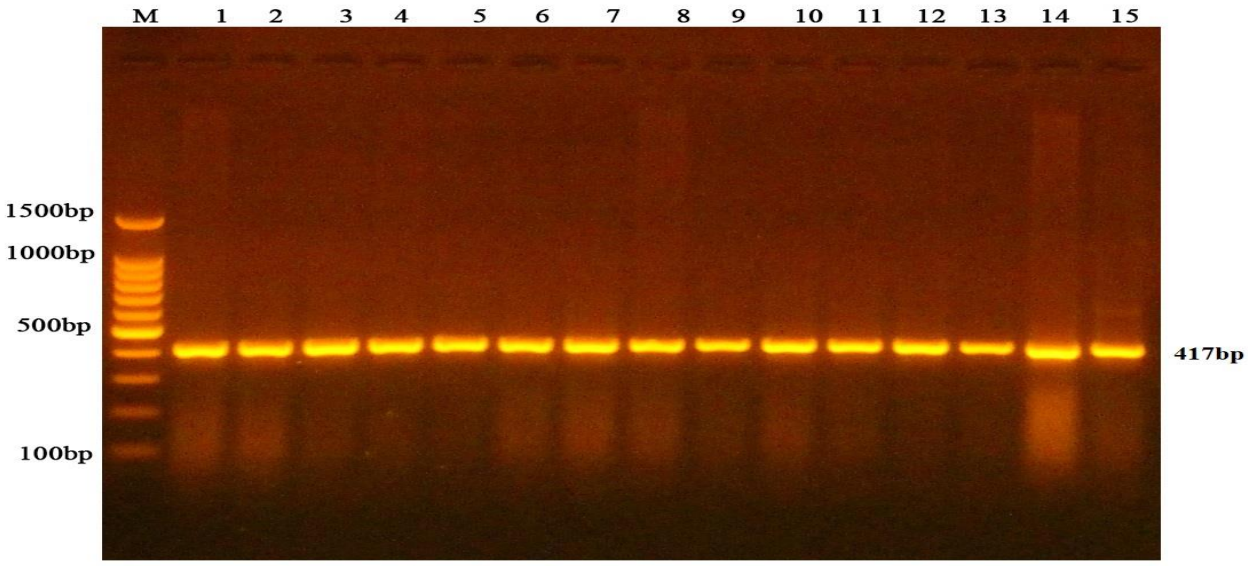


Figure (4-6): Amplification of *FlaA* gene of *Proteus* samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes (1-15) represent *P.mirabilis* isolates

increases eightfold during differentiation; *FlaA* - mutants, on the other hand,are nonmotile and undifferentiated, and do not synthesize flagellin (Tuson *et al*.,2013

The detection rate of *zapA* gene in the present study was 100% as all isolates showed detectable gene as shown in figure (4-6). These results were similar to the results obtained by (AL-Jumaa, 2011; Abd. AL-Monaam, 2013; AL-Mayahi, 2017; Ali and Yousif, 2015) in that all (100%) *P. mirabilis* produce protease. However, the current result is partially inconcordant with those reported by (AL-Salihi, 2012; El-Baghdady *et al*., 2009; Al-Duliami, *et al*., 2011). Who discovered that the detection rate of *P. mirabilis* isolates was 0%, 41.9, and 45.8%, respectively.

Protues mirabilis produces a metalloprotease from the serralysin family of zinc proteases, which is encoded by the *ZapA* gene. These proteases may degrade host proteins, releasing amino acids as nutrients, and they can also degrade proteins like IgA, which are involved in host

defense and can also cause tissue harm. *P. mirabilis* mutants lacking IgA protease activity produce normal urease, hemolytic activity, swarmer cell differentiation, flagella production, swarming motility, and biofilm formation. (Wassif *et al.*, 1995; Walker et al., 1999).

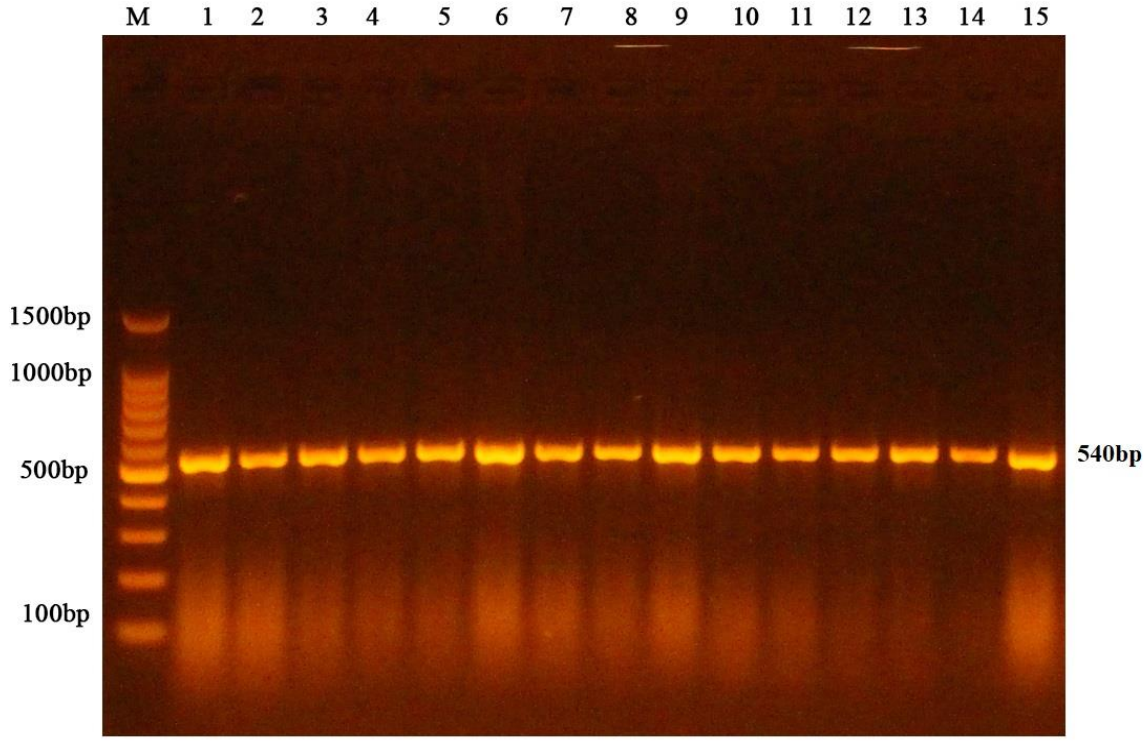


Figure (4.7):Amplification of *ZapA* genes of *Proteus* samples fractionated on 1.5% agarose electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes (1-15) represent *P.mirabilis* isolates.

