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Study of Genotyping Among Clinical Isolates of Proteus mirabilis

A thesis

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

((يرفع الله الذين امنو منكم والذين أوتوا العلم درجات والله بما تعملون خبير))

صدق الله العلي العظيم

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Certification

I certify that this thesis, entitled " ***Study of Genotyping Among Clinical Isolates of Proteus mirabilis*** " at that college of medicine, university of Babylon, as a partial requirement for the degree of ***Master of Science in Medical Microbiology.***

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Dedication

To the soul of the prophet and messenger

Muhammad peace be upon him and his household.

To the fountain of patience, optimism, and hope.

"My Mother"

Iqbal 2022

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*Praise be to "**ALLAH**" and to his prophet "**Muhammad**", my praise and thanks are due for their blessings without which this research would not have been achieved.*

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To all thank you very much

In addition, sorry to those I forgot

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Summary

In this study, a total of 130 specimens from patients suffering from urinary tract infections, vagina, wound and burn infections were obtained from both gender how were attended to Hilla general Teaching Hospital during the period from Septembere to December (2021) in Babylon Province Iraq.

Out of 130 samples, 25 (19.2%) were positive for *Proteus mirabilis* using biochemical assays, the compact VITEK2 system, and ureR gene primer PCR assay. As it was 17 (68%) isolates of bacteria from urinary tract infection patients, 3 (12%) isolates from patients with vagina and wounds patients, and 2 (8%) isolates from burn patients.

At a molecular level, genotyping of *P.mirabilis* isolates was carried out to characterize the genotypic traits by using PCR method targeting the main virulence factors genes. The overall 25 *P. mirabilis* positive samples were screened for three virulence genes included (*hpmA*, *rsbA*, and *pta*) genes.

Specific PCR primer was used for the detection of *ureR* gene. It was found that *ureR* gene was observed in 25(100%) isolates of *P.mirabilis*. Also used for the detection of *hpmA* gene. It was found that *hpmA* gene was observed in 24 bacterial isolates (96%) of *P.mirabilis* which included 17 isolates in urine, 3 isolates in vagina, 2 isolates in wound and 2 isolates in burn.

PCR amplification of *rsbA* gene indicated that only 20 (80%) isolates of *P.mirabilis*, out of 25 total isolates had this gene (including 15 isolates in urine, 2 isolates for each of vagina, wound and one isolates in burn.

pta gene was also detected in *P.mirabilis* by using specific PCR primers. It was found that *pta* gene was observed in 23(92%) isolates of *P.mirabilis* out of 25 which include 15 isolates in urine, 3 isolates for each of vagina, wound, and 2 isolates in burn.

Multilocus sequence analysis is for the taxonomic updating and identification of the genus proteus and re-classification of proteus genospecies. In this study, multilocus sequence analysis (MLSA) approach based on five housekeeping genes (*dnaJ-mdh-pyrC-recA-rpoD*) was used to delineate phylogenetic relationships of species within genus of *Proteus*.

Similarity matrix analysis of studied samples *P.mirabilis* isolate against the reference genome showed high similarity percentage with all samples, where they shared (95-99%) similarity with the reference sequence, that lead to there is a evolutionary variation for studied isolates in comparison with the reference genome. Moreover, the highest percentage of variation was detected in the isolate 11.

All the *P.mirabilis* isolates obtained were subjected to antimicrobial susceptibility testing with 13 antibiotics belong to different classes according CLSI recommendations (20121). Highest rates of sensitivity were observed for antibiotics used in present study include Levofloxacin, Imipenem, Meropenem, Aztreonam, Ciprofloxacin and Amikacin (100%, 100%, 100%, 88%, 88%, 84%) respectively, followed by Cefotaxime, Ampicillin-Sulbactam,

Amoxicillin, Tetracycline and Gentamicin (72%, 64%, 56%, 52%, 48%) respectively, where as there was low sensitivity to Sulphamethoxazole-Trimethoprim and Chloramphenicol (32% and 20%).

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List of Abbreviations

Abbreviation	Complete term
Ala	Alanine
AR	Antibiotic Resistance
Arg	Arginine
armA	aminoglycoside resistance methylase
Asn	Asparagin
Asp	Aspartic acid
AT	AutoTransporter
BHI	Brain heart infusion
cheW	Chemotaxis protein
cldA	cyclomaltodextrin glucanotransferase with a three-domain ABC distribution
Cys	Cysteine
gidA	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG
Gln	Glutamin
Glu	Glutamic acid
Gly	Glycine
His	Histidine
HKGs	house-keeping genes
hmpA	Alpha (α) hemolysin
HRPTECs	Human Renal Proximal Tubular Epithelial Cells
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
MDR	Multidrug resistant
Met	Methionine
MHA	Muller Hinton Agar
MLSA	Multi-locus sequence analysis

MLST	**Multilocus sequence typing**
mrpA	**two component response transcriptional regulatory protein**
npmA	**N(1)-methyltransferase A genes**
PCR	**Polymerase chain reaction**
Phe	**Phenylalanine**
Pro	**Proline**
pta	**Proteus toxic agglutinin**
rmtA	**16S rRNA methylases A genes**
rmtB	**16S rRNA methylases B genes**
rmtC	**16S rRNA methylases C genes**
rmtD	**16S rRNA methylases D genes**
rmtE	**16S rRNA methylases E genes**
Ser	**Serine**
Thr	**Threonine**
Trp	**Tryptophan**
Tyr	**Tyrosin**
ureR	**Urease Regulatory Gene**
UTI	**urinary tract infection**
Val	**Valine**
VEB	**Vietnamese extended-spectrum β-lactamase**

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	with branch lengths (below the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Neighbor-Joining method.
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Chapter one

Introduction & Literature Review

1.1 Introduction

Proteus.mirabilis is a Gram-negative bacterium widely found in the environment and isolated from the intestinal tract of humans and other animals (Drzewiecka, 2016). Urinary tract infection stands out as the most prevalent *P.mirabilis* infection, which has the capacity to cause other diseases in humans (Armbruster *et al.,* 2018). Besides UTIs, this pathogen develops diverse diseases of the respiratory tract and infections of the skin and soft tissue (including postoperative wounds, burns, etc.) (Filipiak *et al.,* 2020).

The medical importance of this bacteria may attribute in its ability to produce a variety of extracellular enzymes such urease, which is responsible for the formation of bladder and kidney stones that protect the bacteria from antibiotic effect, additionally the hemolysin is a cytotoxic toxin for urinary tract epithelial cells (Jansen *et al*., 2003).

Urease is required for urolithiasis, where it contributes in hydrolyzing urea to release ammonia, thereby increasing urinary pH, resulting in precipitation of calcium and magnesium compounds, and urinary stone formation (Armbruster and Mobley, 2012).

P.mirabilis has ability to lyse blood cells and produce alpha (α-hemolytic) hemolysis on blood agar media that is frequently related with pathogenic bacteria, therefore it is considered as essential virulence factors that contribute in their pathogenesis since it can form pores in biological membranes (Afriani *et al*., 2014).

The *rsbA* gene is a regulator of swarming behavior that encodes a sensory, which *rsbA* may function as a protein sensor of environmental conditions. *rsbA* gene is also responsible in biofilm formation and extracellular polysaccharide formation (Zafar *et al*., 2019).

Also *Proteus toxic agglutinin* this protein is present in outer-membrane that facilitates cell to cell aggregation and the α-domain of Proteus toxic agglutinin also has ability to lyse kidney and bladder cells. *pta* gene of *P.mirabilis* had decrease pathology as well as, important colonization in the bladder, kidneys and spleen (Alamuri and Mobley, 2008).

Multi-locus Sequence Analysis (MLSA) has been used for classification at species level in numerous Enterobacteriaceae (Hall *et al*., 2015). Normally, four to seven housekeeping genes were selected for MLSA to determine phylogenetic relationships. It has been advised to use sequence data from more than one gene, to reduce the possibility of ambiguities caused by genetic recombination or specific selection. MLSA is increasingly applied in order to obtain a higher resolution power between species within a genus (Glaeser and Kampfer, 2015).

P.mirabilis organisms sensitive to antibiotics are becoming resistant to drugs by producing beta-lactamases like extended-spectrum beta-lactamases (ESBLs) (Caubey and Suchitra, 2018).

Aim of study

This study aimed to evaluate the genetic diversity of *Proteus mirabilis* isolated from different clinical specimens.

The Objective of study

1- Isolation and detection of *P.mirabilis* from different clinical samples includes (urine, vagina, wound and burn).

2- Molecular diagnosis of *P.mirabilis* by using specific primer for *ureR* gene.

3- Detection of genes virulence factors responsible for hemolysin, swarming and *Proteus* toxic agglutinin of *P.mirabilis* (*hpmA*, *rsbA*, and *pta*) genes by PCR technique.

4- Comparative genome analysis by using MLSA (Multi-locus Sequence Analysis) for five housekeeping genes (*dnaJ-mdh-pyrC-recA-rpoD*).

5- Study antibiotic susceptibility test against *P. mirabilis*.

1.2 Literature review

1.2.1 General Characteristics of *Proteus mirabilis*

Proteus mirabilis are Gram-negative bacilli belonging to the widespread *Enterobacteriaceae* family. The genus *Proteus* was first described by Gustav Hauser in 1885 and divided into two different species, *P. mirabilis* and *P. vulgaris* (Armbruster *et al.,* 2018).

Proteus mirabilis. are rods measuring 1-3μm in length and 0.4-0.8μm in diameter, motile by peritrichous flagella, facultative anaerobic, non-spore forming and non-capsulated with most isolates having fimbriae (Nahar *et al.,* 2014;Drzewiecka, 2016). *Proteus mirabilis* can establish swarming motility on a solid surface and produce biofilms that is one of their important pathogenic factor (Ravanbakhsh *et al.,* 2018).

Members of the family are also readily recognized by the red-brown melanin pigment they form when cultured under aerobic conditions on media containing iron and an aromatic L-amino acid such as phenylalanine, tryptophane, tyrosine or histidine (Kamga *et al.,* 2012). They are negative for arginine and lysine decarboxylase, malonate utilization and acid production from D-sorbitol and L-arabinose (O'hara *et al.,* 2000). Bacteria from the family Proteeae can be differentiated by use of biochemical tests. Those belonging to the genus *Proteus* are lactose negative, urease and phenylalanine-deaminase positive. They also produce hydrogen sulfide and oxidatively deaminated tryptophane (Janda and Abbot, 2006).

P.mirabilis grow over a wide temperature range below 42^{o}C but optimally at 34-37^{o}C, most strains particularly if grown at 22-30^{o}C, do not form discrete colonies on nutrient agar (Manos and Belas, 2006). *P.mirabilis* can be found in the human intestine as part of the microflora but can cause disease when becoming in contact with urea in the urinary tract, *P.mirabilis* prevalent in hospitals and care facilities and responsible for ninety percent 90% of *Proteus* infections (Armbruster *et al*., 2017).

Proteus strains form several sorts of discrete colony on MacConkey agar. Phase A colonies are smooth, morphologically they consist of regular bacillary forms 5-6 µm long, which on other media give rise to discontinuous swarming. Phase B colonies are smaller, smoother, and more conical. They consist of highly pleomorphic cells (coccobacilli, filaments and giant cells) that do not swarm on other media. Phase C colonies are flat, rough, and larger than phase A colonies. They consist of long filaments that give rise to continuous swarming on other media. The dominant type's specific O antigen is not found in phase B cells. (Senior, 2010).

In liquid medium, *P. mirabilis* exist as a short (1.5-2 µm.), motile rod with peritrichous flagella typical of members of the family Enterobacteriacaceae. When transferred from liquid to an agar surface, the cell undergo a striking change in morphology and physiology, which result in a differentiated morphotype, called a swarmer cell by a mechanism which bacteria elongate dramatically due to an inhibition of septation, reaching 10-40 µm with minimal increase in width. During elongation DNA replication is not affected,

while the synthesis rates of certain proteins, most notably, urease, hemolysin, and flagellin are markedly increased (Morgenstein *et al*., 2010).

P.mirabilis infections are characterized as long term and difficult to treat and can often lead to death due to the capacity of this microorganism to mediate urea hydrolysis, via the urease it produces, causing tissue necrosis and inflammation at the infection site, so that antibiotics is not accessible to the pathogen (Alamuri *et al*., 2009). *Proteus* spp. are common opportunistic pathogens that are highly infectious and cause infections in immunocompromised people. These infections are typically long-lasting and difficult to treat (Drzewiecka and Sidorczyk, 2005).

P.mirabilis infect humans and mostly cause skin wounds and nosocomial infections such as infection of the respiratory tract, eye, ear, nose, neck, burns, sore throat, wound infections and UTI (urinary tract infection) (Drzewiecka, 2016). UTI are among recurrent bacterial infections in human and account for about 20 percent of diseases out of hospital.

1.2.2 Pathogenesis of *Proteus mirabilis*

The genus *Proteus* is usually recorded as an opportunistic microorganism associated with serious invasiveness most of these infections are caused by *P.mirabilis* and it was observed as one of the main causes of urinary tract infections (UTIs), wound infections, respiratory tract infections, otitis media and burn infections (Jacobsen *et al*., 2008). *P.mirabilis* causes 90% of *Proteus* infections and is mostly found in people with compromised immune system (Mandal *et al*., 2015).

The contagious nature of *Proteus* at different human infection sites is usually related to their antibiotic insensitivity that supports their continued existence in different healthcare centers, the great ability of *Proteus* to invade different host tissue is linked to many tools of virulence, like cellular adhesion and diffusion all over the body (Hola *et al*.,2012).

The interaction between *P. mirabilis* and the host defense (immune) system determines the resultant infection. Proteus species have an extracytoplasmic outer membrane, like other gram-negative bacteria, which contains lipoproteins, lipopolysaccharides, polysaccharides and a lipid bilayer. Different components of this membrane interact with the host and host defense mechanisms to determine the organism's virulence. Additionally, the size of the inoculum has a positive correlation with the level of infection. Attachment of *P. mirabilis* to host tissue depends on the activity of its fimbriae (or pili), which are tiny projections on the bacterium surface. The tips of these fimbriae also contain certain compounds and polysaccharides that allow for attachment to specific sites in the

host organism (e.g., endothelium of the urinary tract) or other inanimate surfaces (e.g., medical devices) (Jamil *et al.,* 2022).

P.mirabilis properties involved in the infection process include adhesion to epithelial surfaces, invasion (penetration) of host cells, intracellular multiplication of the pathogen, colonization of the cell tissue or transmission to a new susceptible host, production of enzymes which damage the host defense system, and synthesis of toxins (Schadich and Cole, 2010).

Pathogenicity of *P. mirabilis* is accomplished by two steps, first step the microorganism needs to colonize the infection site and second step it needs successfully to evade host defenses, colonization is done by using two types of fimbriae called Mannose-resistant fimbriae (MRF) and *P.mirabilis* fimbriae (MF) (Saint and Chenoweth, 2003).

There are many mechanisms by which *P. mirabilis* can evade the host defenses: The first is production of protease which functions to cleave the secretory IgA that released by the host in primary response to an infection (AL-Jumaa, 2011).

The second mechanism is evasion immune system through three unique flagellin genes, which have ability to make fusion and form novel flagella capable of tricking the host's defenses (Jansen *et al*., 2003).

The third is through expression of the Mannose-resistant fimbriae that found in some cells but not in others of the same population, and the fourth mechanism is the stone formation depending on urease activity that results in stone formation, and these stones in turn, protect the bacteria from host immune cells and antimicrobial agents, the formation of stones around the organisms prevents antibiotic treatment effect (AL-Jumaa, 2011).

Once *Proteus mirabilis* attach to the target site, a cascade of events is initiated in the host cell, including interleukin IL-6 and IL-8 secretion in addition to apoptosis and epithelial cell desquamation. *Proteus mirabilis* also produce urease, which has been shown to be associated with an increased risk of pyelonephritis and upper UTIs. *Proteus mirabilis* also hydrolyze urea to ammonia, thereby alkalinizing the urine. Through the production of urease and ammonia, *Proteus* can produce an environment where it can survive. Additionally, alkaline urine will decrease the solubility of both organic and inorganic compounds, encouraging precipitation and struvite (e.g., magnesium ammonium phosphate and calcium carbonate-apatite) stone formation (Jamil *et al.,* 2022).

Like other Gram-negative bacteria, *Proteus mirabilis* release endotoxin (part of the Gram-negative bacterial cell wall) when invading the bloodstream; thereby triggering additional host inflammatory responses which can ultimately result in sepsis or systemic inflammatory response syndrome (SIRS), a severe condition with a 20% to 50% associated incidence of mortality.

1.2.3 Virulence Factors of *Proteus mirabilis*

Proteus mirabilis have many virulence factors helping in their adhesion, growth, colonization and invasion into infected tissues and thus progressing of the pathogenesis. These virulence factors include flagella, capsule, fimbriae, outer membrane proteins, lipopolysaccharides (LPS), biofilm formation, and several enzymes such as, haemolysin, metalloprotease, amino acid deaminase, urease (Struble *et al*., 2009; Saleh *et al.*, 2019). *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. A number of studies 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 (Abbas *et al.*,2015).

Three potential toxins have been characterized for their important role in virulence. These are hemolysin, Proteus toxic agglutinin *pta*, and *zapA* metalloprotease. Hemolysin could play a role in the spread of infection into the kidneys and the initiation of acute pyelonephritis. Pta is an auto-transporter that performs serine protease activity on the surface of bacteria (Adams-Sapper, *et al.*, 2012;Sader *et al.*, 2014).

The *pta* protein contributes to the colonization of the bladder and kidney. In vitro and in vivo UTI studies have demonstrated the additive effects of hpmA and pta, particularly with respect to cystitis and possibly interstitial nephritis (Alamuri *et al.*, 2009).

Other significant virulence factors include toxins *HpmAB*, iron and zinc uptake systems, proteases and flagella. Urease which hydrolyses urea to ammonia and carbon dioxide. This activity is a substantial source of nitrogen for bacteria and also contributes to the formation of crystalline biofilm that blocks the lumen which is considered as one of the most important virulence factors of *P.mirabilis* (Armbruster and Mobley, 2012; Czerwonka *et al.*, 2016).

Swarming motility has been associated with virulence of various important human pathogens such as *P.mirabilis*. Many of these pathogens experience major shifts in the expression levels of virulence factors and other pathogenicity related traits correlating with formation of swarm cells. For example, swarming *P. mirabilis* displays increased virulence by hemolysin, ureolytic and proteolytic activities, and invasion behavior in comparison with nonmotile cells. The swarming phenotype also contributed to pathogenicity of *P.mirabilis* in infection models (Rütschlin and Böttcher, 2020).

In general the presence of flagella on the surface of pathogenic and opportunistic bacteria facilitate the colonization and dissemination from the initial site, the association of motility with the virulence of flagellated *P.mirabilis* bacilli has been demonstrated. The ability of *P.mirabilis* to express virulence factors, including urease, protease, and hemolysin, and to invade human urothelial cells is coordinately regulated with swarming differentiation (Liaw *et al*., 2005).

1.2.3.1 Urease Regulatory Gene (*ureR* gene)

The urease enzymes is produced by all strains of *Proteus* species and it is also considered as diagnostic and differential feature which characterizes the members of this genus from the rest of the intestinal family members (Ali and Yousif, 2015). Urease is an important virulence factor in the pathogenicity of *Proteus* and urease production is a prominent characteristic of the genus *Proteus* (Kamil and Jarjes, 2019).

Urea is considered as the main nitrogenous excretory product in humans and many of animals, urease (urea amidohydrolase) catalyzes the hydrolysis of urea to ammonia and carbon dioxide, which lead to an increase in the urine pH and non-physiological alkalization of urine (Stickler *et al*., 2006). While pH of urine is naturally neutral or slightly acidic, also enables the bacterium dependent on urea as a source of nitrogen resulting in an increase of nutrients for the bacteria, which is required for DNA and protein synthesis (Alamuri *et al*., 2009). The gene clusters encoding this enzyme have been cloned from numerous bacterial species including *Proteus mirabilis*. The urease gene cluster includes three structural genes, *ureA, ureB,* and *Urease C* (*ureC*), besides four accessory genes, *ureD, ureE, ureF,* and *ureG,* and a positive transcriptional activator, *ureR* (Shoket *et al.,* 2014).

Urease operon expression is positively activated by UreR. *UreR*, a dimer of identical 293-amino-acid polypeptides, binds urea, causing the protein to bind avidly to both the *UreR* and *UreD* promoters. RNA polymerase is recruited by *UreR*, thus activating transcription (Dattelbaum *et al*., 2003).

1.2.3.2 Hemolysin *Proteus mirabilis* Type Alpha (*hpmA* gene)

The hemolysin is typically synthesized as precursor proteins, it is covalently changed to yield an active hemolysin, *P.mirabilis* with a range of hemolytic activity and there was no difference in the colonization and renal tissue damage in a mouse model of ascending urinary tract infection (Nielubowicz, 2010).

The hemolysin enzyme acts on destroying the leukocyte membrane by making small holes in the membrane of the leukocyte and epithelial cell and its presence is a very important factor in providing the bacteria with iron; and because of its cytotoxic, it leads to the destruction of the kidney tissue of the host (Forbe *et al*., 2007). The most important cytotoxins in this uropathogen are *hpmA* and *hlyA* hemolysins and *Pta* cytotoxic agglutinin that have been implicated in the pathogenicity of the host (Mirzaei *et al*.,2019).