The gene (*Esp*) which encodes for enzymes responsible for Biofilm formation in *Proteus mirabilis* using PCR technique represented 15(40%) his result did disagree with the researcher Sayal *et al*.,(2017)who found (50%)were positive. Regarding the *Esp* gene, the current results found that none of the tested *P. mirabilis* isolates (40%)had detectable *Esp* gene. Upon reviewing the literature, *P. mirabilis* had a number of colonizing factors; so following initial attachment, it produces mannose-resistant *Proteus*-like (MR/P) pili, that facilitate biofilm formation and

colonization of the bladder and kidneys, and are crucial for catheter-associated biofilm formation (Jacobsen *et al*., 2008; Jacobsen and Shirtliff, 2011). Other pili encoded include *P. mirabilis*-like fimbriae (PMFs), which are important for bladder and kidney colonization, and non-agglutinating fimbriae (NAFs), which are able to attach to uroepithelial cells in *vitro* (Armbruster and Mobley, 2012). In addition to pili, *P. mirabilis* encodes two autotransporters, TaaP (trimeric autoagglutinin autotransporter of *Proteus*) and AipA (adhesion and invasion mediated by the Proteus autotransporter), which are important for bladder and kidney infection, respectively (Flores-Mireles *et al*., 2015). AIPAC can adhere to bladder and kidney cells. Whereas, TaaP is required for bladder infection. Importantly, both autotransporters bind to extracellular-matrix proteins: AipA preferentially binds to collagen I, and TaaP to laminin, which might provide an explanation for their different tissue tropisms (Jacobsen *et al*., 2008).

Adherence is a key event initiating each step in UTI pathogenesis; Different bacteria use different bacterial adhesins recognize receptors on the bladder epithelium (uroepithelium) and mediate colonization. *Protues mirabilis* following colonization of the urethra it migrate to the bladder, where a complex host–pathogen interactions that determine the survival of bacteria by invading the bladder epithelium, producing toxins and proteases to release nutrients from the host cells, and synthesizing siderophores to obtain iron. By multiplying and overcoming host immune surveillance, the *P. mirabilis* subsequently ascend to the kidneys, again attaching via adhesins or pili to colonize the renal epithelium and then producing tissue-damaging toxins. Consequently, it able to cross the tubular epithelial barrier to access the blood stream, initiating bacteraemia

(Alamuri *et al*., 2010; Pellegrino *et al*., 2013; Flores-Mireles *et al*., 2015).

Generally, the differences in detection rates of the *P. mirabilis* genes can be attributed to various factors including, the type of specimens, location, patient characteristics, strain isolation and identification techniques plus the molecular technique used.

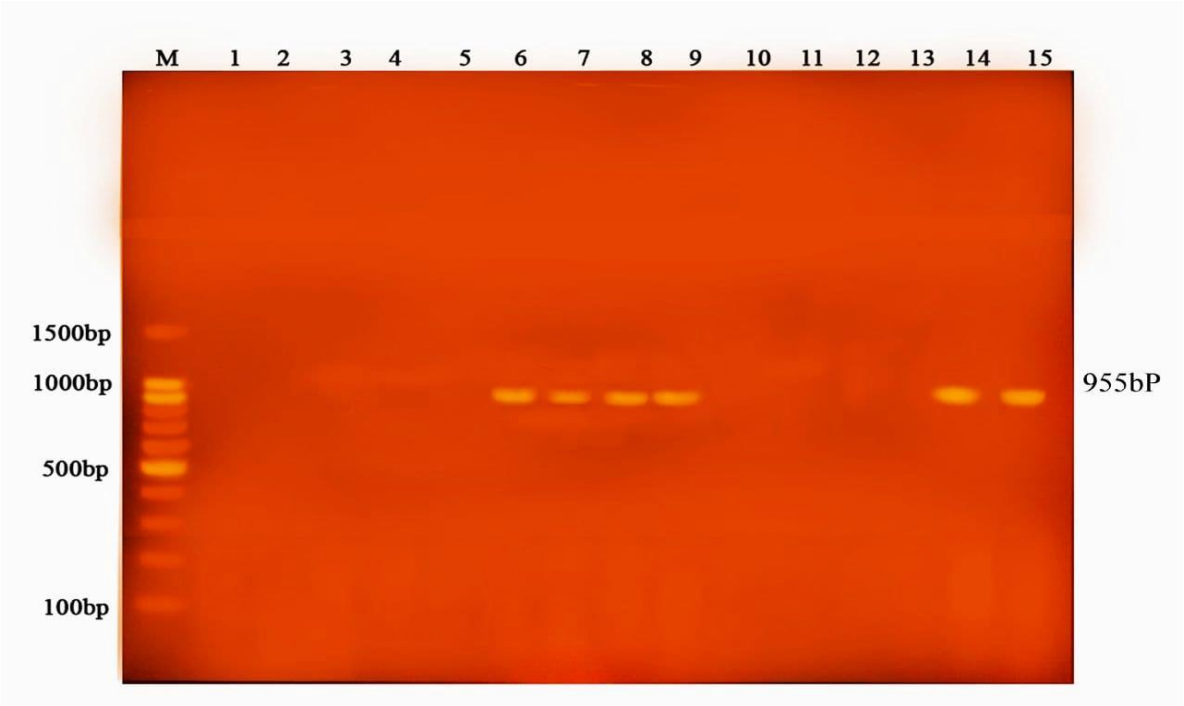


Figure (4.8) Amplification of *Esp* gene of *Proteus* samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes (1-15) represent *P.mirabilis* isolates.