The hemolytic activity produced by *P. mirabilis* is associated to hemolysin *hpmA*. This hemolysin is associated to the cell, calcium-independent, former of pores, codified by two genes, *hpmA* and hpmB, that codify the *hpmA* (166 kDa) and *hpmB* (63 kDa) proteins, respectively. *hpmA* hemolysin is responsible for tissue damage and is activated when its N-terminal peptide is cleaved, resulting in active *hpmA* (140 kDa), and *hpmB* is responsible for *hpmA* activation and transport. Evidence suggests a correlation between the hemolytic activity and the invasive and cytotoxic capacity in the *Vero* cells by *P.mirabilis*, increasing the virulence of the infections (Cestari *et al*., 2013).

The increase in hemolysin *hpmA* production is coordinately regulated during the cell differentiation for the swarmer form and during infection, and is correlated with the invasiveness of *P. mirabilis* strains. Hemolysin *hpmA* produced by *P.mirabilis* is considered a very important virulence factor. Although it is not considered essential during the initial infection stages, it is important in the final stages when the bacteria are already colonizing the kidneys (Cestari *et al*., 2013).

Detecting and characterizing *P.mirabilis* hemolysin *hpmA* has required to elucidate its importance as a virulence factor, in addition to its probable relationship with other factors produced by *P. mirabilis* that together contribute to cytotoxicity in the UTIs of humans (Lazm *et al*., 2018). This toxin facilitate Proteus in the kidney and also cause pyelonephritis in urinary tract (Zafar *et al*., 2019).

P.mirabilis strains synthesizing cell associated hemolysin had a lower 50% lethal dose (LD50) than non hemolytic strains when injected into mice, it was also shown that hemolytic activity is correlated with cell invasiveness of *Proteus* strains, *hmpA* was also cytotoxic against a variety of target cell lines: human B-cell lymphocyte, human monocyte, Vero and cultured human renal tubular epithelial cells. *HpmA* production is considered an important virulence factor of uropathogenic *P.mirabilis*. Thus, it is important to detect the genes and verify hemolysin expression in a collection of uropathogenic P. mirabilis (Cestari *et al*., 2013).

hpmA mediates lysis of a broad range of cell types from numerous host species and appears to be the primary *P.mirabilis* virulence factor responsible for cytotoxicity to human renal proximal tubular epithelial cells (HRPTECs). Deletion of *hpmA* dramatically decreases cytotoxicity, and allows for internalization of *P.mirabilis* by HRPTECs in *vitro*. Because HRPTECs form a protective barrier for the kidney parenchyma, it was hypothesized that hemolysin may be a critical virulence factor that mediates spread of P.mirabilis into the kidneys and development of pyelonephritis (Armbruster *et al*., 2018).

However, deletion of *hpmA* does not appear to impact tissue colonization or damage during independent challenge in the murine model of ascending UTI, indicating that either hemolysin has less of an impact during experimental infection than the in *vitro* cell culture studies suggest, or the activity of other virulence factors mask its contribution in *vivo*. In agreement with this finding, *hpmBA* was not identified as a fitness factor for UTI or CAUTI in any of the genome-wide transposon mutagenesis studies (Armbruster *et al*.,2017). likely because of a combination of complementation in *trans* by the other hemolysin-producing transposon mutants present during infection and possibly production of other cytolysins with similar functions (Armbruster *et al*., 2018).

1.2.3.3 Regulator of Swarming Behavior gene (*rsbA* gene)

RsbA gene (regulator of swarming behavior also called *rcsD*) plays an important role in initial and stabilization of swarming, and appearing disease bacteria in urinary tract infection, in other words, *rsbA* act as a positive or negative regulator in swarming (Naseri *et al.*,2018).

Generation of hyperswarming or (early) swarming mutants has been used to identify repressors that contribute to swarming. *RsbA* has twice been identified as a repressor of swarming. The *rsbA* gene encodes a phosphotransfer intermediate that is part of the RcsBCD phophorelay system. An *rsbA* mutant has a similar hyperswarming phenotype to that seen during flagellin overexpression. Interestingly, overexpression of *rsbA* also results in precocious swarming. Liaw and colleagues examined the ability of transposon mutants to swarm in the presence of the swarming inhibitor Para-Nitrophenylglycerol (PNPG) and identified a mutant with an insertion in *rsbA*. During swarming, this mutant expresses higher levels of flagellin, as well as other swarming co-regulated virulence factors including hemolysin, protease, and urease (Schaffer and Pearson, 2017).

In *P.mirabilis*, mutations in the Rcs system result in a hyperswarming phenotype. This was first shown with rcsC and rcsD (rsbA) mutants, but has more recently been shown for rcsB mutants. This hyperswarming phenotype is most likely due to an increased expression of flhDC, although direct binding of RcsB to the flhDC promoter has not been established in *P.mirabilis*(Clemmer & Rather, 2008).

An interesting phenotype of mutations in the rcs system is an elongation phenotype in liquid media, a condition normally nonpermissive for elongation. Mutations that cause overexpression of flhDC do not result in a similar phenotype, suggesting that the Rcs regulon may include additional genes involved with cellular elongation (Clemmer & Rather, 2008).

An rsbA-mediated pathway may involve sensing of saturated fatty acids to determine a tendency toward swarming or biofilm formation. That is, in the presence of specific fatty acids (myristic acid, lauric acid, palmitic acid), swarming behavior is inhibited while biofilm formation and extracellular polysaccharide production is enhanced. The *rsbA* mutant is unresponsive to these fatty acids (hyperswarms) and is deficient in biofilm formation under permissive conditions. To further investigate the contribution of the RcsBCD phosphorelay to swarming, Clemmer and Rather constructed an *rcsB* (response regulator) mutant; this strain also had a hyperswarming phenotype. Thus, the RcsBCD system is likely a repressor of swarming behavior (Schaffer and Pearson, 2017).

1.2.3.4 *Proteus* Toxic Agglutinin (*pta* gene)

pta is the first characterized autotransporter that remains membranebound and exhibits both activities cytotoxicity, but not autoagglutination, requires serine protease activity. Generally, secreted autotransporters have cytotoxic properties while membrane-bound autotransporters mediate aggregation or adhesion (Alamuri and Mobley, 2008). Interestingly, *hpmA* and *pta* have an additive effect on cytotoxicity both in vitro and during experimental UTI, particularly with respect to cystitis and possibly interstitial nephritis. *Pta* appears to be the more potent toxin during experimental infection, because the disruption of *pta* has a much greater impact on infectivity than loss of *hpmA* (Armbruster *et al*., 2018).

pta is the cytotoxin that has been implicated in damage of host. The functions associated with *pta* was an essential in the persistence of the microbes in the host. However, *P.mirabilis* caused the cytopathic effects in infected person have been a critical clinical importance *pta* is present in *P.*mirabili*s* that has agglutinin and cytotoxin properties when tested in vitro using human kidney and bladder epithelial cell lines, verifying that auto-transporter proteins could be cytotoxins. *pta* is more active at pH is alkaline, since the activity of the urease enzyme result in a local elevates the pH of urine during infection. Certainly, the transcription and protein levels of *pta* are elevated when *P.mirabilis* is cultured in alkaline conditions. This toxic protein is present in outermembrane that facilitates cell to cell aggregation and the α-domain of *pta* also has ability to lyse kidney and bladder cells. *pta* gene of *P.mirabilis* had decrease pathology as well as, important colonization in the bladder, kidneys and spleen (Alamuri and Mobley, 2008).

1.2.4. Multi-locus Sequence Analysis (MLSA)

Multi-locus Sequence Analysis is based on multi-locus sequence typing (MLST), which was first introduced by Maiden *et al.*, in 1998 as a microbial typing method for epidemiological and population genetic studies of pathogenic bacteria spp. MLST itself is based on the concept of multi-locus enzyme electrophoresis, a molecular typing method applied to populations and epidemiological studies of bacterial species (Glaeser and Kämpfer 2015).

MLST analyses in epidemiological studies are not intended to calculate phylogenetic relationships, but use clustering based on 'allelic profiles' to show the relationship and emergence of sequence types and sequence complexes. However, it has to be pointed out that the resultant trees do not rely on similarities between different alleles. Sequence information supporting the allelic profiles can be used for 'phylogenetic' calculations and, hence, can be used for bacterial identification and classification because it offers the opportunity for provision of a more in depth view of the phylogenetic relationships of prokaryotic species, which originally led to the definition of MLSA (Glaeser and Kämpfer 2015).

The analysis of multiple protein-encoding housekeeping genes has become a widely applied tool for the investigation of taxonomic relationships. The use of information from the comparison and combination of multiple genes can give a global and reliable overview of interorganismal relationships. The committee for re-evaluation of the species definition regarded the sequencing of a minimum of five well-chosen housekeeping genes, universally distributed, present as single copies and located at distinct

chromosomal loci, as a method of great promise for prokaryotic systematics (Gevers et al.,2005).

In comparison with 16S rRNA genes, the higher degree of sequence divergence of housekeeping genes is superior for identification purposes, since the more-conserved rRNA gene sequences do not always allow species discrimination, stated that a small number of carefully selected gene sequences could equal, or perhaps even surpass, the precision of DNA–DNA hybridization for quantification of genome relatedness. In contrast to DNA–DNA hybridization and 16S rRNA gene sequence analysis, multilocus sequence analysis (MLSA) is capable of yielding sequence clusters at a wide range of taxonomic levels, from intraspecific through the species level to clusters at higher levels (Martens *et al.*, 2008). MLSA has been suggested as the best alternative approach to the 16S rRNA gene based phylogeny (Sawabe *et al.*, 2013; Gao *et al.*, 2016).

In order to carry out an MLSA study, several housekeeping loci are sequenced and compared, and evolutionary relationships among the taxa are established. One important advantage of this methodology is the database availability of gene or genomic sequences, in contrast to DNA-DNA reassociation data, the taxonomic standard for circumscribing new species. However, the usefulness of MLSA for describing and circumscribing bacterial species needs to be validated on a case-by-case basis, demonstrating that there is a sufficient degree of congruence between MLSA and DNA–DNA reassociation data (Pascual *et al.*, 2010).

In 2003, Zeigler was already using protein-coding genes to predict results based on whole genome sequencing. Two years later, in 2005, Gevers *et al.,* published an opinion article con-cerning the re-evaluation of prokaryotic species that focused on the impact of multilocus nucleotide sequence-based approaches for prokaryotic taxonomy. They introduced the term MLSA as new designation for sequence analysis of multiple protein-coding genes for taxonomical application to species delineation within agenus. As a result, integration of MLSA into prokaryotic taxonomy should lead to a two-step identification procedure. First, 16S rRNA gene sequence analysis should be applied in order to assign a new strain to the family or even genus level. Second, based on the initial assignment, the isolate should be more exactly assigned within a genus using the MLSA approach. Genes should be used that are both single copies and ubiquitous, at least in the studied taxon, and genes that might be conferred by selective advantages, such as virulence genes, should be avoided. This is in clear contrast to MLST studies, where these genes are recommended to be used if a higher intra-species resolution power is required for epidemiological studies (Maiden, 2006). However, the authors claimed that a universal set of genes would allow a robust and hierarchical classification of all prokaryotic species. They considered that genes other than the16S rRNA gene might be more informative for resolving the phylogenetic relationships within genera or families. Finally, the authors stated that further studies were required in order to obtain more information so that MLSA could be applied sufficiently for species delineation. A genomic evaluation of MLSA by Konstantinidis and Tiedje, (2007).

1.2.5 Antibiotic Susceptibility

Multidrug resistance to *P.mirabilis* could be a result of continuous use of broad spectrum antimicrobials and non adherence to hospital antibiotic therapy, horizontal transfer of drug resistance genes among different bacteria as most patients already have been harboring the resistant organisms in their hospital staying period (Zaman and Shamsuzzaman, 2021).

The pattern of resistance of *P.mirabilis* to β- lactam drugs is complicated and is determined partly by mechanisms of intrinsic resistance and partly by the production of β- lactamases. In general *P.mirabilis* strains that don't form this enzyme are sensitive to amounts of benzylpenicillin that are attainable in urine; they are also very sensitive to ampicillin, carbenicillin, and cephalosporins. However, strains that form β-lactamases are resistant to benzylpenicillin, ampicillin and carbenicillin but often remain sensitive to cephalosporins (Rozalski and Staczek, 2010).

Extended-Spectrum β-lactamases (ESBLs), as one of the major resistance mechanisms among Gram-negative bacteria, have been associated with resistance to different classes of antibiotics such as quinolones, aminoglycosides and trimethoprim-sulfamethoxazole **(**Mirzaei *et al*., 2019)

Resistance of amoxicillin in *Proteus mirabilis* is mostly due to the TEM-1 and TEM-2 Penicillinases. *Proteus mirabilis* has been increased extended-spectrum β-lactamase (ESBL) production. The most predominant enzymes of *Proteus mirabilis* such as TEM, CTX-M,VEB and PER are less common (Miro *et al.,* 2005).

Chloramphenicol is a bacteriostatic antimicrobial. It is a most common broad-spectrum antibiotic, alongside the Tetracycline. it is effective against a variety of Gram-negative and Gram-positive bacteria, including most anaerobic organisms. It prevents the peptidyl bond formation between the amino acids of growing polypeptide chain (Al-Jumaily and Zgaer, 2016).

Trimethoprim and Sulfamethoxazole are combined together due to their synergism effect on bacteria. It's abroad spectrum bacterostatic antimicrobial agent for both Gram positive and Gram negative bacteria. Trimethoprim is a diaminopyrimidine, whereas sulfamethoxazole is a sulfonamide and the Co-trimoxazole inhibits the synthesis of tetrahydrofolic acid, which is necessary for the synthesis of bacterial nucleic acid along with two components of the drug inhibiting different steps in the folate synthesis pathway (Al-Jumaily and Zgaer, 2016).

Fluoroquinolones are used as one of the antibiotic classes for the treatment of UTIs. Unfortunately, extensive misuse of this class of antibiotics resulted in a significant increase in resistance to them in *P.mirabilis* isolates. Furthermore, resistance to other classes of antibiotics like aminoglycosides has been reported among clinical *P.mirabilis* strains. There are different mechanisms for resistance to quinolones and fluoroquinolones including chromosomal mutations in DNA gyrase, and DNA topoisomerase IV, as well as acquisition of plasmid mediated quinolone resistance (PMQR) genes encoding pentapeptide repeat proteins (qnr), aac(6′)-Ib-cr (modified acetyltransferase), and the qepA (efflux pump) **(**Mirzaei *et al*., 2019).

P.mirabilis wild isolates are generally sensitive to fluoroquinolones and even to the antimicrobials belongs to the beta-lactam families, however, the medical isolates of *Proteus* which are more virulent exhibited an increasing rate of resistance to the cephalosporins of broad spectrum and also to fluoroquinolones, which are the corner stone in treatment of UTIs due to their neglected side effect, easy administration and the poor possibility of resistance development (El-Kazzaz, 2021).

Resistance to Aminoglycosides is also mediated at the ribosomal level. In Enterobacteriaceae and nonfermenting Gram-negative bacteria, methylation of the 16S rRNA (the site where aminoglycosides bind and inhibit protein synthesis) by enzymes usually carried on plasmids is mediated by at least seven different genes (*armA, rmtA, rmtB, rmtC, rmtD, rmtE,* and *npmA*) (Zhou *et al.*, 2010).

Aminoglycoside antibiotics are particularly susceptible to modification as they tend to be large molecules with many exposed hydroxyl and amide groups. Aminoglycoside-modifying enzymes confer high levels of resistance to the antibiotic (or antibiotics) that they modify. There are three main classes of aminoglycoside-modifying enzymes: acetyltransferases, phosphotransferases and nucleotidyltransferases. These classes are evolutionarily diverse and vary in the aminoglycosides that they can modify and in the part of the molecule that is modified (Blair *et al.*, 2015).

Amikacin is an aminoglycoside antibiotic used to treat different types of bacterial infection. It is works by binding to the bacterial 30 s ribosomal subunit, causing misreading of mRNA and leaving the bacterium unable to synthesize proteins vital to its growth (Al-Jumaily and Zgaer, 2016).

Ciprofloxacin is a synthetic chemotherapeutic antibiotic of the fluroquinolone drug class. It is member of the broad spectrum antimicrobial agents, which inhibits bacterial DNA and protein synthesis and considered an important drug in thetreatment of urinary tract infection (Al-Jumaily and Zgaer, 2016).

Carbapenemases confer the largest antibiotic resistance spectrum because they can hydrolyze not only carbapenems but also broad-spectrum penicillins, oxymino-cephalosporins, and cephamycins (Opal and Pop-Vicas, 2015). The emergence and spread of carbapenem resistant *Enterobacteriaceae* have become an increasing concern for healthcare services worldwide. Infections caused by these bacteria including *P. mirabilis* cause significant morbidity and mortality (Zaman and Shamsuzzaman, 2021).

Broadly carbapenemases are categorized into three groups: Class A (penicillinases), Class B (metallo-β-lactamases [MBL]), and Class D (Oxacillinases). The New Delhi metallo-β-lactamase (NDM) is one of the class B metallo-β-lactamases. They are present largely in *Enterobacteriaceae*. Decreased susceptibility to imipenem, intrinsic resistance to polymyxins and tetracyclines along with the acquisition of β-lactam resistance traits by *P. mirabilis* seriously limits treatment options (Zaman and Shamsuzzaman, 2021).

Chapter two

Materials and Methods

2. Materials and methods

2.1 Materials

All devices; laboratory tools; chemical materials and reagents; culture media; molecular and diagnostic Kits; used in this study have been explained in the following **Tables: (2-1), (2-2), (2-3), (2-4) and (2-5),** respectively.

2.1.1 Laboratory devices

The main devices and instruments used throughout this study are listed in **Table (2-1).**

Table (2-1) Laboratory devices

No	Instrument	Company	Country
1.	Autoclave	Stermite Olympus A&B	Japan
2.	Centrifuge	Hettich	Germany
3.	Cooling box	Ningbo	China
4.	Digital camera (phone camera)	Samsung	Korea
5.	Distillator	GFL	Germany
6.	Gel Electrophoresis	Cleaver	USA
7.	Incubator, Oven ,Water bath	Memmert	Germany
8.	Light Microscope	Novel	China
9.	Nanodrop	Thermo Scientific™	USA
10.	PCR thermocycler	Prime	UK
11.	Vitek 2 system apparatus	Biomerieux	France

12.	Refrigerator	Concord	Lebanon
13.	UV-transilluminator	Bioneer	Korea
14.	Vortex, Sensitive scale	Ohaus	USA

2.1.2 Laboratory instruments and tools

The main tools and instruments used throughout this study are listed in **Table (2-2).**

Table (2-2) Laboratory tools

No	Instrument	Company	Country
1.	1000ul,200ul,10ul Aerosol Barrier+ Tips	Promega	USA
2.	Conical centrifuge tubes 30ml	Alfa	Jordan
3.	Conical flasks , Cylinders	Jlassco	India
6.	Glass slide, Cover slide	Leitz	Germany
7.	Disposable Petri dishes	Grenier	Germany
8.	Micropipettes-2-20µl,5-50µl,100-1000µl	Eppendorf	Germany
9.	Flipper rack 64 well	Promega	USA
10.	Loop	Shndon	England
11.	Medical cotton	Hygiene Limited	Jordan
12.	Medical gloves	Broche	China
13.	Eppendorf 1.5 ml , 2.5 ml	Biobasic	Canada
14.	Screw capped bottles 30 ml	DMD-DISPO	Syria

2.1.3 Chemical and Biological Materials

The main chemical materials that used in this study are listed in **Table (2-3)**.

Table (2-3) Chemical Materials

No.	Chemicals	Manufacturer (origin)
1.	Absolute ethyl alcohol	Fluka / Germany
3.	DNA ladder marker	Promega / USA
4.	Loading dye (orange blue), Agarose, Master mix	Promega / USA
5.	Glycerol	Fluka / England
6.	Nuclease free water (1.25) ml	Promega / USA
7.	Tris EDTA (TE)	Bio Basic / Canada
8.	Tris-Borate-EDTA (TBE10X) buffer	Bio Basic / Canada
9.	Nucleic acid staining solution (Green Star^{TM}).	Bioneer / Korea

2.1.4 Culture Media

The media have been used for culture in this study are listed in **Table (2-4).**

Table (2-4) Culture media and their manufacturer

No.	Culture Media	Manufacture (Origin)
1.	Brain Heart Infusion Broth	Himedia / India
2.	Nutrient Broth, Mueller-Hinton Agar	Himedia / India
3.	Blood Agar, MacConkey Agar	Himedia / India
4.	Urea Agar	Quelab / UK

2.1.5 Commercial Kits

The commercial kits have been used in the present study are listed in the **Table (2-5).**

Table (2-5) Commercial kits used in the present study

No.	Type of Kit	Manufacture (Origin)
1.	DNA extraction kit	Geneaid / UK
2.	DNA ladder 1500, 1000 bp, Green master mix	Promega / USA
3.	primers	Microgen / Korea
4.	Gel/PCR DNA Fragments Extraction Kit	Geneaid / UK

Table (2-6) DNA extraction kit components (Geneaid / UK)

No.	Component	Size
1.	Gram + Buffer	30 ml
2.	GT Buffer	30 ml
3.	GT Buffer	40 ml
4.	W1 Buffer	45 ml
5.	Wash Buffer (add Ethanol)	25 ml (100 ml)
6.	Lysozyme	110 mg
7.	Proteinase K (add ddH_2O)	11 MG *2 (1.1 ml)
8.	Elution Buffer	30 ml
9.	CD Columns	100
10.	2 ml Collection Tubes	200

Table (2-7) Master Mix Used in PCR (Promega / USA)

No.	Materials
1.	DNA polymerase enzyme (Taq)
2.	dNTPs (400 µm dATP, 400 µm dGTP, 400 µm dCTP, 400 µm dTTP)
3.	$MgCl_2$ (3mM)
4.	reaction buffer (pH 8.3)

Table (2-8) DNA ladder

No.	Materials
1.	Ladder consist of 11 double-stranded DNA with size 100-1500 bp.
2.	Loading dye has a composition (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA).

Table (2-9) Antibiotic discs and their country of origin according to (CLSI) 2021

No.	Antibiotic	concentration	shortcut	Manufacture(Origin)
Penicillins/ β-lactamase-inhibitor combinations				
1.	Ampicillin-sulbactam	20μg	AMS	Liofilchem / Italy
Macrolides				
2.	Aztreonam	10 μg	ATM	Condalab / Spain
Quinolones				
3.	Ciprofloxacin	5 μg	CPX	Mast / UK
4.	Levofloxacin	5 μg	LVX	Liofilchem / Italy
Folate pathway antagonistic/Co-trimoxazole				
5.	Sulphamethoxazole-trimethoprim	5 μg	SXT	Condalab / Spain
Third generation Cephalosporin				
6.	Cefotaxime	30 μg	CTX	Liofilchem / Italy
Phenicol				
7.	Chloramphenicol	30 μg	C	Liofilchem / Italy
Tetracycline				
8.	Tetracycline	30 μg	TET	Liofilchem / Italy
Carbapenemase				
9.	Meropenem	10 μg	MEM	Condalab / Spain
10.	Imipenem	10 μg	IMP	
Aminoglycoside				
11.	Gentamicin	10 μg	GEN	Condalab / Spain
12.	Amikacin	10 μg	AMK	
Aminopenicillins				
13.	Amoxicillin	15 μg	AMP	Condalab / Spain

2.2. Methods

2.2.1. Patients and Samples Collection

One hundred thirty samples were collected from patients with UTI, vagina, wound and burn attending to Hilla teaching Hospital in the period from Septembere to December (2021). The proper specimens' collections for bacteriological analysis are described below. Those specimens are collected under the help of advisory members to avoid any possible contamination. Each specimen is immediately inoculated onto the blood agar plates, nutrient agar and MacConkey's plates. All plates are incubated at 37ºC for 24 hrs.

2.2.1.1 Ethical approval

The necessary ethical approval from ethical committee of the Hospitals, patients and their followers must obtained. Moreover, all subjects involved in this work are informed and the agreement required for doing the experiments and publication of this work is obtained from each one prior the collection of samples.

2.2.1.2 Exclusion criteria

More than fourteen cases are excluded from the study due to the absence of study criteria when patient use antibiotics after taken the history of patients.

2.2.1.3 Isolation and identification of *proteus mirabilis*

2.2.1.3.1 Urine samples

The specimen were generally collected from patients suffering from UTIs. Mid-stream urine samples were collected in sterilized screw-cap containers, then the urine samples were inoculated on culture media and incubated aerobically at 37°C for 24h (Vandepitte *et al*, 1991).

2.2.1.3.2 Vagina swabs

The samples were generally collected from women. The swabs were inserted into the posterior fornix, upper part of the vagina and rotated there before with drawing them. A vaginal speculum was also used to provide a clear sight of the cervix and the swabs were rubbed in and around the introits of the cervix and withdrawn without contamination of the vaginal wall. Swab for culture should be placed in tubes containing normal saline to maintain the swab moist until taken to laboratory. The swab was inoculated on culture media and incubated aerobically at 37°C for 24h.

2.2.1.3.3. Wound and Burn swab

The samples were generally collected by twice rolating a sterile cotton swab for culture should be placed in tubes containing normal saline to maintain the swab moist until taken to laboratory. The swab was inoculated on culture media and incubated aerobically at 37°C for 24h. All samples were transferred by means of a cooled box to the Faculty of Medicine Laboratory/Babylon University for the purpose of identifying the bacteria and performing laboratory analyzes.

2.2.1.3. Study Design

A Cross-sectional study was designed that include 130 samples, collected from patients with urinary tract infection and vagina, wound, burn infections. Identification of bacterial isolates by chemical methods, VITEK 2 system and identification by PCR assay using specific primers. Detection of four virulence genes, molecular analyses of *p.mirabilis* by Multi-Locus Sequence Analyses MLSA following five housekeeping genes and study of antibiotics susceptibility. As explained in **figure (2-1)**.

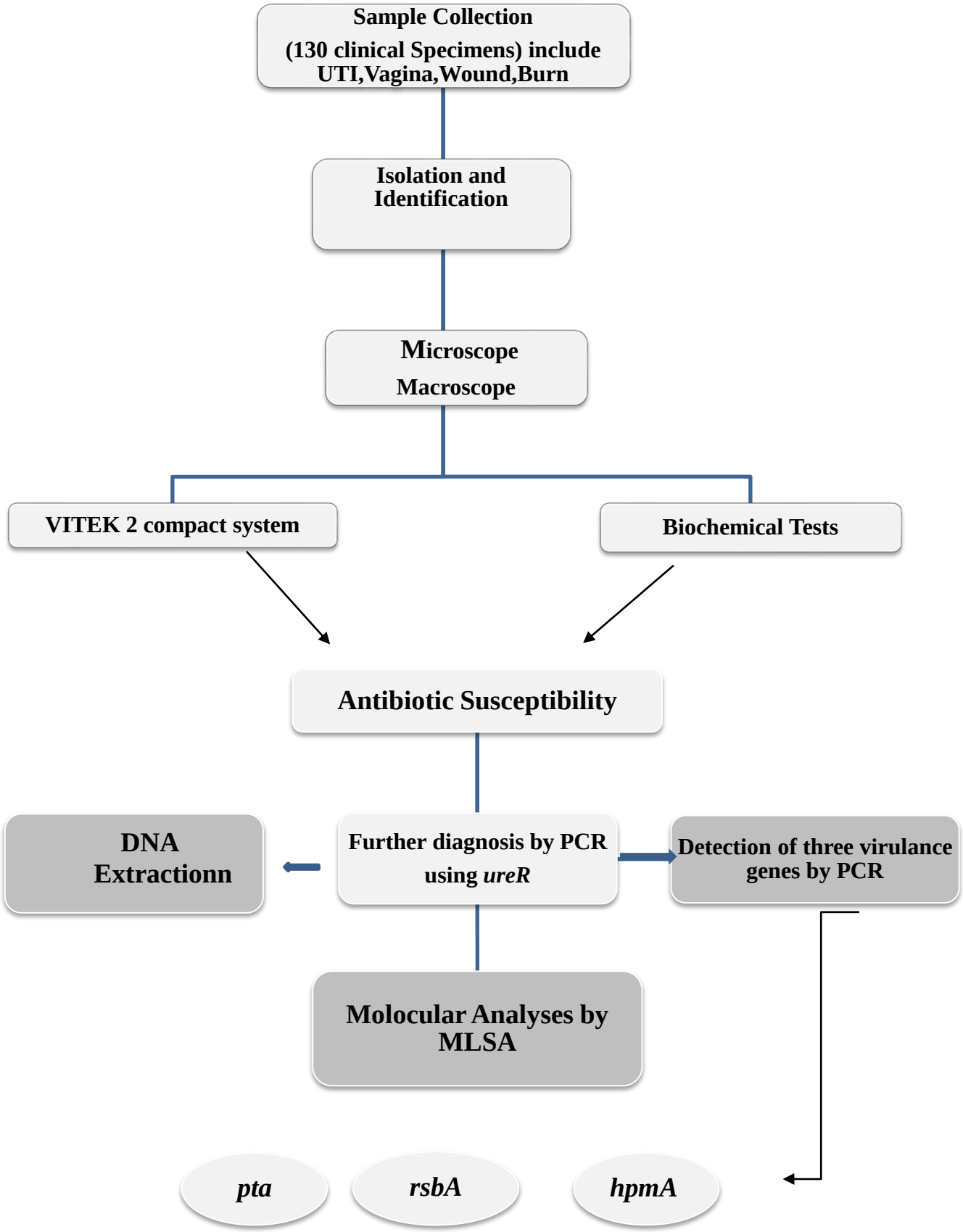

Figure (2-1) Experimental design

2.2.2. Preparation of cultural media

Preparation of culture media occur according to the instructions of the manufacturer company and sterilized by autoclave at (121˚C) for (15) minutes in (1) bar.

No	Media	Purpose of use
1.	**MacConkey agar**	MacConkey agar used for differentiate between lactose fermenters bacteria from non-fermenters and for primary isolation of most Gram-negative bacteria (MacFaddin, 2000).
2.	**Blood agar**	Blood agar preparation by dissolving (40gm) from blood agar base in (1000ml) distilled water. Then sterilized by autoclave at (121˚C) for (15) minutes, and then cooled to (50˚C). add (5%) of fresh human blood or supplemented with (5%) fresh rabbit blood. This medium used to cultivate bacterial isolation and to determine the ability of bacteria to hemolysis blood cell (Russell *et al*., 2006).
3.	**Urea agar medium**	This medium was prepared according to instructions of company by Adding (15ml) of urea solution (sterilized by filtration) to (100ml) urea agar base sterilized by autoclave at (121˚C) for (15) minutes, and cooled at (50˚C), (PH=7.1) then distribute the medium into sterilized

		test tubes and allow to solidify in slant form. This medium was used to detect bacterial ability to produce urease enzyme (MacFaddin, 2000).
4.	**Brain heart infusion broth**	It has been prepared by dissolving (37gm) of medium in (100ml) distilled water. The pH was adjusted at (7.2), and then sterilized by autoclave at (121°C) for (15) minutes (Broth *et al*., 2006).
7.	**Muller-Hinton Agar Medium**	Muller-Hinton agar was prepared according to the manufacturing company (38mg/IL). It was used in anti-bacterial susceptibility testing (MacFaddin, 2000).
9.	**Peptone water medium (pH/8)**	This medium was prepared by dissolving (8gm) peptone powder in (1000ml) of distilled water, then distributed into test tubes, and autoclaved. This medium was used to detect the bacterial ability to decompose the amino acid tryptophan to indole (MacFaddin, 2000).

2.2.3. Solutions

2.2.3.1 Normal Saline Solution

Dissolving (8.5gm) of NaCl in a small volume of distilled water, then completed to 1000ml,fixed pH at (7.2) and sterilized in autoclave at (121˚C) FOR (15) minutes, then kept at (4˚C) (MacFaddin, 2000).

2.2.3.2 Phosphate Buffer Solution

The solution was prepared according to a company (Himedia/ India) by dissolving one tablet in 100 ml of D.W.

2.2.3.3 Urea Solution

Urea solution has been prepared by adding 40 gm. of urea crystal to 100 ml of D.W and then sterilized by filtration with Millipore filter paper (pore size 0.45µm). the suspension was poured in sterilized container and stored at 4◦C until it using to preparation of urea agar media (Joanne, *et al.*, 2008).

2.2.3.4 Sucrose Solution

The solution was prepared by dissolving (1gm) sucrose in (100ml) D.W, and sterilization by filteration (Bubník and Kadlec, 1995).

2.2.3.5 Agarose gel preparation

The agarose gel was prepared according to the method of (Lee et al., 2012) by adding (1gm) agarose to (100ml) of (1x) TBE buffer. Heating the solution to boiling by water bath until dissolving all gel particles. Cooling the solution within (50-60˚C) then mixing with (0.5µg/ml) Green star™ Nucleic Acid Staining Solution.

2.2.3.6 McFarland Tube Standard (0.5)

According to (CLSI, 2010), describe barium sulfate turbidity standard solution equivalent to a (0.5) McFarland standard was prepared as follows:

- A (0.5ml) aliquot of (0.048M) $BaCl_2$ (1.175%w\v) $BaCl_2.2H_2O$) was added to (99.5ml) of (0.18M) H_2SO_4 (1% v\v) with constant stirring to maintain a suspension.
- Correct density of the turbidity standard was verified by using reading the absorbance at (625nm). The absorbance should be (0.08) to (0.1) for the McFarland standard.
- A (4ml) aliquots suspension of barium sulfate distributed into screw cap tubes, which were tightly sealed and stored in dark at room temperature.
- Before each use of barium sulfate turbidity standard, vigorously agitated on mechanical vortex mixer and inspected for uniformly turbid appearance.
- Barium sulfate standard should be replaced or their densities verified monthly.

2.2.4. Laboratory Diagnosis

2.2.4.1. Colonial Microscopic and Macroscopic Examination

A single colony from each primary positive culture on blood, MacConkey and nutrient agar and identify it depending on its morphological properties (colony shape, size, color, borders, and texture) and exam it by light microscope after being stained with Gram's stain. After examination it, biochemical tests were done on each isolates to complete the finale identification (MacFaddin, 2000).

2.2.4.2. Biochemical Tests

2.2.4.2.1. Indole Test

This test uses for determination the ability of organism to produce indole from deamination of tryptophan by tryptophanase. Tube containing a pepton water medium were inoculated with the colony of the tested bacteria and incubated at (37°C) for (18) hours, and then several drops of Kovac's reagent were added to the broth medium. After shaking, the appearance of red color ring formation at the top of broth indicates a positive reaction or result, while no ring or yellow color ring indicates a negative reaction (MacFaddin *etal*., 2000).

2.2.4.2.2.Urease Test

Urease is an enzyme that breaks the carbon-nitrogen bond of amides to form carbon dioxide, ammonia and water. The preparation of media by sterilize urea base agar by autoclave, then cooled it to (50˚C) and add urea supplement to it, then poured it in sterile tubes, for test inoculated it by bacterial culture ,then incubate for (24-48) at hours (37˚C). When reading the result of test the increased of medium pH results from urea break down and ammonia releasing. this pH change is detect by pH indicator that turned pink in basic environment (MacFaddin *et al.,* 2000).

2.2.4.2.3. Hemolysin Test

Hemolysin production was carried out by inoculating bacterial isolate on blood agar medium at (37˚C) for (24-48) hours, An appearance of clear zone around the colonies referred to complete hemolysis (β-hemolysis), greenish zone around the colonies referred to partial hemolysis (α- hemolysis) , while no zone referred to non-hemolysis (γ- hemolysis) (Baron *et al*,. 1994).

2.2.5 Antibiotic Susceptibility Testing Kirby-Bauer

2.2.5.1 Disk Diffusion Test

It was performed by using a pure culture of previously identified bacterial isolates. The most effective antibiotic for each bacterial isolate was determined as recommended by CLSI (2021).

A. The inoculums to be used in this test was prepared by adding 5 isolated colonies grown on MacConkey agar plate to 5 ml of nutrient broth and incubated at 35^{o}C for 18 hours and compared with (0.5) McFarland standard tube for 10 min.

B. A sterile swab was used to obtain an inoculum from the bacterial suspension, this inoculum was streaked on an MHA plate and left to dry.

C. The antibiotic discs were placed on the surface of the medium at evenly spaced intervals with flamed forceps or a disc applicator and incubated for 18 hours.

D. Inhibition zones were measured using a ruler and compared with the zones of inhibition determined by CLSI (2021).

2.2.6. Molecular Study

2.2.6.1 Extraction of Genomic DNA from Bacterial Culture

Genomic DNA was extracted from *P.mirabilis* isolates by using Geneaid Genomic DNA Purification Kit (UK) and done according to company instructions; the bacterial culture has been inoculated in 10 ml brain heart infusion broth medium and incubated at 37ºC overnight in shacking incubator.

1. Bacterial cells (up to 1 x 10^9) were transferred to a 1.5 ml microcentrifuge tube and Centrifuged for 1 min at 14-16,000 x g then discarded the supernatant.

2. One hundred eighty µl of GT Buffer was added then re-suspended the cell pellet by vortex or pipette and 20µl of Proteinase K was added (make sure ddH_2O was added). Then incubated at 60ºC for at least 10min. during the incubation, the tube was inverted every 3min.

3. Two hundred µl of GB Buffer was added then mixed by vortex for 10 seconds and incubated at 70ºC for at least 10 min. to ensure the sample lysate is clear. During incubation, the tube was inverted every 3min. At this time, pre-heat the required Elution Buffer (200 µl per sample) to 70ºC for using in step 7 (DNA Elution).

4.Two hundred µl of absolute ethanol was added and mixed immediately by shaking vigorously. If precipitate is appearing, break it up as much as possible with a pipette then GD Column was placed in a 2 ml Collection Tube and mixture (including any insoluble precipitate) was transferred to the GD Column and centrifuge at 14-16,000 x g for 2 min. The 2 ml Collection Tube containing the flow-through was discarded and then the GD Column was placed in a new 2 ml Collection tube.

5. Four hundred µl of W1 Buffer was added to the GD Column and Centrifuge at 14-16,000 x g for 30 seconds then the flow-through was discarded. The GD Column was placed back in the 2 ml Collection tube.

6. Six hundred µl of Wash Buffer (with ethanol) was added to the GD Column then centrifuge at 14-16,000 x g for 30 seconds, the flow-through was discarded and the GD Column was placed back in the 2ml Collection tube and centrifuged again for 3min at 14-16,000 x g to dry the column matrix.

7.The dried GD Column was trransferred to a clean 1.5 ml microcentrifuge tube and 100µl of pre-heated Elution Buffer was added into the center of the column matrix then leave it on for 3min to allow Elution Buffer to be completely absorbed and centrifuged at 14-16,000 x g for 30 seconds to elute the purified DNA.

2.2.6.2. Estimation of DNA Concentration

The extracted genomic DNA was checked by using Nanodrop which measures DNA concentration (ng/µl).

2.2.6.3. PCR Amplification

Identification of *P.mirabilis* was confirmed by PCR to determine the specific serogroups according to (Levy *et al.*, 2008) method with minor modifications.

2.2.6.4. The mixture of PCR reaction

Amplification of DNA was carried out in final volume of 25 µl as explained in **Table (2-10).**

Table (2-10) Contents of the Reaction Mixture

No.	Contents of reaction mixture	Volume
1.	Go Taq Green master mix	12.5 µl
2.	Upstream primer	1.5 µl
3.	Downstream primer	1.5 µl
4.	DNA template	3 µl
5.	Nuclease free water	6.5 µl
	Total volume	25 µl

2.2. 6.5 Amplified Products by Agarose Gel Electrophoresis

The PCR amplification of products were analyzed by Agarose gel electrophoresis using 1% agarose gel prepared by dissolving 1g of agarose mixed with 100ml of 10x Tris Borate EDTA (TBE) buffer (10ml TBE + 90ml sterile distilled water) heated to boil on hot plate. The agarose gel was cooled down to 45°C where 250µl of Green star^{TM} Nucleic Acid Staining Solution (1/200 fold) was added (Jegasothy, *et al.*, 2000). The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was powered gently into the tray, and allowed to solidify at room temperature for 30min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber which was filled with TBE buffer covering the surface of the gel, 5µl of DNA sample was transferred into the signed wells in agarose gel, and in one well we put the 5µl DNA ladder mixed with 1µl of loading buffer. The electric current was allowed at 70volts for 30min. UV transilluminator was used for the observation of DNA bands, and gel was photographed using a digital camera.

2.2.6.6. Primer Sequences

Molecular assay in this study includes 9 genes; one for diagnosis, three for virulence, and five for MLSA. The oligonucleotide primers for all genes used in this study were obtained from previous studies and pubmlst.org, each one has specific nucleotide sequences and product size. The primer sequences and PCR conditions that used are listed in Tables (2-11), (2-12), (2-13) and (2-14).

Table (2-11) Primer used in PCR assays for the detection of *P.mirabilis,* and their expected amplicons

Genes	Primer sequence (5′-3′)	Size bp	Reference
***ureR* F**	GT GAG ATT TGT ATT AAT GG	225	(Zhang *et al*., 2013)
***ureR* R**	ATA ATC TGG AAG ATG ACG AG		

Table (2-12) Virulence genes primers sequences with their amplicon size base pair (bp)

Genes	Primer sequence (5'-3')	Size bp	Reference
***rsbA* F**	TTGAAGGACGCGATCAGACC	467	(Badi *et al.*,2014)
***rsbA* R**	ACTCTGCTGTCCTGTGGGTA		
***hpmA* F**	GTTGAGGGGCGTTATCAAGAGTC	709	(Cestari *et al*., 2013)
***hpmA* R**	GATAACTGTTTTGCCCTTTTGTGC		
***Pta* F**	AAAAGGCCAGGTGTTTGATG	181	(Design this study)
***Pta* R**	CGGGCCATAGTTGTTGCTAT		

2.2.6.7. Dissolving and Preparation of Primers

All primer pairs used in this study were dissolved using TE Buffer, 1X (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA. Firstly, the primer stock tube prepared and then the working solution would prepare from primer stock tube. According to the instruction provided by primer manufacturer (Bioneer/Korea) the TE buffer were added to get 100 Pico mole/microliter concentration of primer stock solution. The working solution prepared from stock by dilution with TE buffer to get 10 Pico mole/microliter and kept in 20 ºC.

Table (2-13): The PCR amplification conditions performed with a thermal cycler were specific to each single primer set depending on their reference procedure, as follows

Gene	Initial denaturation	Denaturation	Annealing	extension	Final extension	Cycle
ureR	94°C / 4 m	94°C / 40 s	58^{o} C / 60s	72°C / 20s	72°C/10 m	30
rsbA	94°C / 5 m	94°C / 60 s	58^{o} C / 45s	72°C / 60s	72°C/7 m	30
hpmA	95°C / 5 m	95°C / 30 s	62^{o} C / 30s	72°C / 20s	72°C/5 m	30
Pta	95°C / 5 m	95°C / 30 s	57^{o} C / 30s	72°C / 20s	72°C/5 m	30
dnaJ	95°C / 5 m	95°C / 30 s	55^{o} C / 45s	72°C / 60s	72°C/8 m	30
mdh	95°C / 5 m	95°C / 30 s	55^{o} C / 45s	72°C / 60s	72°C/8 m	30
pyrC	95°C / 5 m	95°C / 30 s	52^{o} C / 30s	72°C / 60s	72°C/8 m	30
recA	95°C / 5 m	95°C / 30 s	52^{o} C / 30s	72°C / 60s	72°C/8 m	30
rpoD	95°C / 5 m	95°C / 30 s	52^{o} C / 30s	72°C / 60s	72°C/8 m	30

2.2.7. Multi-Locus Sequence Analysis (MLSA)

P.mirabilis MLSA scheme uses internal fragments of the following five house-keeping genes:

- *mdh* (Malate dehydrogenase)
- *pyrC* (Pyrimidine biosynthetic enzyme)
- *recA* (recombinase A)
- *rpoD* (RNA polymerase sigma factor)

were used for MLSA. Primer and PCR conditions were available on MLSA database https://www.researchsquare.com/static/img/article/icon-cite.svg with minor modifications regarding selection of PCR and sequencing primers, ashown in Table (2-14).