4.16 Detection of (*FlaA* & *ZapA*)genes of *Proteus mirabilis* using Multiplex PCR.

The result of multiplex PCR of *FlaA* & *ZapA* genes revealed that all isolates of *P. mirabilis* were carried this gene with a percentage(100%) each of the size of the product of(*FlaA* & *ZapA*) genes were 417 bp &540 pb, respectively. of DNA ladder (100 bp) indicating detected genes (Figure 4.9).

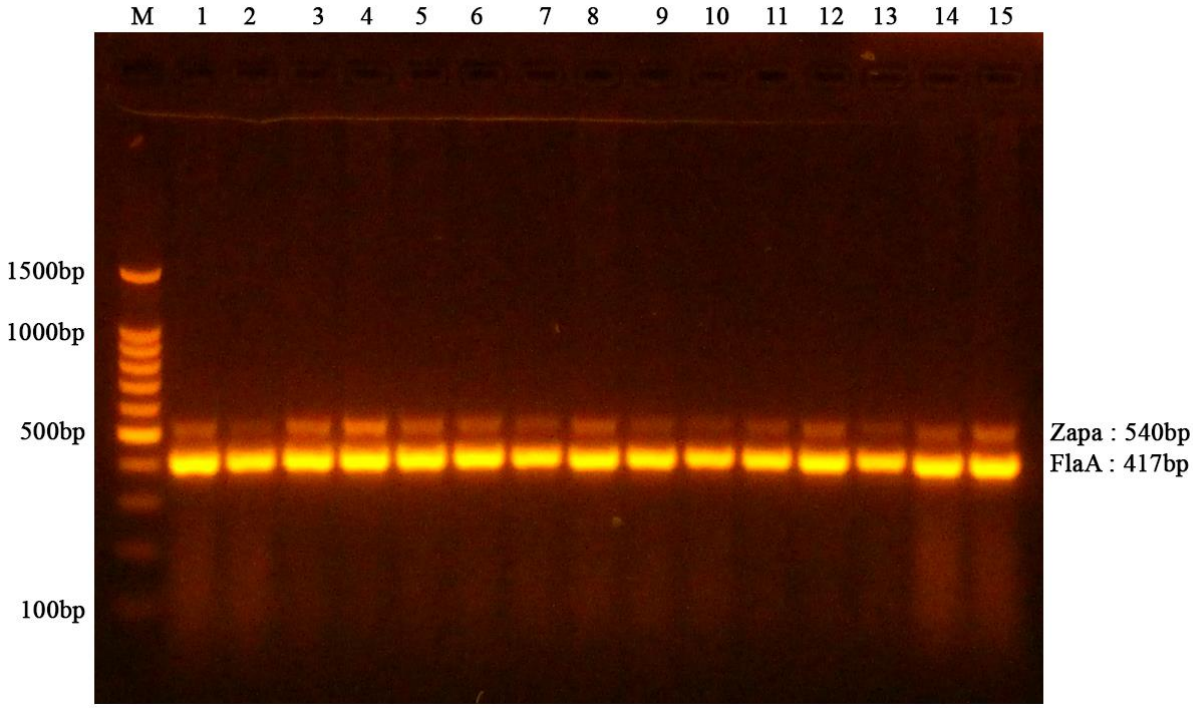


Figure (4.9) Amplification of *ZapA*& *FlaA* genes of *Proteus* samples fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker.

4.17. Gene Sequencing of *Protues mirabilis*:

The sequencing reactions indicated the exact position of the analyzed samples after performing NCBI blast analysis. This engine showed a high similarity of more than 99% between the sequenced samples and the expected target that covered a portion of the *ZapA* locus within the *P. mirabilis* sequences.

After positioning the 540 bp amplicons' sequences within the *ZapA* locus of *P. mirabilis* sequences, the details of these sequences were highlighted, starting from the position of the forward primer to the position of the reverse primer within the same sequences as appeared in appendix (5).

The alignment results of seven samples, with size product (540 bp) revealed the detection of three variations in comparison with the referring sequences of the GenBank. Table (4-14) shows that the variations in gene sequence were represented by three substitutions of amino acids in three positions of *ZapA* for samples (S2, S5,S6,S7, S8, S2 and S14).

The observed variations were further analyzed to identify whether such nucleic acid substitution induces possible alterations in its corresponding position in the encoded amino acids from the *ZapA* region. All nucleic acid sequences were translated to their corresponding amino acid sequences using the Expasy translate suite. It was found that the detected nucleic acid substitutions of G349A, G439T and G159A were found in the amino acids Gly (G), Asp (D), and His (H) in the positions 34, 64, and 128, respectively.

Table (4-14): The pattern of the observed mutation in the 540 bp of the *ZapA* amplicons in comparison with the NCBI referring sequences (GenBank acc. no. CP046048.1).