Table (2-14) Primers sequences used for *P.mirabilis* MLSA and their amplicon size base pair (bp)

Genes	Primer sequence (5'-3')	Size bp	Reference
dnaJ F	ATGAAATATCACCCAGAYCG	790	(Dai *et al.*, 2020)
dnaJ R	ACACGRCCATCMAGWGTT		
mdh F	GCAAAGAAACGGGCATRTT	769	(Dai *et al.*, 2020)
mdh R	CRGGTGGTATTGGTCAGG		
pyrC F	TGATTGGCATGTTCACTT	745	(Dai *et al.*, 2020)
pyrC R	GATTCTTTGCGATGTTGT		
recA F	CTRTACCAWGCACCMGCTT	807	(Dai *et al.*, 2020)
recA R	AGGKTCTATCATGCGTCT		
rpoD F	CGGGAAGGTGAAATTGAT	775	(Dai *et al.*, 2020)
rpoD R	CGATAGACATACGACGGT		

2.2.7.1 PCR amplification

PCR reactions were carried out according to Stepan *et al.*, (2011) by using the following cycling conditions 94°C for 30s; followed by 30 cycles of 95°C for 30s; 55°C 30s and 76°C for 30s, with a final extension of 75°C for 2 min followed by hold at 4°C. A 5 µl of the PCR products were loaded into 2% agarose gels in 1 X TAE with loading dye, and run at 100V in 1X TAE for 60min. Images of the gels were captured using a gel documentation system.

2.2.7.2. MLSA Analysis

All raw data were analyzed in University of Babylon colliege of medicine at Microbiology department using several bioinformatic software and tools. Phylogenetic trees were constructed by MLSA of the concatenated sequence of five HKGs fragments (*dnaJ-mdh-pyrC-recA-rpoD*) and the five individual HKGs. Comparison analyses of the sequences against the reference genome were conducted with BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). Clustal-W was used to perform multiple alignments of the nucleotide sequences. The phylogenetic analysis was performed using MEGAX for Neighbor-Joining method (Saitou and Nei, 1987;Kumar *et al.*, 2018). QIAGEN CLC Genomics Workbench version 20.0 was used to draw the Alignment sequences overview.

Chapter Three

Result & discussion

3. Results and Discussion

3.1. Isolation and Identification of *Proteus mirabilis*

The samples were then submitted to culturing on the blood agar plates, nutrient agar and MacConkey agar plates for isolation and identification of *Proteus mirabilis.*

All sample were subjected to aerobic culturing on different media and it was out of the total 130 samples 95(73.07%) samples, were showed positive bacterial culture. No growth was seen in other 35(26.9%) samples, which indicate the presence of microorganisms that may be cultured with difficulty such as virus, fungi and other agent or may be due to difference in the size and nature of the samples.

Among 95 positive cultures, only 25(19.2%) of samples show positive results *P.mirabilis*. These isolates then subjected to molecular detection methods using specific primer based on *ureR* gene as genetic marker for confirmed identification of *P.mirabilis* by PCR the results revealed that 25(100%) were positive for *ureR* as shown in Table (3-1). Kamil and Jarjes, (2021) They were isolated 47 *P.mirabilis* out of Fifty-one *Proteus* isolates from patients with symptomatic infections (urine, wounds swabs, burn swabs, vaginal swabs, ear swabs, eye swabs, and sputum).

Table (3-1) prevalence of *Proteus mirabilis* among other etiological agents associated with sample isolated

No. Sample	Culture			Molecular diagnosis By *ureR* gene	
	Proteus mirabilis	Negative results	Other Bacteria	Positive results	Negative results
130	25(19.2%)	35(26.9%)	70(53.8%)	25 (100%)	0 (0%)

Attallah and Farhan, (2020) were diagnosed with 20(8.33%) isolates of *P.mirabilis* of the total of 240 samples. Mohanad *et al.,(*2017) were found that only 25(20%) of *P.mirabilis* were isolated from 125 sample. Mahdi and Al-Deresawi, (2014) who found (92.30%) of *P.mirabilis* were isolated from 310 samples. Also Alsherees *et al*., 2016) his record 45(75%) out of 60 isolates are diagnosed as *P.mirabilis* isolated from different site of infections.

The variation in *Proteus* isolation between studies may be attributed to many factors such as sanitary practices in hospitals and staff, environmental conditions, isolation and identification techniques, social and cultural level of patients, or may be due to differences in the size of samples; all these factors may employ togather and play an important role in inhibit or stimulate the growth and distribution of pathogenic bacteria in hospitals.

3.1.2. Distribution of *P.mirabilis* Isolated from Different Clinical Samples

The result of this study was observed that (25) isolates were recorded related to *P.mirabilis*, collected from the following site, 17(68%) isolates obtained from urine samples, 3(12%) isolates from vagina samples, 3(12%) isolates from wound samples, 2(8%) isolates from burn samples as shown in Table (3-2).

Table (3-2): *P. mirabilis* isolated from different sites of infection

Site of infection	No. of smples	No. of P. mirabilis	%
Urine	55	17	(68%)
Vagina	30	3	(12%)
Wound	25	3	(12%)
Burn	20	2	(8%)
Total	**130**	**25**	**100%**

This table indicate that *P.mirabilis* are highly isolated from urine at percentage (68%) followed by vagina and wound at percentage (12%) for each one and burn at percentage (8%).

Following *E.coli* and *K.pneumoniae*, *P.mirabilis* is the third most common etiological factor of urinary tract infection (UTI) (Peng *et al.*, 2016; Armbruster *et al.*, 2018). Nearly 50–60% of all women suffer from an episode of UTI at least once in their lifetime. If the predisposing factors which are responsible for the occurrence of UTI are not timely diagnosed and treated, then it is also common for UTI episodes to reoccur. Untreated UTI can result in serious complications such as kidney damage, renal scarring, and renal failure (Ahmed *et al.*, 2019).

Enterobacteriaceae are increasingly playing a greater role in the many hospital acquired infections. Surgical wounds sites with high bacterial contaminants constitute a serious problem in the hospital especially in surgical practice where clean operations can become contaminated and subsequently infected. The degree to which surface wounds are infected by surrounding bacteria contaminants have become clinically important (Mohammed *et al.*, 2013).

Abbas *et al*., (2015) has reported that 17(12.6%) out of 135 isolates are diagnosed as *P.mirabilis* isolated from different site of infections including urine, vaginal, wounds and burns swab which similar to our results.

The results of bacterial isolation showed that 17(68%) of *P.mirabilis* are isolated from urine samples. These results parallel with the results of Abbas *et al*.,(2015) and Alsherees *et al*.,(2016) they found 11(64.7%), 33(78.6%) respectively of *P.mirabilis* are isolated from urine sample. However, this results resembles with result obtained by Al-Jumaily & Zgaer, (2016) and Lamees *et al*., (2018) they found 20(40%), 53(40.15) respectively isolates of *P.mirabilis* from urine of UTI patients. Also Al-Bassam and Al-Kazaz (2013) and Ram *et al*., (2019) found 9 (10.7), 65 (39.2%) respectively isolates of *P.mirabilis* from urine of UTI patients.

The bacteria has numerous virulence factors that are important for causing UTI and several of these factors appear to be more important for establishing infection in different areas of the urinary tract. These virulence factors include adherence capability, urease production and flagella (Alsherees *et al*., 2016).

The results obtained by the present study showed that three isolates 3 (12%) of *P.mirabilis* are isolated from wound infections. This study resembles with the results of Abbas *et al*., (2015) who isolated *P.mirabilis* from patients with wounds infections in percent (5.9%) of the total *P.mirabilis* isolates. The current study is dismatching with those obtained by Al-Bassam and Al-Kazaz (2013) that isolates of *P.mirabilis* in (24%) from wound of the total *P.mirabilis* isolates. Alsherees *et al*., (2016) who isolated (56.9%) of *P.mirabilis* from patients with wounds infections. Also Oladipo *et al*., (2014) that *P.mirabilis* shown more common in wound sample, which isolated (88.9%) *P. mirabilis* in wound.

Infection of wound is the successful invasion, proliferation by one or more species of microorganisms anywhere within the body's sterile tissues, sometimes resulting in pus formation. Development of wound infection depends on the interplay of many factors. Wound infections may occur following accidental trauma and injections, but post-operative wound infections in hospital are most common (Mohammed *et al.*, 2013).

The results obtained by the present study show that two isolate 2(8%) of *P.mirabilis* are isolated from burn infections. However, this study resembles with the results of Zafar *et al*., (2019) who isolated *P,mirabilis* from patients with burn infections (3%) and surgical wound infections (5%) of the total *P.mirabilis* isolates. Mohammed *et al.*, (2013) were reported in Nigeria that *P.mirabilis* was more frequently isolated from surgical wounds 19(47.4%) than burn 19(21%).

In the present study *P.mirabilis* was isolated in 3(12%) as causative agent of vaginitis. This result is disagreeing with Pal *et al*., (2014) and Alwash *et al*., (2008) were isolated *P.mirabilis* from vagina at percentage (3.1%), (4%) respectively. While Lamees *et al*., (2008) and Abbas et al., (2015) that isolated (11%), (5.9%) respectively *P.mirabilis* from vaginal swabs, this result was agrement with present study.

Vaginitis means an inflammation of the vagina that may concern in discharge, irritating or discomfort. The cause is usually a change in the standard balance of vaginal bacteria or an infection. *E. coli* and *Proteus* are the major bacteria establish in the postmenopausal states (Alwash *et al*., 2008). Iraq has a great infection burden and the genito-urinary infections are very projecting, this may be due to a less reasonable personal/ community hygiene for some of the economically later word populations (Abass *et al*., 2014).

Changes in vaginal microflora that show a critical role in promising vaginal colonization (Aiyegoro *et al*., 2007), and my suggest that the reason intestinal bacteria are associated with urinary tract and vagina infections is due to the proximity of the anal opening to the vagina and urethra, so contamination from the anus lead the bacteria can also be subsequently found the vagina area though this is a lot less common. The comparative more resistance of Gram-negative bacteria than Gram–positive to the inhibitory effect of vaginal liquid and by their capabilities to adherence to the epithelial cells of the urinary tract by their pili (Inabo and Obanibi, 2006).

3.1.3. Identification of *P. mirabilis*

The identification of *P.mirabilis* is done according to (Forbes *et al*., 2007), by depending on Colonial morphology (Shape, swarming, odor and lactose fermententation on MacConkey) and Microscopic examination that include the morphology of bacterial cells is investigated by Gram-stain to observe a shape, arrangement of cells and type of reaction with Gram-stain. After staining, specific biochemical tests are done to each isolates for the final identification.

Table (3-3) Identification Test of *P. mirabilis*

Proteus mirabilis	**Values**
Culture media	
MacConkey agar	Pale, non-lactose fermenter
Blood agar	Swarming,hemolysis (α- hemolysis)
Odor of colonies	(fishy odor)
Morphology	
Shape	Rods
Size	1-3 µm in length 0.4 - 0.8 µm in width
Biochemical Tests	
Indole	Negative
Urease	Positive

3.2. Confirmation and Diagnosis of *P. mirabilis* by PCR using specific primer

In this study, for more confirmation to the identity of these isolates, species specific primers have been used to amplify urease gene, (*ureR*) from *P.mirabilis* isolates, that responsible for the production of urease enzyme and regarded as a diagnostic feature of these species using PCR. All of the isolates of *P.mirabilis* were used to produce a specific size of the 225-bp fragment of the *ureR* gene 25(100%) as shown in Figure (3-1), and Table (3-4).

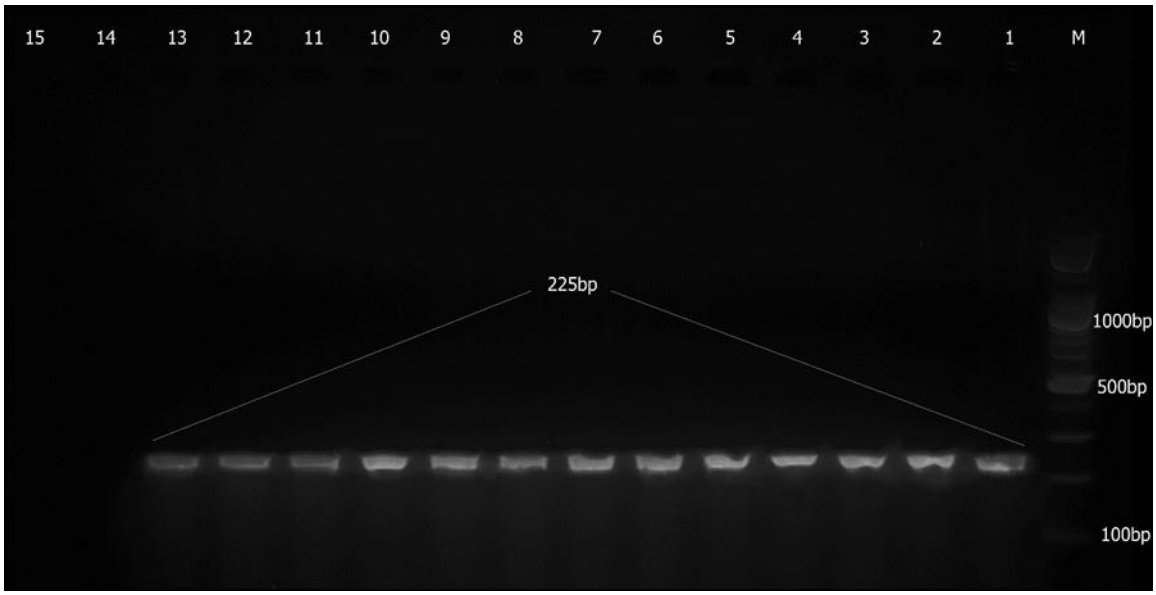

Fig.(3-1): 1% Agarose, 70 V for 30 Min gel electrophoresis of PCR products obtained by using ureR-specific primer. lanes (1-5) urine, (6-8) vagina, (9-11) wound, (12-13) burn represent the identified ureR gene products with 225bp, Lane M represent 1000bp DNA ladder.

Table (3-4): Identification of *ureR* gene of *P. mirabilis* in patients from different sample sources

Results	*ureR* N (%)Positive	*ureR* N(%)Negative
Urine	17 (100%)	0(0%)
Vagina	3 (100%)	0(0%)
Wound	3 (100%)	0(0%)
Burn	2 (100%)	0(0%)
Total	25/25	0/25

The results recorded that all isolates were positive for culture and biochemical diagnosis, gave positive results for (*ureR*) gene, urease synthesis *(ureR*) gene were used in this study for rapidly and confirmatory diagnosis of *P.mirabilis* by PCR using specific primer.

In this study specific target was obtained and utilized in conventional PCR,which was proven more rapid convenient and accurate for identification of *P.mirabilis* than previous methods. The results PCR approach demonstrated that comparation genetic methodology was successful identifying specific target (Kamil and Jarjes, 2021).

Alatrash and Al-Yasseen (2017) also showed that all P. mirabilis isolated from patients with urinary tract. infections were able to produce urease and possess ureR that encode to urease by appearing of amplicon with molecular weight 359 bp when electrophoresed on 1% agarose gel. Furthermore, according to Latif *et al.*, (2017), Kamil and Jarjes (2021), the results of PCR amplification to specific *ureR* primers indicated that (100%) of *P. mirabilis* isolates gave positive result at 225bp. In this study, the results were compatible to their findings as (100%) of the *P. mirabilis* isolates yielded *ureR* amplicon products with 225 bp.

In the study, for more confirmation to the identity of these isolates, species specific primers have been used to amplify urease gene, (*ureR*) from *P.mirabilis* isolates and (*ureC*) from *P.vulgaris*, that responsible for the production of urease enzyme and regarded as a diagnostic feature of this species using PCR (Kamil and Jarjes, 2021).

Several studies referred to use of *ureR*-based molecular method for identification of *P. mirabilis* such as Zhang *et al.*, (2013) who designed a species specific primers depending on the conserved *ureR* sequence of *P. mirabilis* to identify this species using PCR, a 225-bp DNA product was amplified from this species and detected on an agarose gel. As well as, Adnan *et al.*, (2014) who presented *ureR* as a high discriminatory power for identification of *P. mirabilis* using the PCR technology.

The urease enzymes are produced by all strains of *Proteus* species and it is also considered as diagnostic and differential feature which characterizes the members of this genus from the rest of the intestinal family members (Ali and Yousif, 2015). Urease is a hallmark of infections with *Proteus* species and it is considered as one of the most important virulence factor of *Proteus*. Many studies demonstrate the high ability of *Proteus* species to produce urease (Kamil and Jarjes, 2021).

3.3. Antibiotic Susceptibility Results of *Proteus mirabilis*

All the identified *P.mirabilis* isolates from urine, vagina, wound and burn samples were subjected to *in vitro* antibiotic resistant test by modified Kirby-Bauer disc diffusion method. Selective antibiotics are used to show their effect on *P.mirabilis* isolates such as Sulphamethoxazole-Trimethoprim, Chloramphenicol,Cefotaxime, Ampicillin-sulbactam, Amoxicillin, Tetracycline, Gentamicin, Amikacin, Aztreonam, Ciprofloxacin, Levofloxacin, Imipenem and Meropenem.The results are shown in Table (3-5) and Figure (3-2). The results compare according to Clinical Laboratory Standard Institute guidelines (CLSI, 2021) as resistant.

Highest rate of resistant is seen to almost antibiotics used in present study, 20(80%) isolates were resistant to Chloramphenicol, 17(68)% isolates were resistant to Sulphamethoxazole-Trimethoprim, 13 (52%) isolates were resistant to Gentamicin, 12(48%) isolates were resistant to Tetracyclin, 11(44%) isolates were resistant to Amoxillin, 9 (3 6 %) isolates were resistant to Ampicillin-sulbactam, 7(28%) isolates were resistant to Cefotaxime, 4 (1 6 %) isolates were resistant to Amikacin, 3 (1 2 %) isolates were resistant to Aztreonam and ciprofloxacin for each, while the isolates was highly sensitive to Levofloxacin 25(100%), Imipenem 25(100%) and Meropenem 25(100%).

Table (3-5) :Percentage of antibiotics sensitive and resistance by *P. mirabilis* against 13 types of antibiotics by Kirby-Bauer disc diffusion according to CLSI 2021 (n=25)

No.	Antibiotic	Shortcut	Susceptible (No.) %	Resistant(No.) %
Penicillins/ β-lactamase-inhibitor combinations / Aminopenicillins				
1.	Ampicillin-sulbactam	AMS	16 (64%)	9 (36%)
2	Amoxicillin	AMP	14 (56%)	11 (44%)
Macrolides				
3.	Aztreonam	ATM	22 (88%)	3 (12%)
Quinolones				
4.	Ciprofloxacin	CPX	22 (88%)	3 (12%)
5.	Levofloxacin	LVX	25 (100%)	0 (0%)
Folate pathway antagonistic/Co-trimoxazole				
6.	Sulphamethoxazole-trimethoprim	SXT	8 (32%)	17 (68%)
Third generation Cephalosporin				
7.	Cefotaxime	CTX	18 (72%)	7 (28%)
Phenicol				
8.	Chloramphenicol	C	5 (20%)	20 (80%)
Tetracycline				
9.	Tetracycline	TET	13 (52%)	12 (48%)
Carbapenemase				
10.	Meropenem	MEM	25 (100%)	0 (0%)
11.	Imipenem	IMP	25 (100%)	0 (0%)
Aminoglycoside				
12.	Gentamicin	GEN	12 (48%)	13 (52%)
13.	Amikacin	AMK	21 (84%)	4 (16%)

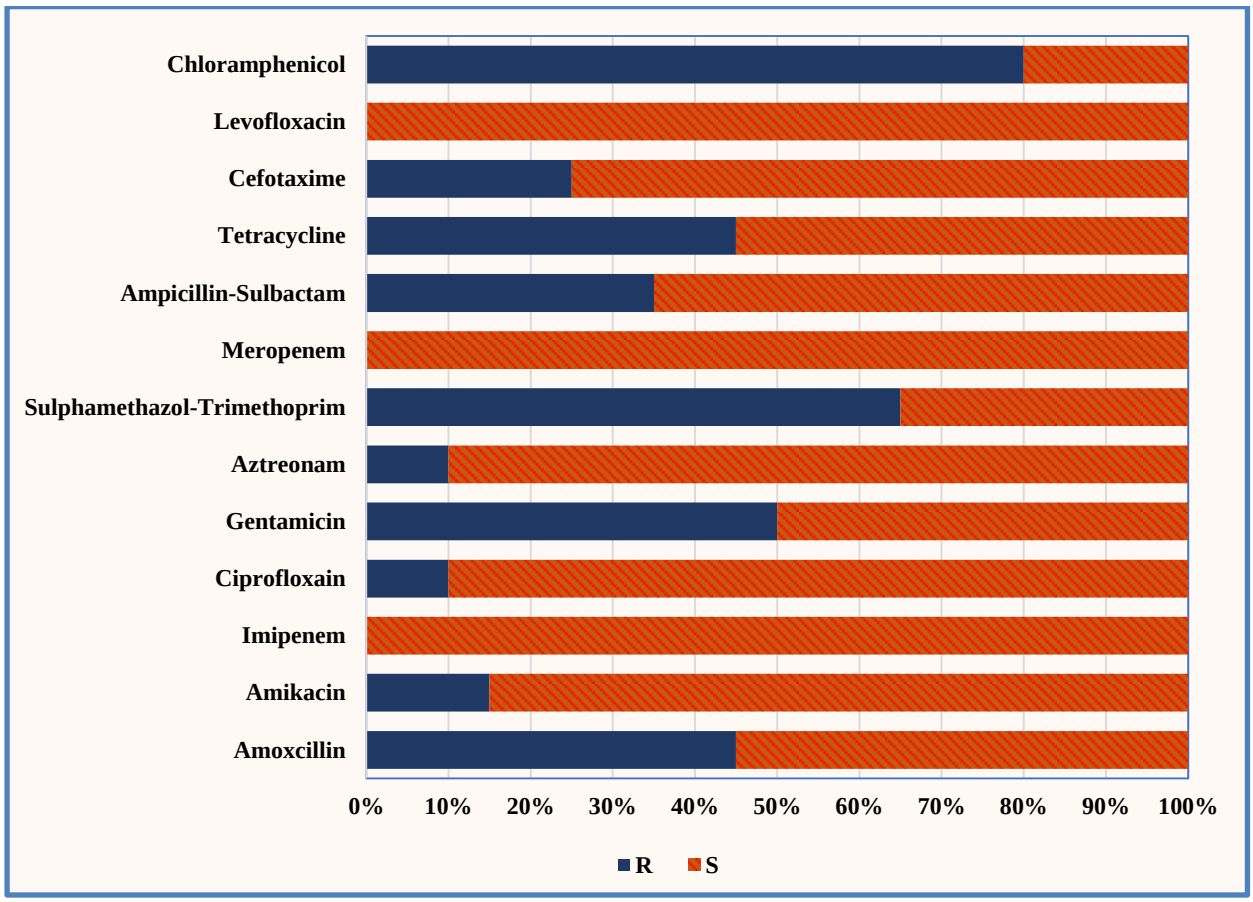

Fig (3-2): Antimicrobial Susceptibility Testing for (25) *Proteus mirabilis* isolates

Proteus mirabilis is an opportunistic pathogen responsible for variety of infections, mostly prevalent is the urinary tract infections (Serry *et al*., 2018). This study aims to investigate the antimicrobial resistance pattern of *P.mirabilis* isolated from patients with urinary tract infections, vagina, burn and wound infections.

Antibiotics, representing both naturally as well as chemically synthesized entities, emerged as a powerful tool in counteracting infectious diseases, following serendipitous discovery of penicillin from Penicillium notatum by Alexander Fleming in 1928. Widespread usage of antibiotics that imposes strong selection pressure for resistance development (ability to withstand effects of antibiotics) took a strong grip over the health care system globally as concerns regarding resistance to available drug regime restrict therapeutic options available to treat the disease (Sultan *et al*., 2018).

Synthesis of β-lactamases is the predominant mechanism for resistance to β-lactamantibiotics (Pal *et al*., 2016). Results showed that 9 isolates (36%) of *P.mirabilis* isolates were able to produce Ampicillin-Sulbactam (β-lactamase) and this result was in disagreement with Serry *et al*., (2018) who found that *P.mirabilis*, were produced β-lactamase in percentages (85.1%). Many studies were reported different percentages of β-lactamase production for *P.mirabilis*, Hussein, (2013) showed that *P.mirabilis* isolates produced β-lactamase (39%), while Al-Duliami *et al*., (2011) found that *P.mirabilis* isolates were able to produce β-lactamase in (45.8%).

Most *P.mirabilis* isolates were susceptible to common antibiotic classes, but recent studies in different countries have indicated that antibiotic resistance among *P.mirabilis* isolates is increasing. The *β*-lactam resistance patterns of the *P.mirabilis* isolates have reported the production of various classes of extended-spectrum *β*-lactamases (ESBLs) (Chen *et al*., 2017).

P.mirabilis have shown resistance to β-lactam antibiotic group. The effect of Amoxicillin on bacteria is planned as shown in the results which shown that (44%) of isolates were resistant to this antibiotic agent. These results of *P. mirabilis* resistant to this antibiotic are compatible with Mirzaei *et al*., (2019) who found (44.5%), of the isolates were resistant to Amoxicillin. While Mohammed *et al.*, (2013), Philips, (2014) and Zafar *et al*., (2019) reported that of *P.mirabilis* were (68%), (78.6%), (93.7%) respectively resistant to Amoxicillin. The β-Lactam resistance is noticed by Pagani *et al*., (2002) who found that the Amoxicillin resistance in *P. mirabilis* was almost always (52%).

Those resistant to Amoxicillin are recognized to their production of β-lactamase enzymes that hydrolysis the β-lactam ring and it made inactive, failure of antibiotic to binding to Penicillin binding protein (PBP) target site and low affinity binding of antibiotic to (PBP) also confer resistance to these antibiotics (Ang *et al*., 2004). Evolution and spread of a multidrug-resistant *P.mirabilis* clone with chromosomal AmpC-type β-lactamase was reported in Europe (D'Andrea *etal.*,2011).

The isolates have (68%), (80%) resistance to Trimethoprim/Sulfamethoxazole, Chloramphenicol respectively. Al-Bassam and Al-Kazaz (2013), Nahar *et al*., (2014) and Al-Jumaily & Zgaer, (2016) were showed the resistance of *P.mirabilis* isolates to Trimethoprin/Sulphamethazol were (65%), (66.7%) and (72%) respectively. Mohanad *etal.*, (2017) the resistance (100%), (84%) respectively to Chloramphenicol and Trimethoprim-Sulfamethaxazole.

This result was agreement to the result of (Serry *et al*., 2018) where resistance rates of (78.8%), (72.2%) were observed with Sulphamethoxazole-Trimethoprim and Chloramphenicol.

Most Trimethoprim/Sulfamethoxazole resistance genes reside within integrons, horizontally transferable genetic elements which play an important role in their dissemination. Type 1 integrons contains an integrase gene (*int*) that allows integration of new genes into the bacterial genome as gene cassettes. One class of genes commonly inserted in this manner are genes which confer resistance to trimethoprim. The sulfamethoxazole resistance gene is also associated with type 1 integrons. Type 2 integrons cannot acquire new gene cassettes due to a non-functional *int* gene (Blahna *et al*., 2006). Other common horizontally transferable elements are transposons and plasmids, on which Sulfamethoxazole resistance genes *sul2* and *sul3* are often found (Perreten and Boerlin, 2003).

It acts as a broad spectrum antibiotic resistance mechanism for Chloramphenicol involves enzymatic inactivation via acetylation mediated by Chloramphenicol Acetyltransferases (CATs). Apart from enzyme inactivation Chloramphenicol resistance mechanisms, also involves inactivation by phosphotransferases, target site mutation, permeability barriers and efflux pumps (Sultan *etal*., 2018).

The *P.mirabilis* isolates recovered were resistant to Tetracycline (48%) which disagrees with Mohanad *et al.*, (2017) and Serry *et al*., (2018) they found 100% resistance to tetracycline. While resistance rate of (92%) was reported by Zafar *et al*., (2019). Mechanisms of resistance for Tetracycline hold three key strategies: energy-dependent efflux pumps (ABC efflux pumps), ribosomal protection proteins (RPPs), or enzymatic inactivation (Sultan *et al*., 2018). Tetracycline is bacteriostatic especially in large doses and it is short acting. In small doses oral antibiotics do not reduce the number of organisms but they affect their function. The antibiotic can also inhibit various enzyme activities such as lipase by interfering with protein synthesis combining with bacterial ribosome (Andrews, 1990).

In respect to Aminoglycosides, this study demonstrated 52% resistance of *P.mirabiis* isolates to Gentamicin. This result was parallel to the result of Al-Jumaily & Zgaer, (2016), Mohanad *et al.*, (2017) and Al-Hamdani and Al-Hashimy (2020) they showed that (55.8%), (64%), (70%) respectively of *P.mirabilis* isolates from different clinical sample were resistance to Gentamicin. While Serry *et al*., (2018), Zafar *et al*., (2019) and Hussein *et al.*, (2020) reported that the resistance to Gentamicin was (42.6%), (21.8%), (20.6%) respectively isolates of *Proteus mirabilis*.

Among aerobic bacteria, aminoglycoside resistance is most commonly due to enzymatic inactivation through aminoglycoside-modifying enzymes. These may be coded by genes on plasmids or chromosomes. Several aminoglycoside-modifying enzymes have been shown to be carried on transposons (Opal and Pop-Vicas, 2015).

In the present study, the *P.mirabilis* isolates were sensitive to Amikacin in 84%, and this result was in agreement with the results of Al-Jumaily & Zgaer, (2016), Mohanad *et al.*, (2017) and Zafar *et al*., (2019) as the found that the percentage sensitivity of Amikacin were (92.6%), (88%), (82.8%) respectively. Singla *et al.,* (2015) found the percentage of susceptibility to Amikacin was (87% to 98), which considered an agreement to this result. The rate of entry of Aminoglycoside molecules into bacterial cells is a function of their binding to a usually non saturable anionic transporter, where up on they retain their positive charge and subsequently are "pulled" across the cytoplasmic membrane by the internal negative charge of the cell (Opal and Pop-Vicas, 2015).

ESBL, AmpC and Carbapenemase genes, resistance genes to other antibiotic families such Quinolones (qnr, aac6'Ib) and to Aminoglycosides (APH, AAC, AAD, methylases) are more and more frequently identified in *P.mirabilis*. These genes are carried on mobile genetic elements such as plasmids, transposons, mobile genomic islands, and on integrons that can be mobilized through various mobile elements (Girlich *et al*., 2020).

The Carbapenems was demonstrated by the sensitive to Imipenem and Meropenem with percentage (100%). Rashmi, (2015) who found (100%) sensitive to Imipenem of *P.mirabilis*. Also Al-Jumaily & Zgaer, (2016), Mohanad *et al.*, (2017) and Al-Hamdani and Al-Hashimy (2020) they found sensitive (97.2%), (96%), (70%) respectively to Imipenem. However, the results conducted by Serry *et al*., (2018) stated that lower resistance rates of *P.mirabilis* isolates against Imipenem, Meropenem (8.5%), (6.4%) respectively. Mohanad *et al.*, (2017) who found sensitive (100%) to Meropenem.

While Prakash and Saxena, (2013) they showed that Meropenem sensitivity was 100% against *Proteus.* But Al-Bassam and Al-Kazaz (2013) they found lower resistance (15%), (20%) to Imipenem and Aztreonam respectively. Also Mirzaei *et al*., (2019) who found lower resistance rates (11.8%) to Imipenem, (4.5%) to meropenem.

P.mirabilis isolates present an elevation in the resistance level to imipenem due to many reasons: the loss of outer membrane porins, decreased expression of PBP1a or reduced binding of imipenem by PBP2 (Girlich *et al.,* 2015). ImpR (outer membrane protein) over expression resulted in increased carbapenem MICs in the laboratory strain and clinical isolates. Development of resistance against imipenem in *P. mirabilis* is also due to the absence of 24 kDa OMP (Tsai *et al*., 2015; Zaman and Shamsuzzaman, 2021).

Carbapenems are family of β-lactam antibiotics that unique because they are relatively resistant to hydrolysis by most β–lactamases, this family has many members such as Imipenem and Meropenem, which are broadly active antibacterials that are used for infections caused by difficult to treat or multidrug-resistant bacteria often used as "last-line agents" (Papp-Wallace *et al*., 2011).

Regarding Quinolones antibiotics, results indicated that (88%) of the isolates were sensitive to Ciprofloxacin. This result was in agreement with the results of Mohanad *et al.*, (2017) detected that the antibiotic sensitive was (100%). But Hussein *et al.,* (2020) and Al-Jumaily & Zgaer, (2016) reported that the sensitive to ciprofloxacin was (69.8%), (67.4%) respectively.

In the present study, the *P.mirabilis* isolates were sensitive to Levofloxacin in 100%. However, the results conducted by Serry *et al*., (2018) stated that of *P.mirabilis* isolates against levofloxacin, ciporofloxacin (31.9%), (38.3%) respectively. Also Mirzaei *et al*., (2019) who found lower resistance rates (15.5%) of *P.mirabilis* isolates against ciporofloxacin. Ciprofloxacin, a fluoroquinolone that inhibits DNA gyrase necessary for completing DNA replication, was used to grow the *P.mirabilis* as it is a widely used drug to treat bacterial infection (Sharma *et al*., 2010).

Ciprofloxacin was bactericidal drug, they were affected against Gram negative and Gram positive bacteria and the resistance to fluoroquinolones was through chromosomal mutation or alternation affecting the ability to fluroquinolones to permeate to bacterial cell wall (Rashmi *et al*., 2005). Ciprofloxacin is one of broad spectrum antibiotics in their effect on pathogenic bacteria; recently it is the most important drug for UTIs treatment (Gilho Lee, 2013).

Regarding to Third Generation Cephalosporin antibiotics, the results showed that the resistance against Cefotaxime was 28% and these results were in agreement with Mohanad *et al*.,(2017) who found that (28%) of the isolates were resistant to Cefotaxime. While Al-Hasso and Khalaf, (2013) they found that all *P. mirabilis* isolates were resistance against Cefotaxime. Also Serry *et al*., (2018), Dehnavi and Zarif, (2017) they found that (51.1%), (50%), respectively of the isolates were resistant to Cefotaxime. These results of resistance to this antibiotic are dismatching with that of Al-Hamdani and Al-Hashimy (2020) who found that (90%) of *P.mirabilis* were confer resistant to Cefotaxim.

In this study *P.mirabilis* isolates showed high sensitivity against Azetronam it was revealed that 22 isolates (88%) showed sensitive to this antibiotic. These results were confirmed to that reported by Al-Bassam, (2010) and Al-Jumaily & Zgaer, (2016) they found (81%) , (81.7%) sensitivity to this antimicrobial. Also Serry *et al*., (2018), Mirzaei *et al*., (2019) they found lower resistance rates (14.9%), (10%) respectively of *P.mirabilis* isolates against Azetronam.

The different *Proteus* species differs in their susceptibility pattern to different antibiotics. *P.mirabilis* has intrinsic resistance to nitrofurantoin and tetracycline but is generally susceptible to the amino- and ureido-penicillins (ampicillin, amoxicillin, and piperacillin), cephalosporins (cefazolin, cefoxitin, cefuroxime, cefotaxime, ceftazidime, ceftriaxone, ceftizoxime, and cefepime), aminoglycosides (amikacin, gentamicin, and tobramycin), imipenem, ciprofloxacin, and trimethoprim-sulfamethoxazole. Multidrug-resistance of *P.mirabilis* calls for regular review of antimicrobial sensitivity pattern among clinically isolated *P.mirabilis* in order to be able to decide which antibiotic to be prescribed (Bahashwan and El Shafey, 2013).

3.4 Genotyping

3.4. Molecular detection of virulence genes in *P. mirabilis*

P.mirabilis expresses several virulence factors involved in pathogenesis such as adhesions, swarming motility, urease, hemolysin, proteases, and lipopolysaccharide endotoxins (Armbruster and Mobley, 2012).

The genotypic characters were tested for all *P.mirabilis* isolates in this study in order to detect of some virulence factors. The specific primers were used for screening the presence of (*hpmA*, *rsbA*, and *pta*) genes. DNA was extracted from activated pure culture of *P.mirabilis* bacteria using genomic DNA Bacteria Kit (geneaid). Detection of DNA bands using Agarose gelelectrophoresis (1%). Then conventional PCR were used in this study to detect the presence of the important virulence genes that related with causing UTI and vagina , wound, burn infection (*hpmA*, *rsbA* and *Pta*) of the *P.mirabilis* bacteria using specific primers as shown in Table (3- 6).

Table (3-6): Distribution of Virulence factors *(hpmA, rsbA* and *Pta*) among *P.mirabilis* isolates

genes	No. Isolates	(%)
hpmA	24	96%
rsbA	20	80%
Pta	23	92%

3.4.1. Molecular Detection of (*hpmA* gene) in *P. mirabilis*

Polymerase chain reaction technique was used in investigation of the genes responsible for the virulence factor in *P.mirablis* through the use of fragment of the DNA with limited number of nucleotides (oligonucleotide), which act a primers specialized for virulence genes in *P.mirablis* and it include *hpmA*. *hpmA* gene which responsible for producing hemolycin. *P.mirabilis* α-hemolysin is different from other *Proteus spp*., its organized by (*hpmA*), that encode for the *hpmA* protein (Lazm *et al*. 2018). In this study, *hpmA w*as found in 24 isolates in a rate of (96%) shown in Figure (3-3), and Table (3-7).

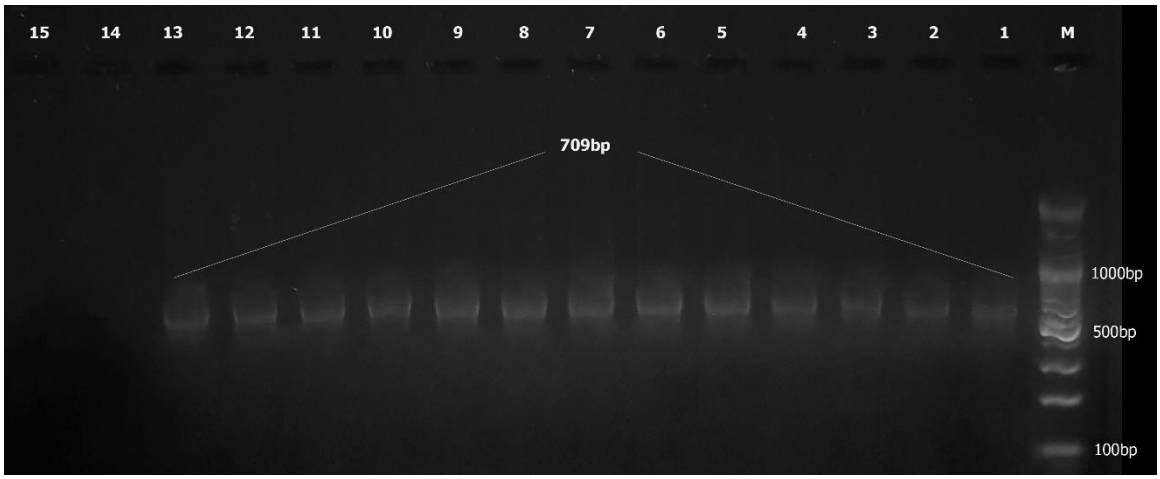

Fig. (3-3): 1% Agarose, 70 V for 30 Min gel electrophoresis of PCR products obtained by using *hpmA* -specific primer. Lanes (1-5) urine, (6-8) vagina, (9-11) wound, (12-13) burn represent the identified *hpmA* gene products with 709bp, Lane M represent 1000bp DNA ladder.

Table (3-7): Identification of *hpmA* gene of *P. mirabilis* in patients from different sample sources

Results	*hpmA* N (%)Positive	*hpmA* N (%)Negative
Urine	17 (100%)	0(0%)
Vagina	3 (100%)	0(0%)
Wound	2 (94%)	1(6%)
Burn	2 (100%)	0(0%)
Total	24/25	1/25

These results is similar to Cestari *et al*., (2013) found the ratio of this gene in *P.mirablis* isolates (96.24%) presented amplification for the *hpmA* gene by PCR. This result is consistent with Al-Hamdani and Al-Hashimy (2020) and Hussein *et al.*, (2020) as they reported that the rate of this gene in *P.mirablis* isolates was (90%), (98.4%), respectively. Also Ali and Yousif, (2015) and Lazm *et al*., (2018) they found all of the isolates (100%) had the ability to produce hemolysin. Fraser *et al.*, (2002) who described the *hpmB*/A locus that codifies hemolysin *hpmA* as a well-conserved region in *P. mirabilis* isolates.

P.mirabilis composes most (97%) of the *Proteus* urinary tract isolates, this suggests that *hpmA* was the predominant *Proteus* hemolysin and might play a role in extra intestinal infections caused by *Proteus spp*. *hpmA* gene was a suitable and efficient molecular marker for the distinction of *P.mirabilis* and could used as an alternative molecular tool for examining phylogenetic relationships of the *P.mirabilis* and a powerful tool for the study of different microorganisms (Lazm *et al*., 2018).

These authors also observed that some *Proteus* spp. samples can express hemolysin *hlyA* similar to that of *E.coli*. Hemolysin *hpmA* production is considered an important virulence factor of uropathogenic *P.mirabilis*. Thus, it is important to detect the genes and verify hemolysin expression in a collection of uropathogenic *P. mirabilis* (Cestari *et al*., 2013)

The function of hemolysin is to form pores in target host cells. It was proposed that hemolytic activity helps *P.mirabilis* to spread into the kidneys during infection. This is probably mediated through the increased ability of hemolytic *P.mirabilis* cells to invade host tissue (Chalmeau *et al*., 2011).

The hemolysin is encoded by *hpmA*, while a second gene, *hpmB*, encodes a membrane transporter; both genes are highly conserved across *P.mirabilis* isolates. The levels of hemolysin in *P.mirabilis* correlate with its ability to invade cultured kidney cells, and an isogenic *P.mirabilis hpmA* mutant is minimally invasive in cultured cells (Schaffer and Pearson, 2017).

Secreted pore-forming toxins are a common feature of pathogenic bacteria. In particular, hemolysins are secreted pore-forming toxins that insert into eukaryotic cell membranes, causing efflux of sodium ions and cell damage. Two hemolysins have been described for members of the Proteus genus, one that is calcium dependent and similar to the α-hemolysin of E.coli (hlyA), and another that is calcium independent (Armbruster *et al*., 2018).