Sample	Native	Allele	Position in the PCR fragment	Position in the reference genome	Amino acid position	Type of variant
S2, S14	G	A	159	1128653	H167	Missense
S2, S5, S6, S8, S12, S14	G	A	349	1128843	D103	Silent
S7	G	T	439	1128933	G73	Silent

A comprehensive phylogenetic tree was generated in the present study, which was based on the observed nucleic acid variation detected in all investigated samples. This phylogenetic tree contained all amplicons of *ZapA* sequences, alongside other relative reference sequences. The total number of the aligned nucleic acid sequences in this comprehensive

tree was 113. This generated comprehensive tree indicated the presence of major bacterial organisms, *Proteus mirabilis*. Furthermore, two other related species of the same genus were also observed, namely *Proteus vulgaris* and *Proteus cibarius*. As it was inferred from *ZapA* gene-based tree, close phylogenetic correlations between *Proteus mirabilis* with both *vulgaris* and *Proteus cibarius* sequences were observed. Alongside *Proteus* sequences, *Serratia marcescens* was added as an out group sequence to serve as a reference group to give further determination between the evolutionary relationship among the ingroup *Proteus mirabilis*, *Proteus vulgaris*, and *Proteus cibarius* organisms. Due to many clades occupied by *Proteus mirabilis* sequences, a high diversity of *Proteus mirabilis* sequences were inferred from this tree. It was found that a portion of *Proteus mirabilis* sequences was directed toward the outgroup sequences of *Serratia marcescens*, which were usually identified in the UTI-infecting organisms. This is due to the high diversity of *Proteus mirabilis* that led to multiple positioning of these sequences in many clades in the cladogram. Nevertheless, the currently generated neighbor-joining comprehensive tree showed extremely close genetic distances among all the investigated S1– S15 samples. These close genetic connections were attributed to the small genetic distances observed among these samples. Though small genetic distances were observed among S1– S15 samples, they were clustered to four main phylogenetic clades, S1 / S3 / S4 / S7 / S8 / S9 / S110 / S12 / S13 / S14 clade, S5 / S6 / S11 clade, S2 / S14 clade, and S7 clade within the *Proteus mirabilis* sequences. No genetic variation was observed in the S1 / S3 / S4 / S7 / S8 / S9 / S110 / S12 / S13 / S14 clade. Thus, it was exerted an entire homology beside variable strains of *Proteus mirabilis* sequences. Beside this clade, the S7 clade was located. S7 clade was

positioned in the immediate vicinity to the GenBank accession number of CP047352.1, which was belonged to the Chinese *Proteus mirabilis* strain ZA25.

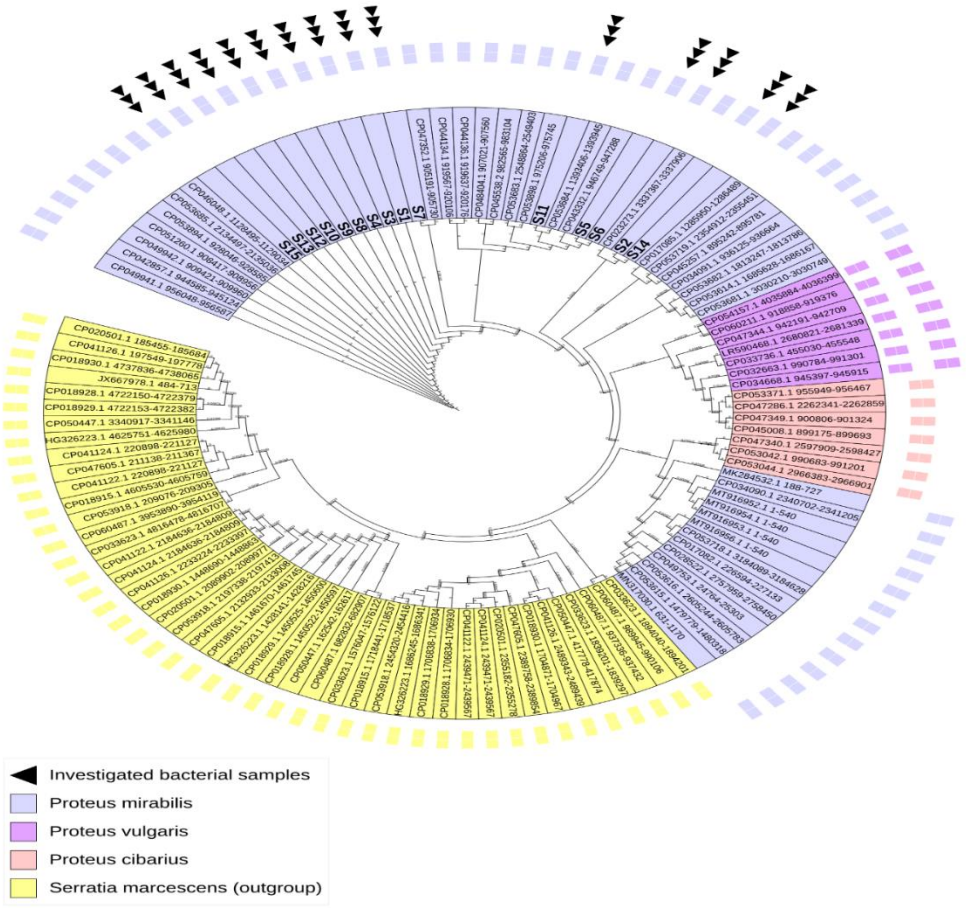


Figure (4-10): The comprehensive phylogenetic tree of genetic variants of the *ZapA* locus within fifteen *Proteus mirabilis* bacterial isolates. The variably colored numbered refer to Genbank acc. numbers of deposited reference sequences. The numbers in the nodes of the tree refer to the degree of phylogenetic positions among the comprehensive tree categorized organisms. The symbol "S" refers to the code of the investigated samples.

The reason behind the slight deviation of S7 from the standard *P. mirabilis* clade is attributed to the presence of the G439A variant. This entailed a possible evolutionary contribution of this variant in this deviation to this Asian strain. The same thing was also observed for the S2 / S14 clade, which exerted another deviation toward the GenBank

accession number CP023273.1 that belonged to the Sweden *Proteus mirabilis* strain CCUG 70746 strain. As in the case of the S7 clade, the G159A variant in the S2 / S14 clade had forced this isolate to take this slightly deviated position. However, the G349A variant may not be involved in causing any tilt in the currently investigated isolates. It was inferred from this tree that both G439A and G159A variants may also refer to the possible geographical origins for these isolates. Most importantly, the presence of the observed nucleic acids variations of G159A and G439T may entail a possible role for the *ZapA* genetic fragment in the adaptation made by *Proteus mirabilis* toward the clinical sources from which they were obtained. Accordingly, the currently utilized *ZapA*-based amplicons can be useful in the resolution of genetic differences among the currently investigated S1 – S15 samples. Therefore, this comprehensive tree has provided an inclusive tool about the ability of such genetic sequences to efficiently describe *P. mirabilis* variations using this genetic fragment. This, in turn, gives an additional indication of the power of the currently utilized *ZapA* -specific primers to identify the phylogenetic grouping of the currently investigated isolates of *P. mirabilis*.