3.4.2. Molecular Detection of (*rsbA* gene) in *P. mirabilis*

Swarming (*rsbA*) gene was detected by PCR technique using specific primers for this gene. The results showed that 20(80%) of *P. mirabilis* isolates gave positive result at (467bp) in PCR amplification of *rsbA* gene, as shown in the Figure (3-4), and Table (3-8).

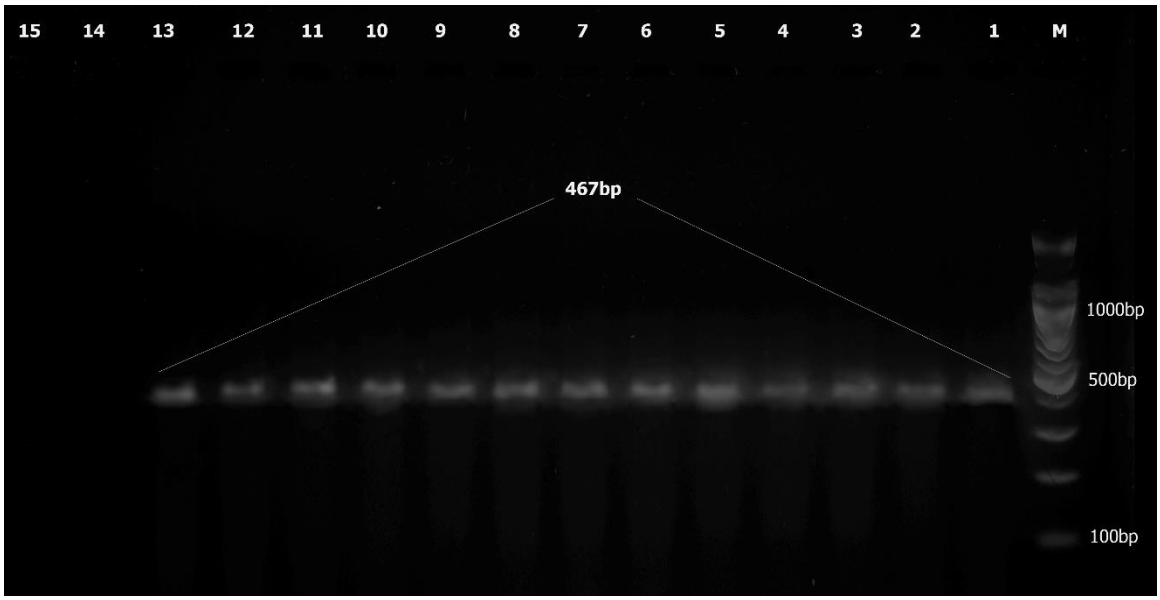

Fig. (3-4): 1% Agarose, 70 V for 30 Min gel electrophoresis of PCR products obtained by using rsbA-specific primer. lanes (1-5) urine, (6-8) vagina, (9-11) wound, (12-13) burn represent the identified rsbA gene products with 467bp, Lane M represent 1000bp DNA ladder.

Table (3-8): Identification of *rsbA* gene of *P. mirabilis* in patients from different sample sources.

Results	*rsbA* N (%)Positive	*rsbA* N(%)Negative
Urine	15(88%)	2(12%)
Vagina	2 (67%)	1(33%)
Wound	2 (67%)	1(33%)
Burn	1 (50%)	1(50%)
Total	20/25	5/25

The results is consistent with the previous studies , Pathirana, *et al*., (2018) they found (80%) *P.mirabilis* isolates. Also the results of the present study nearly agreement with the results obtained by Dehnavi and Zarif, (2017) who found that (63%). But the results of the present study nearly disagreement with the results obtained by and Abbas *et al*., (2015) they found that (53%) *P.mirabilis* isolates display *rsbA* genes band so the foundation is common in *P.mirabilis.* While Al-Hamdani and Al-Hashim, (2020) and Hussein *etal*., (2020) they found that (100%) *P.mirabilis* isolates.

Some studies reported that the Arabic coffee may has an inhibitory effect on repressor of secondary metabolites gene *(rsbA*) that regulated *P.mirabilis* swarming and lead to a reduction in production of other virulence factors or inhibit genes expression of other virulence factors (Laftaah, 2012).

The compounds that inhibit the swarming may attributed to complex with flagellar proteins of swarming cells and cause its disintegration or impair formation of flagella and motility**.** The enhanced or inhibited swarming of *P.mirabilis* by compounds may attributed to many reasons include; these compounds may acts as extracellular signals or intracellular signals, may serve as cell-cell communication signals that interact with some of membrane sensor

proteins or may affect membrane fluidity, these compounds may interact with activity of *rsbA* proteins or through either an *rsbA*-dependent or *rsbA*-independent pathway to regulate swarming and virulence factor expression in *P. mirabilis* or these compounds may have an inhibitory effect on *rsb*A gene that regulated *P. mirabilis* swarming (Wang *et al.,* (2006). There are several theories have been suggested to explain the mechanism of swarming of bacteria. The negative chemotaxis, accumulation of secondary metabolites in the colony vicinity, impairment of flagellation, enhanced growth rate (Laftaah, 2012).

One of the prominent features of *P. mirabilis* is the ability to swarm on solid surfaces. Even though several genes are associated with the swarming phenomenon as *cheW*, *gidA* and *cldA* genes, the *rsbA* gene is important for the swarming regulation (Pathirana, *et al*., 2018).

In this study all isolates were harbor *rsbA* gene that related to the bacterial movement and the showed variation in swarming expression by *P.mirabilis* which might be due to different reasons related to the strains themselves (e.g. strain variation, their origin etc) or growth and incubation conditions such as incubation period, pH, temperature, nutrition ingredients in media or expression of certain related swarming genes. This explanation corresponds with Senior's experiment when he observed a strong correlation between the ability of *P.mirabilis* to swarm growth and the ability to produce protease. *P.mirabilis* has the ability to promote infection of a host during swarming and highly motile because the swarming cells could migrate through the urinary tract and cause many infections (Saleh *et al.*, 2020).

3.4.3. Molecular Detection of *(pta* gene*)* in *P. mirabilis*

Pta is recognized as virulence factor in *P.mirabilis*. The PCR technique was used investigation of the *pta* gene through the use of fragment of DNA with a limited number of oilgonucleotide which act as primer specialized a virulence gene of *P. mirabilis*. The result of the current study was shown that *pta* gene was present in 23 isolates out 25 isolates of *P.mirabilis* at rate (92%) from different samples sources with long length in (181bp) as shown in Figure (3-5) and Table (3-9). This result is consistent with result obtained by Lamees, (2017) who were found that the *pta* gene is present in almost isolates of *Proteus mirabilis* in rate at (93.75%).

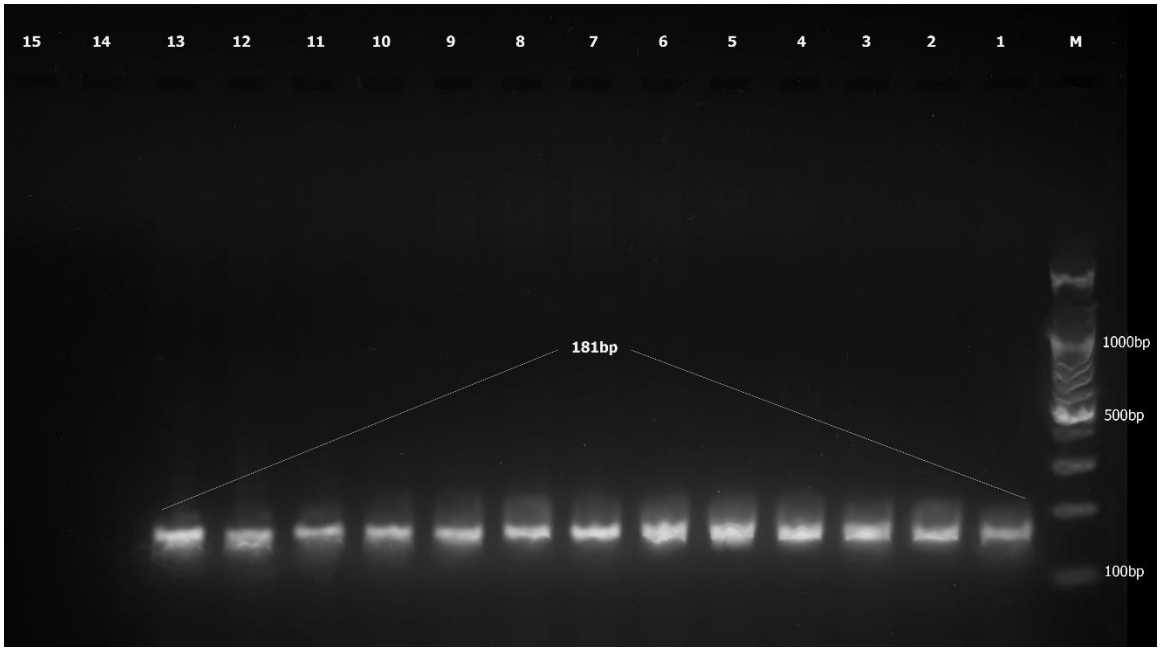

Fig. (3-5): 1% Agarose, 70 V for 30 Min gel electrophoresis of PCR products obtained by using Pta-specific primer. Lanes (1-5) urine, (6-8) vagina, (9-11) wound, (12-13) burn represent the identified *Pta* gene products with 181bp, Lane M represent 1000bp DNA ladder.

Table (3-9): Identification of *Pta* gene gene of *P. mirabilis* in patients with different sample sources.

Results	*Pta* N (%)Positive	*Pta* N(%)Negative
Urine	15 (88%)	2(12%)
Vagina	3 (100%)	0(0%)
Wound	3 (100%)	0(0%)
Burn	2 (100%)	0(0%)
Total	23/25	2/25

Also Al-Duliami *et al*. (2011) as they reported that 95.8% of *Proteus* spp. were positive to agglutination test. Armbruster and Mobley, (2012) who were found the expression of *pta was* distinguished in urinary isolates *Proteus mirabilis* negative *pta* gene had reduced pathology as well as, defect in colonization of kidneys and bladder *Proteus mirabilis* may not be as homogeneous as once thought as genetic variance is possible, especially when pathogen specific factor (e.g auto transporter).

The *Proteus mirabilis* negative *pta* gene had reduced pathology, as well as a serious colonization defect in urine, kidneys, and spleen (Engel *et al.*, 2007). The irregular adhesin–toxin was early distinguished as an outer-membrane surface expressed protein that is recognized by the mouse immune system (Nielubowicz *et al.*, 2008), and the loss of Proteus toxic agglutinin results in a critical colonization imperfection in the kidneys and bladder, and in addition lessened pathology (Alamuri and Mobley, 2008).

Inactivation of Proteus toxic agglutinin results in a greater diminishment in cytotoxicity. Proteus toxic agglutinin describe as cytotoxin with no bacterial homologues that works optimally in the alkalinized urinary tract, a characteristic of urease-mediated urea hydrolysis during *Proteus mirabilis* infection (Alamuri *et al.,* 2009). Proteus toxic agglutinin as essential virulence factor in the uropathogen Proteus mirabilis. The bacteria have ability to cause necrosis to invade tissue though the ability to produce toxin as *Pta* toxin (Choubini *et al.,* 2016).

Because Proteus toxic agglutinin is not only a key cytotoxin in *Proteus mirabilis* but also a surface exposed protein that is a critical for establishing an infection in the host. *P. mirabilis* produce Pta, which is cause damage of tissue and dissemination to the kidneys, initiating acute pyelonephritis (Jacobsen *et al.*,2008; Cestari *et al.,* 2013).

Proteus toxic agglutinin is a bi-functional outer-membrane autotransporter that mediates cell–cell accumulation and furthermore contains a catalytically active α-domain equipped of lysing bladder and kidney cells (Flannery *et al.,* 2009). Moreover, Proteus toxic agglutinin hole the host cell membrane, leading to outflow of the cytosol, osmotic stress and de-polymerization of actin filaments; the structural integrity of the cell is therefore compromised, resulting in bladder and kidney damage (Armbruster and Mobley, 2012).

3.5. A Multi-Locus Sequence Analysis for Identification of the *P.mirabilis*

MLSA has been used for classification at the species level in numerous Enterobacteriaceae (Colston *et al.*, 2014). MLSA has the advantage of being more convenient and more conducive to the whole genome sequencing method. Normally, four to seven housekeeping genes were selected for MLSA to determine phylogenetic relationships. It has been recommended that researchers use sequence data from more than one gene to reduce the possibility of ambiguities caused by genetic recombination or specific selection. MLSA is increasingly applied to obtain a higher resolution power between species within a genus and provides a perspective for the genotypic taxonomic analyses of genus Proteus (Glaeser and Kampfer, 2015).

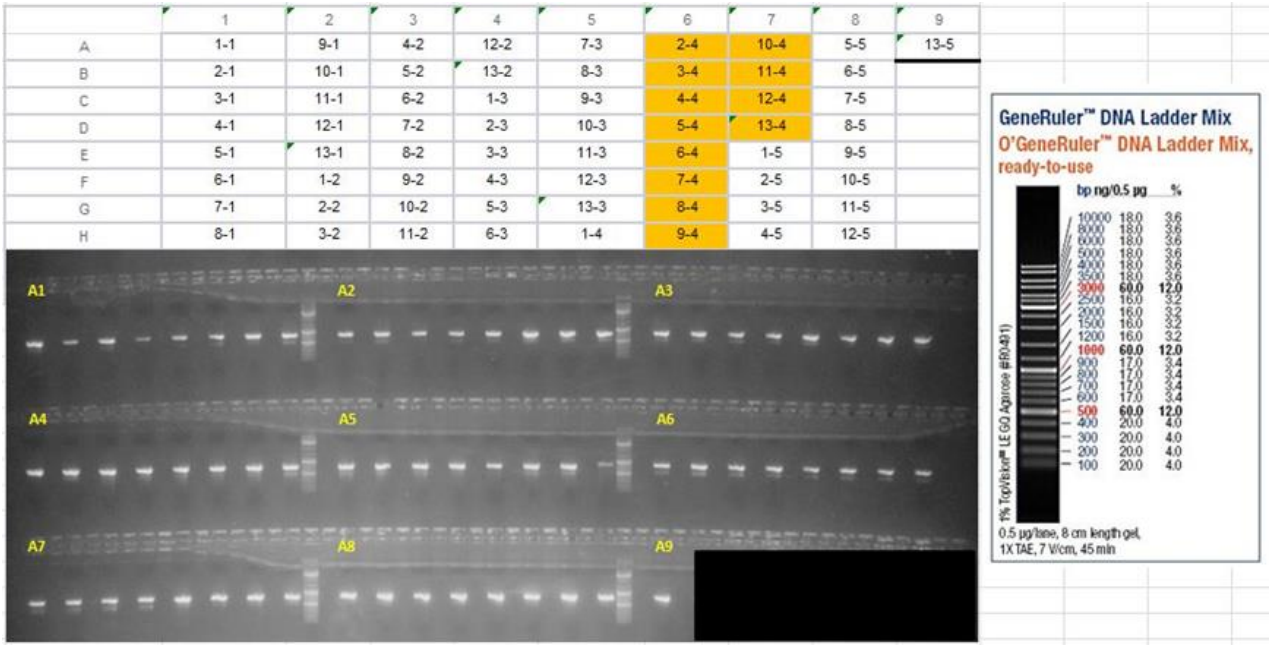

Fig. (3-6): 1.5% Agarose, 100 V for 30 Min gel electrophoresis for 5 housekeeping genes for 13 *P. mirabilis* isolates

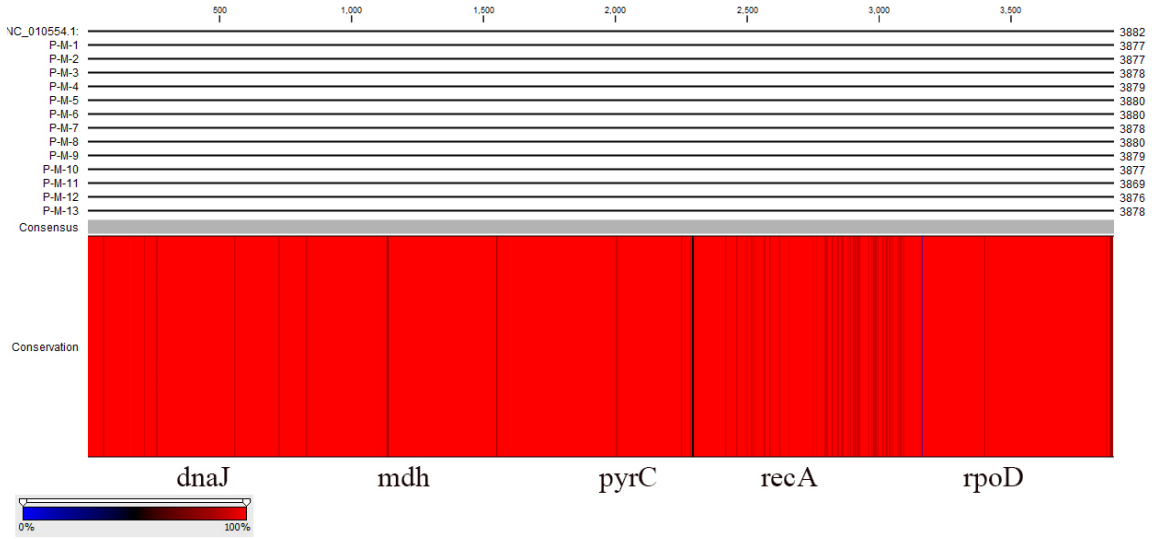

Fig. (3-7): Whole concatenated genes (dnaJ, mdh, pyrC, recA and rpoD) overview against the reference genome of *Proteus mirabilis*. Similarity differences were illustrated above by color of conservation.

In this study, amplicon obtained by PCR of *P.mirabilis* collected, the five housekeeping genes (*dnaJ*, *mdh*, *pyrC*, *recA*, and *rpoD*) have a good corresponding relationship of consistency among different species. As shown in Fig. (3-8), similarity matrix analysis of studied samples of P. mirabilis against the reference genome showed high similarity percentage with all samples, where they shared (95-99%) similarity with the reference sequence, that lead to there is a evolutionary variation for studied isolates in comparison with the reference genome. Moreover, the highest percentage of variation was detected in sample 11, and that may be due to the different isolation place from other samples or other reasons related to environmental conditions and time of isolation. In general, this results reveal the high capacity of this bacteria to develop through the time.

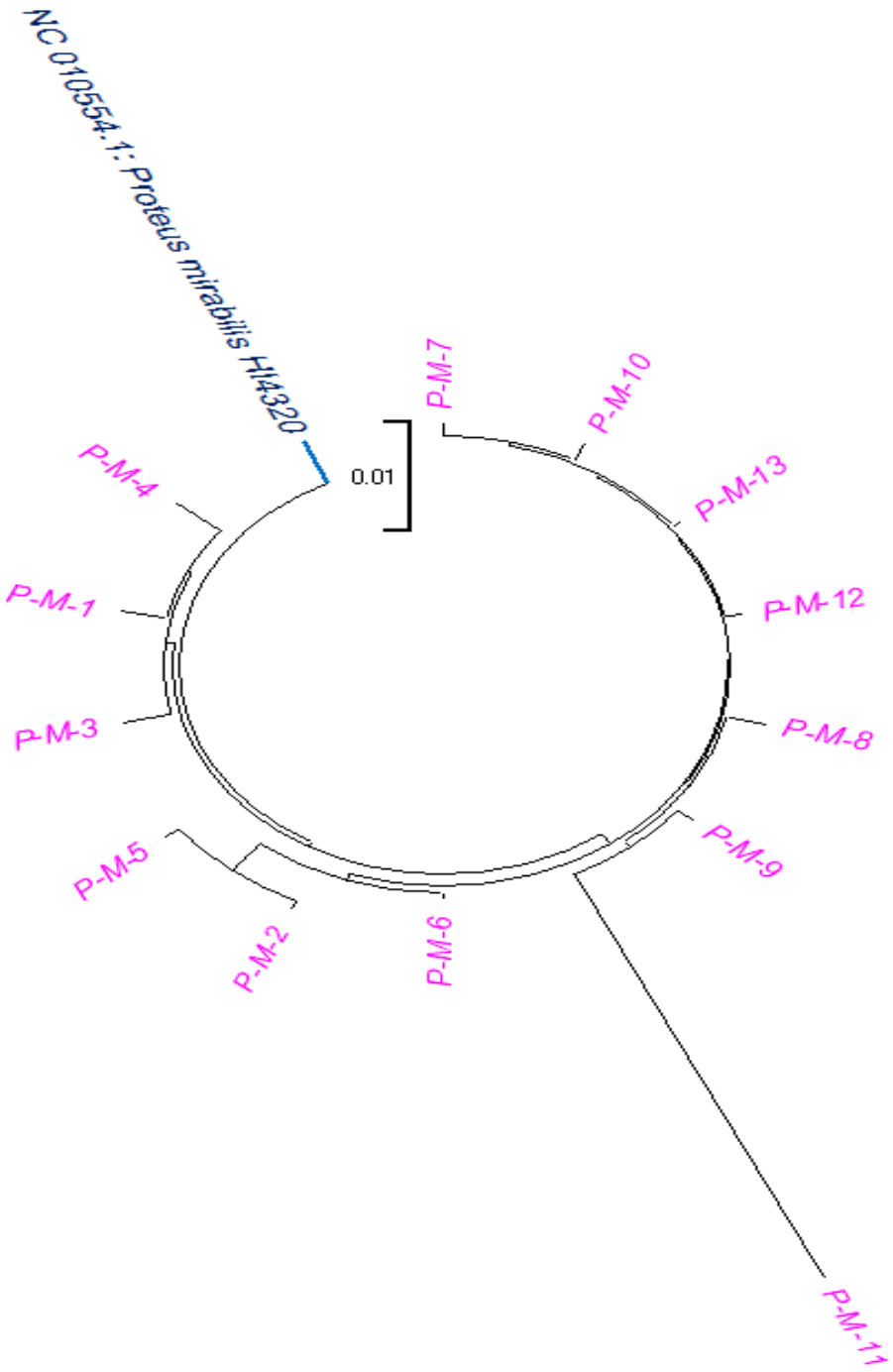

Fig. (3-8): Phylogenetic tree of all studied *Proteus mirabilis* strains based on concatenated dnaJ, mdh, pyrC, recA and rpoD gene sequences. The tree is based on a total of 3890 positions in the final dataset. The tree is drawn to scale in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Neighbor-Joining method.

Multi-locus sequence analysis (MLSA) based on concatenated segments of housekeeping genes is used in phylogenetic studies to resolve taxonomic relationships among closely related species (Mulet *et al.,* 2010; Gonz_alez *et al.,* 2013; Tambong *et al.,* 2017).

Multi-locus sequence analysis (MLSA) is a powerful high-resolution method which provides data on genetic changes in housekeeping genes and could be served as a valid technique for the study of epidemiological relationships (Kämpfer and Glaeser, 2012). In fact, MLSA is able to compare the primary DNA sequences of multiple conserved proteincoding loci in order to assess the diversity and relationship between different isolates and to determine the sources and evolutionary alterations of different taxa. To obtain a higher resolution of the phylogenetic relationships of species within a genus or genera within a family, multilocus sequence analysis (MLSA) is currently a widely used method. In MLSA studies, partial sequences of genes coding for proteins with conserved functions (‘housekeeping genes’) are used to generate phylogenetic trees and subsequently deduce phylogenies.

Multilocus sequence analysis (MLSA) has become the standard for phylogenetic analyses of bacterial species and has been shown to be a powerful molecular method for microbial population genetic studies. This method consists of the analysis of multiple (usually four to eight) conserved housekeeping genes, which encode proteins that are essential for the survival of the organism (Cooper and Feil, 2004; Gevers *et al.,* 2005; Almeida *et al.,* 2010).

MLSA relies on the concatenation of aligned DNA sequences from each gene. Mutations within housekeeping genes are largely assumed to be selectively neutral, and therefore are more likely to correctly reflect the phylogeny of the strains (Gevers *et al.,* 2005; Hajri *et al.,* 2012). In addition, the concatenated sequence data could reduce the weight of horizontal gene transfer 'HGT' (Macheras *et al.,* 2011) and/or recombination (Timilsina *et al.,* 2015), which may provide more reliable phylogenetic relationships among closely related strains (Glaeser and Kämpfer, 2015). MLSA has been successfully used to describe the genetic structure of several phytopathogenic bacteria (Castillo and Greenberg, 2007; Young *et al.,* 2008; Almeida *et al.,* 2010; Trantas *et al.,* 2013; Tancos *et al.,* 2015; Constantin *et al.,* 2016).

In MLSA, fragments of approximately 500 bp length of usually five marker genes are sequenced and the combined sequence profiles are used for the phylogeny analysis (Jolley and Chan, 2004). MLSA is more useful in strain level identification within a species. For MLSA, a set of housekeeping genes are considered based on the sequence variability among the particular species of bacteria. In certain cases, the MLSA approach also uses highly variable genes that have direct implications in its phenotypic characteristics.

Zeigler, (2003) even suggested that sequence analysis of less than five suitable housekeeping genes might be sufficient for a reliable classification. Multilocus sequence analysis (MLSA), using the sequences of multiple protein-coding genes for genotypic characterization of a group of prokaryotes (Gevers *et al.*, 2005), is used increasingly to study and elucidate taxonomic relationships between species (Wertz *et al.*, 2003; Ade´kambi and Drancourt, 2004; Holmes *et al.*, 2004; Naser *et al.*, 2005; Thompson *et al.*, 2005). MLSA is distinct from multilocus sequence typing (Maiden *et al.*, 1998), a method that is used mostly in epidemiology to characterize strains at an infraspecific level by comparing allelic mismatches in housekeeping genes.

Finally, an actual example will be given of how MLSA has been applied for the re-evaluation of prokaryotic genera and species within genera. In this case, the application of MLSA to members of the family Enterobacteriaceae will be illustrated. Brady *et al.*, (2008) established a MLSA scheme that has been applied to date in several studies for clarifying the problematic taxonomic situation within the family Enterobacteriaceae, which contains several paraphyletic in distinguishable genera based on the 16S rRNA gene phylogeny. As a consequence of an MLSA study, different species were reclassified into new genera and known genera were emended.

Table (3-10): Polymorphisms in *DnaJ* gene of 13 *Proteus mirabilis* clinical strains with different sources compared to the reference strain

No	Source	Nucleotide Change	Base Number	Amino Acids	Type of Mutation
1	Urine	TAT→TGT	192307	Tyr → Cys	Missense
		GCG→GTG	192355	Ala → Val	Missense
		GTA→GCA	192462	Val → Ala	Missense
2	Urine	ACC→ACT	192444	Thr→Thr	Silent
		ACT→ACC	192648	Thr→Thr	Silent
		GCA→GCG	192738	Ala→Ala	Silent
		GTG→GTA	192816	Val→Val	Silent
3	Urine	ATT→GTT	192307	Ile → Val	Missense
		TAG→CAG	192462	Stop→ Gln	Read throgh
		CTG→TTG	192570	Leu → Leu	Silent
		GCG→ ACG	192636	Ala → Thr	Missense
		TTT→CTT	192648	Phe →Leu	Missense
		CGC→AGC	192728	Arg → Ser	Missense
		AGG→GGG	192738	Arg →Gly	Missense
		GCC→ACC	192816	Ala → Thr	Missense
4	Urine	ATT→GTT	192307	Ile → Val	Missense
		GCG→ACG	192636	Ala → Thr	Missense
		TTT→CTT	192648	Phe →Leu	Missense
		CGC→AGC	192728	Arg → Thr	Missense
		AGG→GGG	192738	Arg → Gly	Missense
		GCC→ACC	192816	Ala → Thr	Missense
5	Urine	CTG→TTG	192444	Leu → Leu	Silent
		TTT→CTT	192648	Phe →Leu	Missense
		AGG→GGG	192738	Arg →Gly	Missense
		GCC→ACC	192816	Ala → Thr	Missense
6	Burn	CTA/TTA	192204	Leu→Leu	Silent
		ATT/GTT	192307	Ile → Val	Missense
		TAG/CAG	192462	Stop→ Gln	Read throgh
		CTG/TTG	192570	Leu→Leu	Silent

		GCG→ACG	192636	Ala → Thr	Missense
		TTT→CTT	192648	Phe →Leu	Missense
		CGC→AGC	192728	Arg → Ser	Missense
		AGG→GGG	192738	Glu →Gly	Missense
		GCC→ACC	192816	Ala → Thr	Missense
7	Burn	CCT→CTT	192204	Pro → Leu	Missense
		TAT→TGT	192307	Tyr → Cys	Missense
		GTA→GCA	192462	Val → Ala	Missense
		CCT→CTT	192570	Pro → Leu	Missense
		AGC→AAC	192636	Thr → Asn	Missense
		CTT→CCT	192648	Leu → Pro	Missense
		GCG→GAG	192728	Ala → Glu	Missense
		CAG→CGG	192738	Gln → Arg	Missense
8	Vagina	CTA→TTA	192204	Leu → Leu	Silent
		ATT→GTT	192307	Ile → Val	Missense
		TAG→CAG	192462	Stope→ Gln	Read throgh
		CTG→TTG	192570	Leu → Leu	Silent
		GCG→ACG	192636	Ala → Thr	Missense
		TTT→CTT	192648	Phe →Leu	Missense
		CGC→AGC	192728	Arg → Ser	Missense
		AGG→GGG	192738	Arg →Gly	Missense
9	Vagina	CTA→TTA	192204	Leu → Leu	Silent
		ATT→GTT	192307	Ile → Val	Missense
		TAG→CAG	192462	Stope→ Gln	Read throgh
		CTG→TTG	192570	Leu → Leu	Silent
		GCG→ACG	192636	Ala → Thr	Missense
		TTT→CTT	192648	Phe →Leu	Missense
		CGC→AGC	192728	Arg → Ser	Missense
		AGG→GGG	192738	Arg →Gly	Missense
		GCC→ACC	192816	Ala → Thr	Missense
10	Vagina	CCT→CTT	192204	Pro → Leu	Missense
		TAT→TGT	192307	Tyr → Cys	Missense
		GTA→GCA	192462	Val → Ala	Missense
		CCT→CTT	192570	Pro → Leu	Missense
		AGC→AAC	192636	Ser → Asn	Missense
		CTT→CCT	192648	Leu → Pro	Missense
		GCG→GAG	192728	Ala → Glu	Missense
		CAG→CGG	192738	Gln → Arg	Missense

11	Wound	CTA→TTA	192204	Leu → Leu	Silent
		ATT→GTT	192307	Ile → Val	Missense
		TAG→CAG	192462	Stope→ Gln	Read throgh
		CTG→TTG	192570	Leu → Leu	Silent
		GCG→ACG	192636	Ala → Thr	Missense
		TTT→CTT	192648	Phe →Leu	Missense
		CGC→AGC	192728	Arg → Ser	Missense
		AGG→GGG	192738	Arg →Gly	Missense
		GCC→ACC	192816	Ala → Thr	Missense
12	Wound	CCT→CTT	192204	Pro → Leu	Missense
		TAT→TGT	192307	Tyr → Cys	Missense
		GTA→GCA	192462	Val → Ala	Missense
		CCT→CTT	192570	Pro → Leu	Missense
		AGC→AAC	192636	Ser → Asn	Missense
		CTT→CCT	192648	Leu → Pro	Missense
		GCG→GAG	192728	Ala → Glu	Missense
		CAG→CGG	192738	Gln → Arg	Missense
		TGC→TAC	192816	Cys → Tyr	Missense
13	Wound	CCT→CTT	192204	Pro → Leu	Missense
		TAT→TGT	192307	Tyr → Cys	Missense
		GTA→GCA	192462	Val → Ala	Missense
		CCT→CTT	192570	Pro → Leu	Missense
		AGC→AAC	192636	Ser → Asn	Missense
		CTT→CCT	192648	Leu → Pro	Missense
		GCG→GAG	192728	Ala → Glu	Missense
		CAG→CGG	192738	Gln → Arg	Missense
		TGC→TAC	192816	Cys → Tyr	Missense

Table (3-11):Polymorphisms in *mdh* gene of 13 *Proteus mirabilis* clinical strains with different sources compared to the reference strain

No. strains	Source	Nucleotide Change	Base Number	Amino Acids	Type of Mutation
2	Urine	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent
3	Urine	GTC→GTT	281693	Val → Val	Silent
		GCC→GTC	281551	Ala → Val	Missense
5	Urine	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent
6	Burn	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent
7	Burn	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent
8	Vagina	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent
9	Vagina	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent
10	Vagina	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent
11	Wound	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent
12	Wound	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent
13	Wound	CCC→CCT	281717	Pro→Pro	Silent
		TCC→TCT	281510	Ser→Ser	Silent

Table (3-12):Polymorphisms in *pyrC* gene of 13 *Proteus mirabilis* clinical strains with different sources compared to the reference strain

No. strains	Source	Nucleotide Change	Base Number	Amino Acids	Type of Mutation
1	Urine	CTA→CAA	2385729	Leu → Gln	Missense
		TAA→TGA	2385912	Stop→ Stop	Silent
		TTA→TCA	2386122	Leu →Ser	Missense
		ATT→ACT	2386182	Ile → Thr	Missense
2	Urine	TTT→TTC	2385808	Phe→Phe	Silent
		CTG→CCG	2385843	Leu →Pro	Missense
		ATG→ATA	2386213	Met → Ile	Missense
		GGC→GAC	2386233	Gly → Asp	Missense
3	Urine	CCT→CCA	2385729	Pro→Pro	Silent
		CTT→CTC	2385906	Leu→Leu	Silent
		GTA→GTG	2385912	Val→Val	Silent
		CAT→CAC	2386182	His→His	Silent
		AGG→AGA	2386233	Arg→Arg	Silent
4	Urine	CTA→CAA	2385729	Leu→ Gln	Missense
		TTA→TCA	2385906	Leu→ Ser	Missense
		TAA→TGA	2385912	Stop→ Stop	Silent
		TGG→TGA	2386012	Trp → Stop	Nonsense
		ATT→ACT	2386182	Ile → Thr	Missense
5	Urine	TTT→TTC	2385808	Phe→Phe	Silent
		CTG→CCG	2385843	Leu →Pro	Missense
		TTA→TCA	2386122	Leu →Ser	Missense
		ATG→ATA	2386213	Met → Ile	Missense
		GGC→GAC	2386233	Gly → Asp	Missense
6	Burn	CTA→CAA	2385729	Leu→ Gln	Missense
		CTG→CCG	2385843	Leu →Pro	Missense
		TAA→TGA	2385912	Stop→ Stop	Silent
		GGC→GAC	2386233	Gly → Asp	Missense
7	Burn	CTA→CAA	2385729	Leu→ Gln	Missense
		CTG→CCG	2385843	Leu →Pro	Missense
		TAA→TGA	2385912	Stop→ Stop	Silent
		GGC→GAC	2386233	Gly → Asp	Missense
8	Vagina	CTA→CAA	2385729	Leu→ Gln	Missense
		CTG→CCG	2385843	Leu →Pro	Missense

		TAA→TGA	2385912	Stop→ Stop	Silent
		GGC→GAC	2386233	Gly → Asp	Missense
9	Vagina	CTA→CAA	2385729	Leu→ Gln	Missense
		CTG→CCG	2385843	Leu →Pro	Missense
		TAA→TGA	2385912	Stop→ Stop	Silent
		GGC→GAC	2386233	Gly → Asp	Missense
10	Vagina	CAA→CTA	2385729	Gln → Leu	Missense
		CTG→CCG	2385843	Leu →Pro	Missense
		TAA→TGA	2385912	Stop→ Stop	Silent
11	Wound	CTA→CAA	2385729	Leu→ Gln	Missense
		CTG→CCG	2385843	Leu →Pro	Missense
		TAA→TGA	2385912	Stop→ Stop	Silent
12	Wound	CTA→CAA	2385729	Leu→ Gln	Missense
		CTG→CCG	2385843	Leu →Pro	Missense
		TAA→TGA	2385912	Stop→ Stop	Silent
13	Wound	TAG→AAG	2385729	Stope→ Lys	Read throgh
		TGC→CGC	2385843	Cys → Arg	Missense
		AAT→GAT	2385912	Asn → Asp	Missense

Table (3-13):Polymorphisms in *recA* gene of 13 *Proteus mirabilis* clinical strains with different sources compared to the reference strain

No. strains	Source	Nucleotide Change	Base Number	Amino Acids	Type of Mutation
1	Urine	CGG→CAG	1228533	Arg → Gln	Missense
3	Urine	ACA→ACG	1228722	Thr→Thr	Silent
		GTC→GTG	1228658	Val→Val	Silent
		AGC→AGT	1228596	Ser → Ser	Silent
		GTA→GTG	1228590	Val→Val	Silent
		ACG→CCG	1228116	Thr → Pro	Missense
4	Urine	GGT→GAT	1228622	Gly → Asp	Missense
		GCA→GTA	1228596	Ala → Val	Missense
		TAT→TGT	1228590	Tyr→ Cys	Missense
		CGG→CAG	1228533	Arg → Gln	Missense
6	Burn	AGT→AGA	1228407	Ser → Arg	Missense

7	Burn	GTG→GAG	1228407	Val → Glu	Missense
8	Vagina	AGT→AGA	1228407	Ser → Arg	Missense
9	Vagina	AGT→AGA	1228407	Ser → Arg	Missense
		ACG→CCG	1228116	Thr → Pro	Missense
10	Vagina	GTG→GAG	1228407	Val → Glu	Missense
12	Wound	AGT→AGA	1228408	Ser → Arg	Missense
13	Wound	GTG→GAG	1228408	Val → Glu	Missense

Table (3-14):Polymorphisms in *rpoD* gene of 13 *Proteus mirabilis* clinical strains with different sources compared to the reference strain

No. Strains	Source	Nucleotide Change	Base Number	Amino Acids	Type of Mutation
2	Urine	CAG→CCG	3254724	Gln → Pro	Missense
		CGA→TGA	3254726	Arg → Stop	Nonsense
		CGA→TGA	3255050	Arg → Stop	Nonsense
3	Urine	CAC→TAC	3254777	His→ Try	Missense
		GCA→ACA	3255149	Ala → Thr	Missense
4	Urine	CTT→ TTT	3254597	Leu→Phe	Missense
		AAG→GAG	3255130	Lys→Glu	Missense
		CAG→CCG	3254724	Gln→ Pro	Missense
5	Urine	CAG→CCG	3254724	Gln→ Pro	Missense
		CGA→TGA	3254726	Arg → Stop	Nonsense
		CGA→TGA	3255050	Arg → Stop	Nonsense
6	Burn	CAG→CCG	3254724	Gln → Pro	Missense
11	Wound	CAG→CCG	3254724	Gln→ Pro	Missense

Conclusion

And

Recommendation

Conclusions

This study reached the following conclusion:

1.Using of (*UreR*) gene for earlier identification of *P.mirabilis* infections.

2. High sensitivity for levofloxacin , imipenem , meropenem , aztreonam , ciprofloxacin and amikacin indicates that these drugs are currently have a good activity for *P.mirabilis.*

3. It was found that the presences of some virulence factor genes in *P. mirabilis* as (*hpmA* and *Pta*) at high percentage increase pathogenicity of this pathogen.

4. The *rsbA* showed the lower percentage among *P. mirabilis* isolates.

5. The MLSA scheme provides a rapid, economical and precise identification of Proteus strains (95-99%).

Recommendations

1. More genetic studies on gene level are needed to determine the virulence factors of *Proteus* by using genetic methods such as: PCR, and sequencing techniques.

2. Using modern rapid molecular assays to identify *P. mirabilis.*

3. Re-evaluation of antibiotics currently used to treat UTI, vagina, wound, burn infection especially after the bacteria have developed resistance to most of them.

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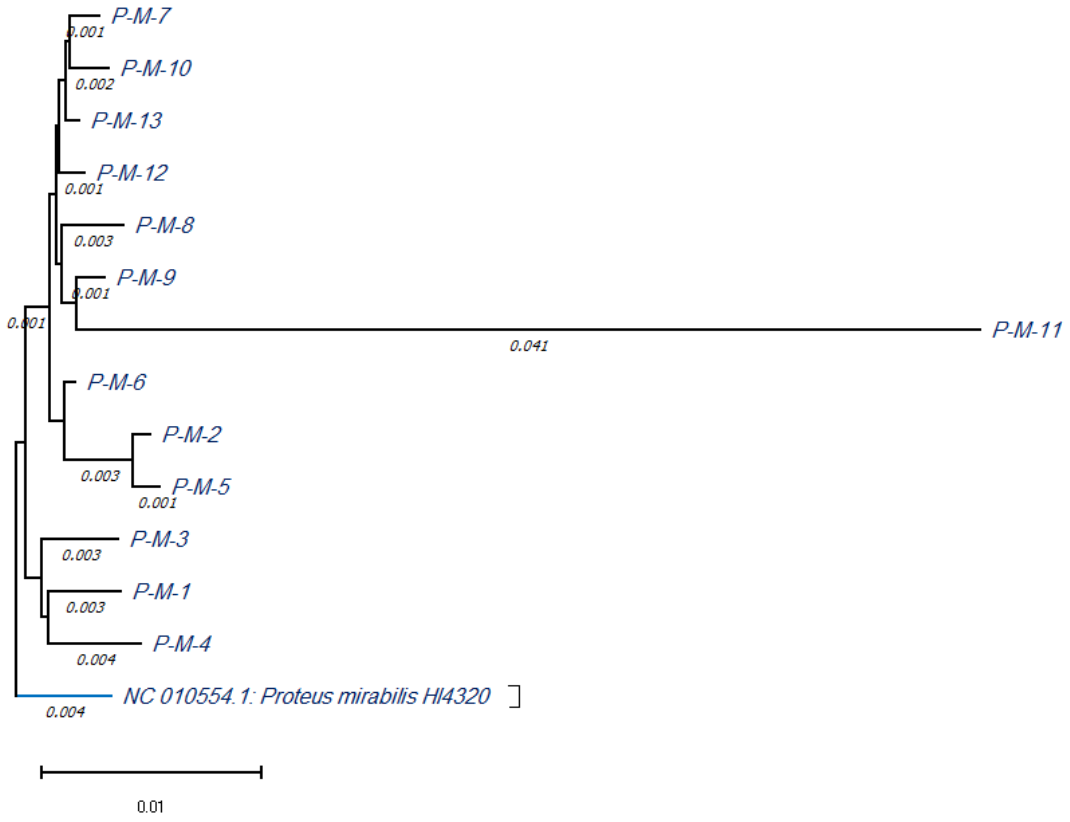
Appendices

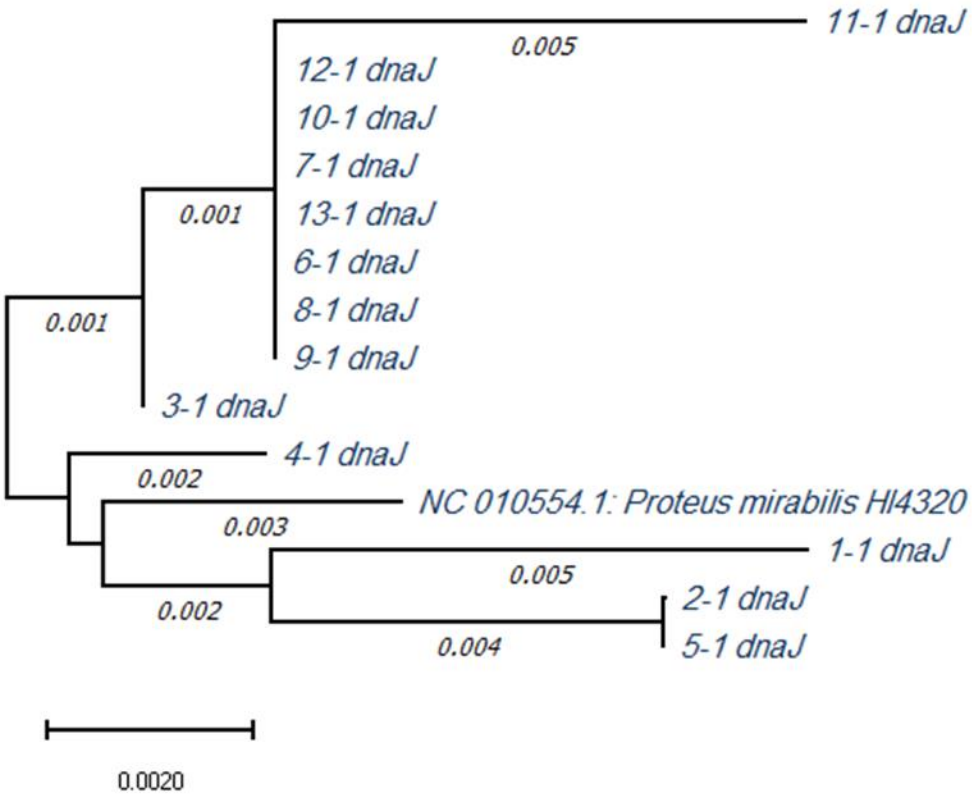
Appendix (1): Similarity Matrix of Whole concatenated genes (dnaJ, mdh, pyrC, recA and rpoD) against the reference genome of *Proteus mirabilis*. Similarity differences were illustrated above by color of conservation.