(Conclusions and Recommendations)

Conclusions:

1- Twenty five isolates of *P. mirabilis* were recovered from 300 patients who suffering from urinary tract infections.

2- The age group (20-29) years old, reported the highest infection rate with uropathogenic *P. mirabilis* among other age groups.

3- All 25 isolates expressed the virulence factors: hemolysin, urease, siderphore and CFA (I,II, and III) by conventional methods.

4- Eleven isolates of *P. mirabilis* were strong biofilm formers.

5- Seventeen isolates were multi drug resistant, whereas eight isolates were extended drug resistant.

6- Using singleplex PCR technique, all isolates of *P. mirabilis* had the virulence genes: *FlaA, Mrp, Urec* and *Zap*. Whereas, six isolates only had *Esp* gene.

7- Using multiplex PCR technique, all isolates of *P. mirabilis* had the virulence genes: *FlaA* and *Zap*.

8- Three isolates of *P. mirabilis* showed amino acids substitutions in *Zap* gene; G159A, G349A, and G439T.

9- It was found that a portion of *Proteus mirabilis* sequences was directed toward the outgroup sequences of *Serratia marcescens*, which were usually identified in the UTI-infecting organisms.

Recommendations:

We are recommended the followings:

1- Further study of uropathogenic *P. mirabilis* using immunological and other molecular detection methods.

2- Study the sequence of other genes of MDR and XDR *P. mirabilis* isolated from UTI and other systemic infections.

3- Bacteriological, immunological and molecular study of other species of *Proteus* isolated from different infections.

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Appendices

Appendix (1)

Appendix 1: Information Form of Patients

Sample number:

Patient name.

Age.

Sex:

Living:

Sample type.

Date of sample collection:

Appendix (2): Chart Report of VITEK-2 System for identification the Results

bioMérieux Customer: M.Sc.Manal
System #:

Laboratory Report

Printed Sep 21, 2020 04:13 CDT
Printed by: Labadmin

Patient Name: Dina Nazar 3, .
Isolate: 303-1 (Approved)

Patient ID: a303

Card Type: GN Bar Code: 2411143203508975 Testing Instrument: 0000148FFB2A (VK2C8812)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0013000340542211
Organism Quantity:

Selected Organism: Proteus mirabilis

Comments:	

Identification Information	**Card:** GN		**Lot Number:** 2411143203	**Expires:**	Jan 12, 2021 12:00 CST
	Completed: Sep 20, 2020 09:34 CDT		**Status:** Final	**Analysis Time:**	4.05 hours
Organism Origin	VITEK 2				
Selected Organism	99% Probability	**Proteus mirabilis**			
	Bionumber: 0013000340542211			**Confidence:**	Excellent identification
SRF Organism					
Analysis Organisms and Tests to Separate:					
Analysis Messages:					
Contraindicating Typical Biopattern(s)					

Biochemical Details

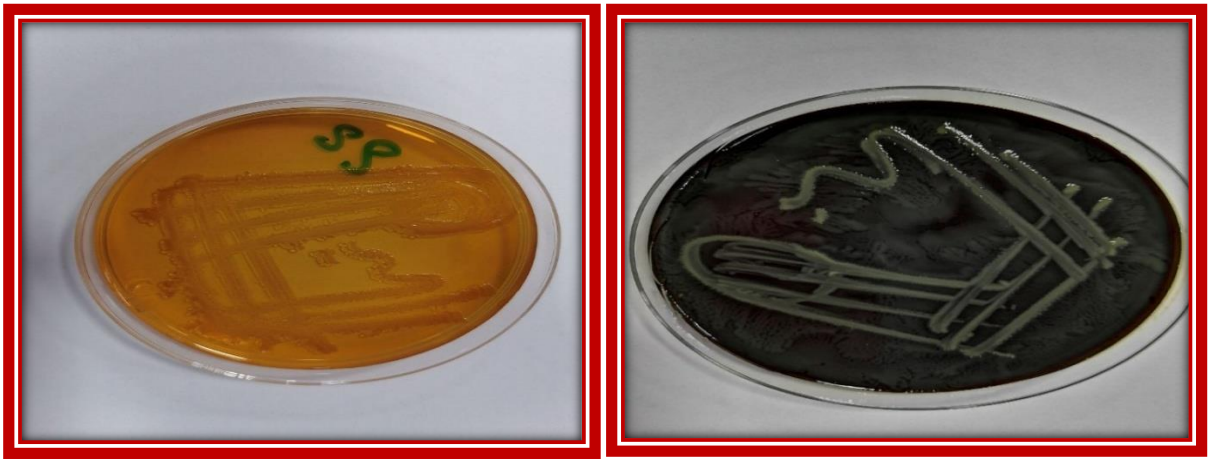
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	+	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	+	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	+
46	GlyA	-	47	ODC	+	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	+	64	ILATa	-			

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

Page 1 of 1

Appendix (3)

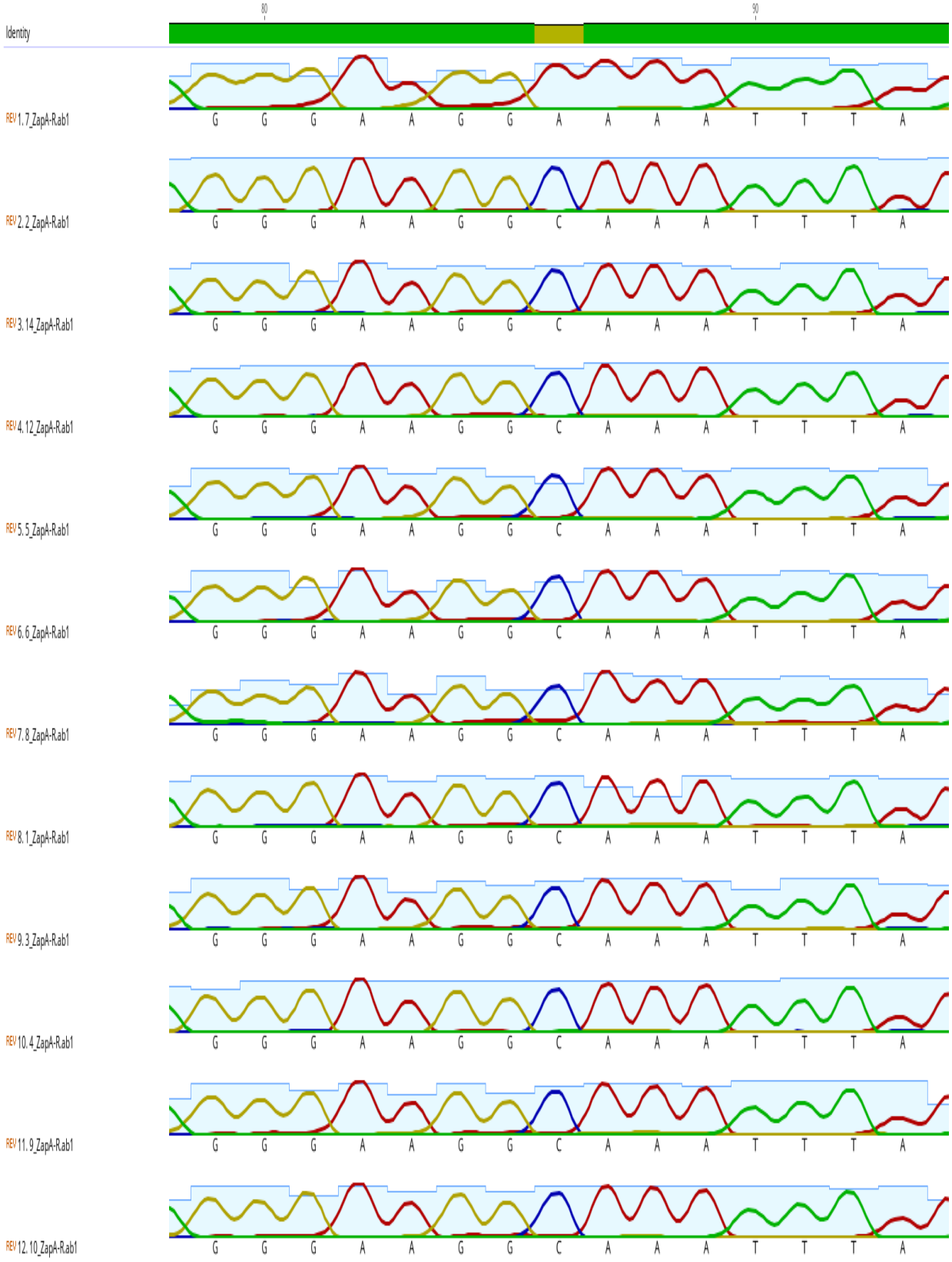


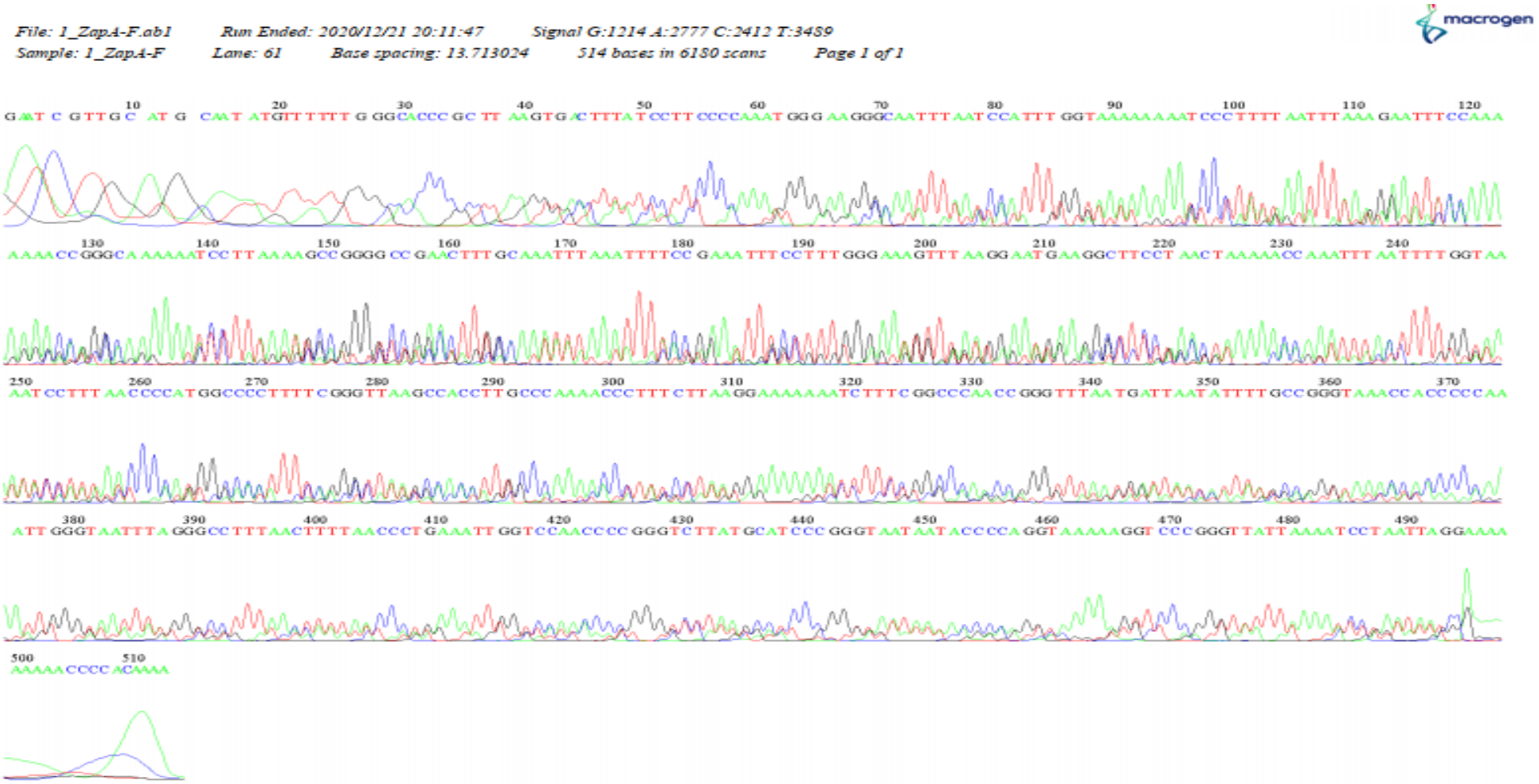
(1) **(2)**

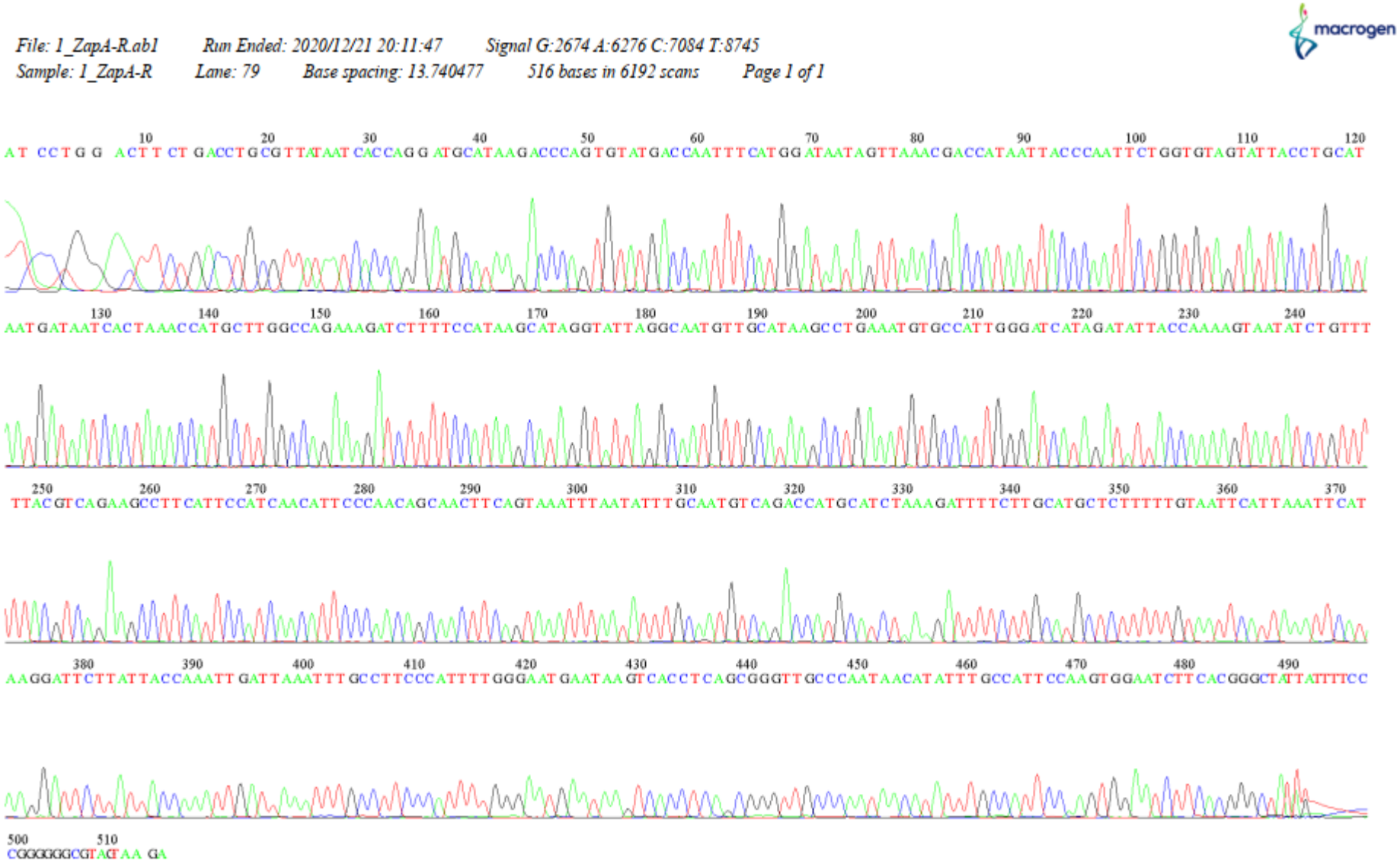
Shows *Protues mirabilis* cultured on (1) MacCongy agar medium and (2) blood agar medium

Appendix **(4)** **Biochemical results of *Proteus mirabilis* isolates**

Appendix (5) Results of sequential analysis of gene results gene ZapA




File: 1_ZapA-F.ab1 Run Ended: 2020/12/21 20:11:47 Signal G:1214 A:2777 C:2412 T:3489
Sample: 1_ZapA-F Lane: 61 Base spacing: 13.713024 514 bases in 6180 scans Page 1 of 1
macrogen