	NC_010554.1	P-M-1	P-M-2	P-M-3	P-M-4	P-M-5	P-M-6	P-M-7	P-M-8	P-M-9	P-M-10	P-M-11	P-M-12	P-M-13
NC_010554.1	100 %	99%	99%	99%	99%	99%	99%	99 %	99%	99 %	99 %	95 %	99%	99%
P-M-1	99%	100 %	99%	99%	99%	99%	99%	99 %	99%	99 %	99 %	95 %	99%	99%
P-M-2	99%	99%	100 %	99%	99%	100 %	100 %	99 %	99%	99 %	99 %	95 %	99%	99%
P-M-3	99%	99%	99%	100 %	99%	99%	99%	99 %	99%	99 %	99 %	95 %	99%	99%
P-M-4	99%	99%	99%	99%	100 %	99%	99%	99 %	99%	99 %	99 %	95 %	99%	99%
P-M-5	99%	99%	100 %	99%	99%	100 %	100 %	99 %	99%	99 %	99 %	95 %	99%	99%
P-M-6	99%	99%	99%	99%	99%	100 %	100 %	100 %	100 %	100 %	100 %	96 %	100 %	100 %
P-M-7	99%	99%	99%	99%	99%	99%	100 %	100 %	99%	100 %	100 %	95 %	100 %	100 %
P-M-8	99%	99%	99%	99%	99%	99%	100 %	99 %	100 %	99 %	99 %	95 %	99%	99%
P-M-9	99%	99%	99%	99%	99%	99%	100 %	100 %	99%	100 %	99 %	96 %	100 %	100 %
P-M-10	99%	99%	99%	99%	99%	99%	100 %	100 %	99%	100 %	100 %	95 %	100 %	100 %
P-M-11	95%	95%	95%	96%	95%	95%	96%	96 %	96%	96 %	96 %	100 %	96%	96%
P-M-12	99%	99%	99%	99%	99%	99%	100 %	100 %	100 %	100 %	100 %	96 %	100 %	100 %
P-M-13	99%	99%	99%	+99 %	99%	99%	100 %	100 %	100 %	100 %	100 %	96 %	100 %	100 %

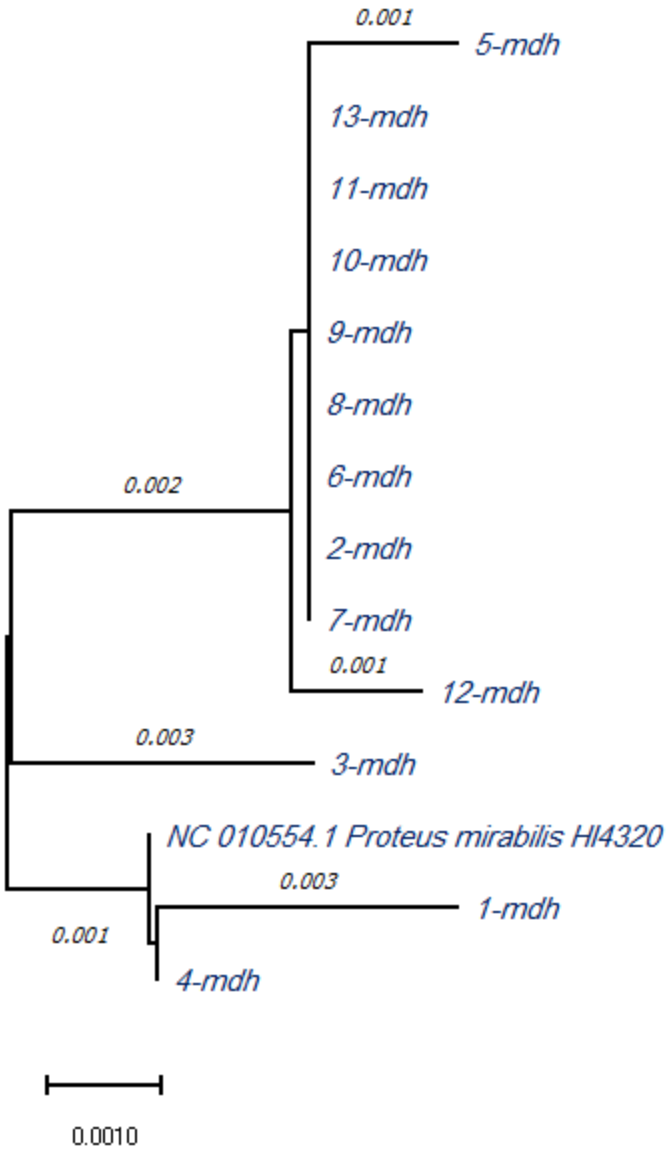
Appendix (2): Phylogenetic tree of all studied Proteus mirabilis strains based on concatenated *dnaJ, mdh, pyrC, recA* and *rpoD* gene sequences. The tree is based on a total of 3890 positions in the final dataset. The tree is drawn to scale, with branch lengths (below the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Neighbor-Joining method.



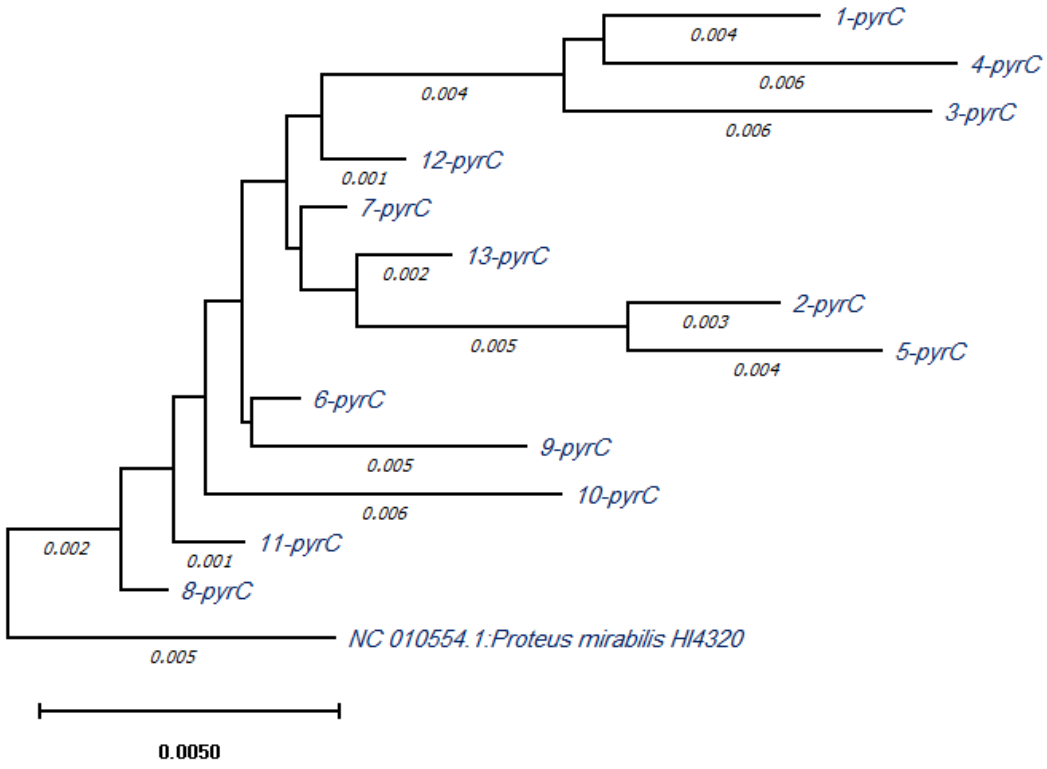
Appendix (3): Phylogenetic reconstructions of Proteus mirabilis strains based on *dnaJ* gene and their identification with the reference. Strain number of each species is shown in parentheses. The scale bar indicates substitutions per site. The evolutionary distances were computed using the Neighbor-Joining method.



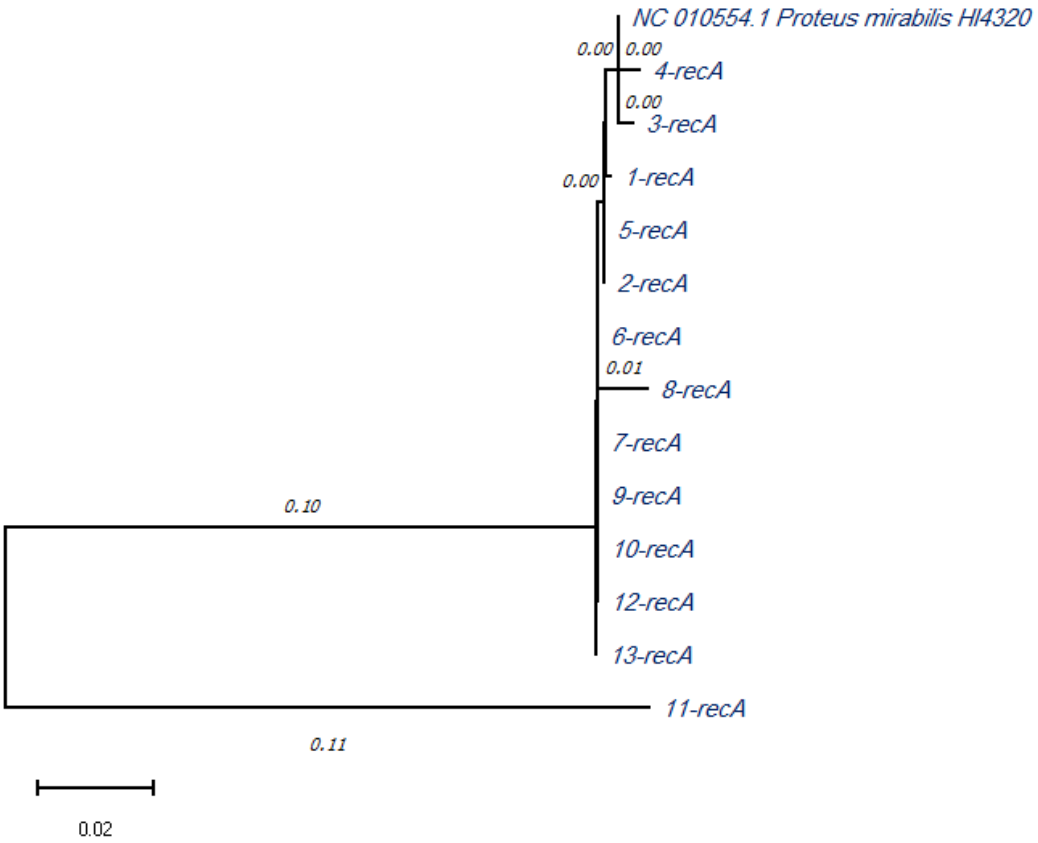
Appendix (4): Phylogenetic reconstructions of Proteus mirabilis strains based on *mdh* gene and their identification with the reference. Strain number of each species is shown in parentheses. The scale bar indicates substitutions per site. The evolutionary distances were computed using the Neighbor-Joining method.



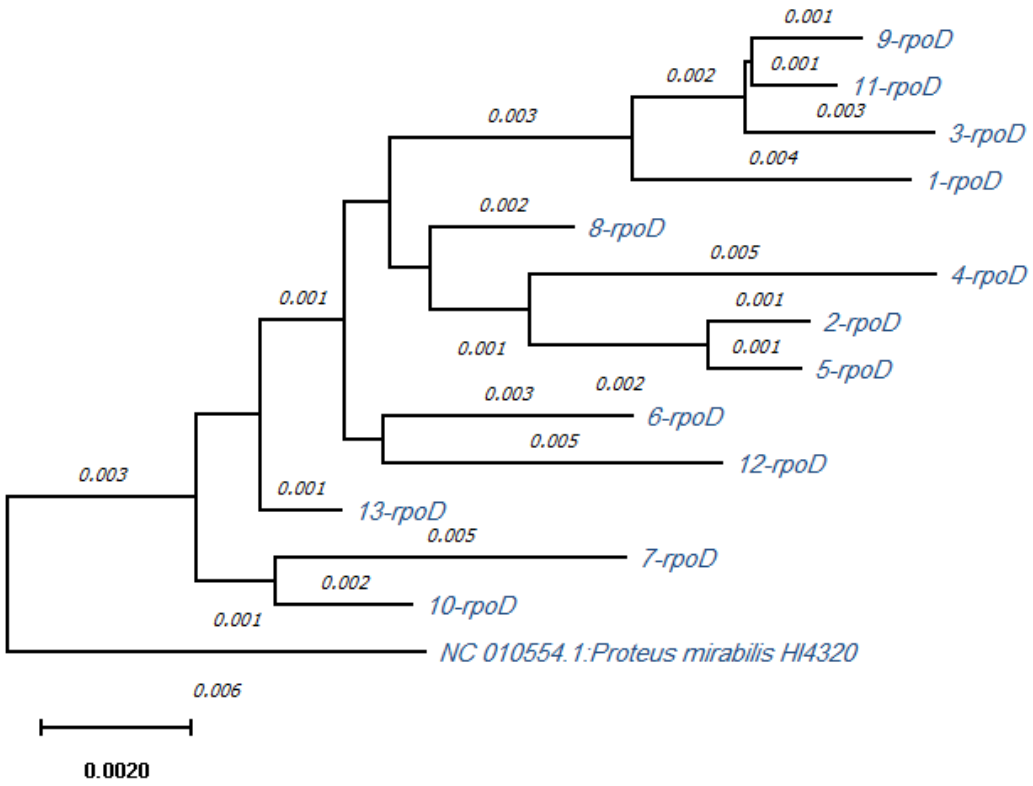
Appendix (5): Phylogenetic reconstructions of Proteus mirabilis strains based on *pyrC* gene and their identification with the reference. Strain number of each species is shown in parentheses. The scale bar indicates substitutions per site. The evolutionary distances were computed using the Neighbor-Joining method.



Appendix (6): Phylogenetic reconstructions of *Proteus mirabilis* strains based on *recA* gene and their identification with the reference. Strain number of each species is shown in parentheses. The scale bar indicates substitutions per site. The evolutionary distances were computed using the Neighbor-Joining method.



Appendix (7): Phylogenetic reconstructions of *Proteus mirabilis* strains based on *rpoD* gene and their identification with the reference. Strain number of each species is shown in parentheses. The scale bar indicates substitutions per site. The evolutionary distances were computed using the Neighbor-Joining method.



الخـــــــــلاصة

في هذه الدراسة تم الحصول على (130) عينة من مرضى يعانون من التهاب المسالك البولية ، التهاب المهبل ، الجروح و الحروق من كلا الجنسين ومن مستشفى الحلة التعليمي العام في مدينة الحلة خلال الفترة من أيلول إلى كانون الأول (2021) في محافظة بابل.

من أصل 130 عينة ، كانت 25 (19.2٪) موجبة لـ *Proteus mirabilis* باستخدام اختبارات الكيمياء الحيوية ، ونظام VITEK2 المضغوط ، ومقايسة PCR باستخدام بوادئ الخاصه بجين ureR. اذ كانت 17 (68٪) عزله بكتريا من مرضى التهاب المسالك البولية و 3 (12٪) عزله لكل من المرضى الذين يعانون من التهاب المهبل ومرضى الجروح و 2 (8٪) معزله من مرضى الحروق .

استخدم اساس PCR للتحري عن جين (*ureR*) . وقد لوحظ أن جين (*ureR*) وجود في 25 عزلة (100٪) من *P. mirabilis* التي تضم جميع عزلات التهاب المسالك البولية و التهاب المهبل و الجروح و الحروق .

على المستوى الجزيئي، تم إجراء التنميط الجيني لعزلات *P. mirabilis* للتحري عن جينات الضراوة باستخدام طريقة تفاعل البلمرة المتسلسل(PCR) ، شملت هذه الدراسة التحري عن ثلاثة جينات مهمة لعوامل الضراوة وهي (*hpmA, rsbA,* and *pta*).

تم التحري عن الجين *hpmA* ، باستخدام بوادئ PCR المحددة للحمض النووي الجيني لكل العزلات. اشار تفاعل البلمرة الى وجود 24 (96٪) عزله من *P. mirabilis* والتي تشمل (17) عزله من مرضى التهاب المسالك البولية و (2) عزله لكل من مرضى الجروح والحروق و (3) من مرضى الذين يعانون من التهاب المهبل. كذلك اشاره تفاعل البلمره المتسلسل للجين (*rsbA*) الى أن 20 (80٪) عزلة فقط من *P. mirabilis* من اجمالي 25 عزله تحتوي هذا الجين والتي تضم (15) عزله من مرضى التهاب المسالك البولية و (2) عزله لكل من المرضى الذين يعانون من التهاب المهبل ومرضى الجروح و (1) عزله من مرضى الحروق.

كما تم الكشف عن الجين (*pta*) في *P. mirabilis* باستخدام البادئ PCR المحددة. وقد وجد أن الجين (*pta*) لوحظ في 23 عزلة (92٪) من *P. mirabilis* التي تضم (15) عزلة من مرضى التهاب المسالك البولية و (3) عزله لكل من المرضى الذين يعانون من التهاب المهبل ومرضى الجروح و(2) عزله من مرضى الحروق .

تحليل تسلسل متعدد البؤرة هو لتحديث تصنيف الجنس *Proteus* وتحديده وإعادة تصنيف أنواع جينات *Proteus*. في هذه الدراسة ، تم استخدام نهج تحليل تسلسل متعدد التركيز (MLSA) على أساس خمسة جينات Housekeeping Genes (HKGs) لتحديد العلاقات التطورية للأنواع داخل جنس *P. mirabilis* .

أظهر تحليل مصفوفة التشابه لعينات تمت دراستها من *P.mirabilis* مقابل الجينوم المرجعي نسبة تشابه عالية مع جميع العينات ، حيث يتشتركوا بنسبة (95-99٪) مع التسلسل المرجعي ، مما أدى إلى وجود تباين تطوري للعزلات المدروسة مقارنةً بالعزلات الجينوم المرجعي. علاوة على ذلك ، تم اكتشاف أعلى نسبة تباين في العينة(11).

تم إخضاع جميع عزلات *P. mirabilis* التي تم الحصول عليها لاختبارات الحساسية للمضادات الحيوية مع (13) مضاد حيوي تنتمي إلى فئات مختلفة وفقًا لتوصيات CLSI (2021).

لوحظت أعلى معدلات الحساسية للمضادات الحيوية المستخدمة في الدراسة الحالية لمضادات ليفوفلوكساسين، ايميبينيم ، ميروبينيم، أزتريونام، سيبروفلوكساسين وأميكاسين بنسبة 100٪، 100٪، 100٪، 88٪ ،88٪ و84٪ على التوالي، تليها نسبة اقل من الحساسية لمضادات الجيل الثالث من سيفوتاكسيم ، أمبيسيلين- سب بكتام، أموكسيسيلين ، تتراسيكلين ، جنتاميسين ، سلفاميثوكسازول- ترايميثوبريم وكلورامفينيكول بنسبة 72%، 64%، 56%، 52% ، 48%، 32٪ ، 20٪ على التوالي .

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل/ كلية الطب

دراسة التنميط الوراثي بين سلالات سريرية لبكتريا *Proteus mirabilis*

رسالة مقدمة إلى

مجلس كلية الطب / جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير في العلوم/ الأحياء المجهرية الطبية

من قبل

اقبال حربي كاظم جلعوط

بكالوريوس علوم حياة/جامعة بابل/ 1998

دبلوم عالي أدله جنائية/ 2018

اشراف

الأستاذ الدكتورة

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1444 هـ 2022م