File: 1_ZapA-R.ab1 Run Ended: 2020/12/21 20:11:47 Signal G:2674 A:6276 C:7084 T:8745
Sample: 1_ZapA-R Lane: 79 Base spacing: 13.740477 516 bases in 6192 scans Page 1 of 1
macrogen
10 20 30 40 50 60 70 80 90 100 110 120
AT CCTGG ACTTCT GACCTGCGTTATAAT CACCAGG ATGCATAAGACCCAGTGTATGACCAATTTCATGGATAATAGTTAAACGACCATAATTACCCAATTCTGGTGTAGTATTACCTGCAT
130 140 150 160 170 180 190 200 210 220 230 240
AATGATAATCACTAAACCATGCTTGGCCAGAAAGATCTTTTCCATAAGCATAGGTATTAGGCAATGTTGCATAAGCCTGAAATGTGCCATTGGGATCATAGATATTACCAAAAGTAATATCTGTTT
250 260 270 280 290 300 310 320 330 340 350 360 370
TTACGTCAGAAGCCTTCATTCCATCAACATTCCCAACAGCAACTTCAGTAAATTTAATATTTGCAATGTCAGACCATGCATCTAAAGATTTTCTTGCATGCTCTTTTTGTAATTCATTAAATTCAT
380 390 400 410 420 430 440 450 460 470 480 490
AAGGATTCTTATTACCAAATTGATTAAATTTGCCTTCCCATTTTGGGAATGAATAAGTCACCTCAGCGGGTTGCCCAATAACATATTTGCCATTCCAAGTGGAATCTTCACGGGCTATTATTTTCC
500 510
CGGGGGGCGTACTAA GA

الخلاصة

الخلاصة

هدفت هذه الدراسة الى الكشف عن بعض عوامل الضراوة لبكتريا المتقلبة الرائعة *Proteus mirabilis* المعزولة من مرضى اخماج المسالك البولية باستخدام الطرق الجزيئية. تم جمع 300 عينه ادرار من المرضى الذين راجعوا (مستشفى الخالص العام ومستشفى البتول التعليمي ومستشفى بعقوبة التعليمي) للفترة الممتدة مابين الخامس من شهر اب 2020 الى الخامس والعشرين من شهر كانون الثاني 2021 . تم تشخيص المرضى بوساطة استشاري الامراض البولية. معدل عمرالمرضى تتراوح مابين 2–56 سنة. تم عزل وتشخيص بكتريا المتقلبة الرائعة باستخدام الطرق التقليدية والتي شملت(البكتريولوجيه والكيموحيوية واستخدام Vitek) والطرق الجزيئية التي شملت (تقنية تفاعل انزيم متعدد البلمرة ذو الخطوة الواحدة لجين*Esp*,*Mrp*,*Urec* , *ZapA*,*FlaA* وذو الخطوات المتعددة للجينين *FlaA* و *ZapA* وتقنية تتابعات الجين *ZapA*) . تم دراسة حساسية البكتريا تجاه 18 مضاد حيوي باستخدام طريقة انتشار الاقراص في الاكاروطريقة التركيز المثبط الادنى تجاه المضادين السيفبيم والميروبنيم.

باستخدام طرق التحري التقليدية، تم تشخيص 25 عزلة بنسبه 8.3% لبكتريا المتقلبة الرائعة من 300 عينة. سجلت الفئة العمرية (20–29) سنة عمرية اعلى نسبة اصابة بالبكتريا 36%. الاناث اكثر اصابة مقارنة بالذكور وبنسبة 64% والمرضى المناطق الريفية اعلى اصابة وبنسبة 60%. اظهرت جميع العزلات انتاجها لعوامل الضراوة بنسبة 100% حيث كانت النتيجة ايجابية لعوامل الضراوة مثل(الهيمولايسين وانزيم اليوريز والبروتيزوحاملة الحديد مستضد عامل المستعمرة الأول والثاني والثالث وبالنسبة لتكوين الغشاء الحيوي اظهرت 11 عزلة قوية التكوين للغشاء الحيوي بنسبة 44% بينما اظهرت 11 عزلة متوسطة القوة و 3 عزلات ضعيفة التكوين

للغشاء الحيوي وبنسب 44% و 12%على التوالي. اظهرت نتائج حساسية العزلات تجاه المضادات الحيوية قيد الدراسة بأن 17 من25 (68٪) كانت مقاومة للمضادات المتعددة (MDR)، في حين أن 8 (32٪) الباقية كانت مقاومة للمضادات (XDR) ,بالإضافة إلى ذلك ، هناك نتيجة أخرى تتمثل في أن العزلات المكونة للغشاء الحيوي تتمتع بمقاومة قوية أو معتدلة لمعظم المضادات الحيوية المستخدمة في هذه الدراسة.

اظهرت نتائج تفاعل انزيم البلمرة المتعدد ذو الخطوة الواحدة بأن جميع العزلات 100٪ تحتوي على جينات *ZapA* و *Urec* و *Mrp* و*FlaA* بينما 40% من العزلات احتوت على جين *Esp*. اما اظهرت نتائج تفاعل انزيم البلمرة المتعدد ذو الخطوات المتعددة بأن جميع العزلات 100٪ تحتوي على جينات *ZapA* و*FlaA* . اظهرت نتائج تتابعات الجين *ZapA* وجود ثلاث طفرات احلال في العزلات (G159A و G349A وG439T). لوحظ متغير G439Tفي شجرة النشوء والتطور تجاه سلالة المتقلبة الرائعة الصينيةZA25 ، بينما لوحظ G159Aتجاه السلالة السويدية المتقلبة الرائعة ، .CCUG 70746 في المقابل ، لم تحدث متغيرات G349A أي انحراف في تحديد المواقع التطورية لعزلات *P. mirabilis* التي تم فحصها حاليًا.

وزارة التعلـــــــيم العـــــــالي
والبحـــــــــث العلمـــــــــي
جامعـــــــــة ديـــــــــالى
كليـــــــــة العلـــــــــوم

التحري الجزيئي عن جينات عوامل الضراوة لبكتريا *Protues mirabilis* المعزوله من أخماج المسالك البولية

رسالة مقدمة الى

مجلس كلية العلوم – جامعة ديالى

وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل

دينا نزار عايش العبيدي

بكالوريوس علوم الحياة (2007) جامعة ديالى

بإشراف

أ.د. زينب محمد نصيف الزبيدي أ. م. د. عباس محيي مزهر العماري

1442 هـ 2021م