Republic of Iraq

Ministry of Higher Education

and Scientific Research

University of Babylon, College of Science

Department of Biology

Molecular Characterization of *bla* $_{TEM}$, *bla* $_{CTX-M}$ and *bla* $_{SHV}$ Genes in *Proteus Species* Isolates

A Thesis

Submitted to the Council of College of Science, University of Babylon as a Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

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B. Sc. Biotechnology /College of Science / University of Babylon (2017)

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2023 A.D **1445A.H.**

بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

وَلَقَدۡ ءَاتَيۡنَا دَاوُۥدَ وَسُلَيۡمَٰنَ عِلۡمٗاۖ وَقَالَا ٱلۡحَمۡدُ لِلَّهِ ٱلَّذِي فَضَّلَنَا عَلَىٰ كَثِيرٖ مِّنۡ عِبَادِهِ ٱلۡمُؤۡمِنِينَ ١٥

صدق الله العلي العظيم

سورة النمل (الآية ١٥)

Dedication

I dedicate this thesis to:

My father who was proud of me, enthusiastic, and supporting during my education.

My mother for her endless patience and encouragement during this hard work.

My sister who has been supporting and encouraging me.

My brothers and all my family for their love, encouragement, and support.

My supervisor for his great kindness and unlimited care.

My professors and teachers.

My friends and colleagues.

To all with warm regards.

Asraa

Acknowledgments

Foremost, my thanks to Allah, the almighty, for granting me the blessings, and giving me the power and energy to conduct this humble work. I would like to express my deepest gratitude to my supervisor Dr. Hassan Fadhil Naji for his efforts, encouragement, valuable comments, and suggestions. Without his support and careful guidance, this research work would not have been possible. Sincere respect goes to the deanship and its employees, teachers who taught me the right way, and affiliated members of the Department of Biology, College of Science, and the University of Babylon to provide me with an opportunity to confer my M.Sc. I would like to express my heartfelt gratitude to my parents and my family who supported and encouraged me to complete my study. Last but not least, I'm very grateful to all those who assisted and supported me to complete this dissertation.

Asraa

Summary

Proteus are motile, lactose-negative, urease-producing, Gram-negative, rod-shaped bacteria that can distinguish from ordinary enterobacterial bacilli into very elongated rods covered with millions of flagella, generating swarming colonies. They are part of the enterobacteriaceae family.

In this study isolation and identification of *P. vulgaris* and *P. mirabilis* from urine specimens of patients suffering from urinary tract infections (UTIs).Two Hundred and Ten urine specimens were taken from patients at the Imam Sadiq Teaching Hospital, Hilla Teaching Hospital, and Marjan Teaching Hospital in Babylon. The samples of this study were gathered between September 2021 and July 2022.

The current investigation involved 40 isolates of *P. mirabilis* and *P. vulgaris* screened isolates from 210 clinical urine samples (named PM1 to PV40). These isolates were identified depending on the morphological traits of the colonies and cells, biochemical testing, and VITEK 2 compact. It thrived on blood agar, MacConkey agar, and UTI chromogenic agar media. It moved and produced a thin filmy coating of concentric circles that mimic the ripples created by tossing a rock into a lake because of a swarming growth on blood agar. On MacConkey agar, however, it does not swarm and produce colonies that were smooth, light, or colorless.

The antibiotic susceptibility test was performed against 11 types of antibiotics, using disc diffusion method according to Clinical Laboratory Pathogenic Bacteria Standard Institute, CLSI-2022. The majority of isolates exhibited antibiotic resistance, particularly β-lactamase

antibiotics. It was found that most of the bacterial isolates possess multiple resistance to the tested antibiotics. The results showed that the most effective antibacterial against *P. mirabilis* and *P. vulgaris* isolates were erythromycins with high resistance (97.5 %), followed by tobramycin 5 with (85 %), ampicillin with (82.5 %), chloramphenicol (60 %), piperacillin (55 %) and sulfamethoxazole, azithromycin with resistance (52.5 %), while other antibiotics showed lower resistance and high sensitivity such as meropenem, imipenem showed low resistance to *P. mirabilis* and *P. vulgaris* isolates with (35 %), ciprofloxacin (30 %), gentamicin (15 %), and imipenem (12.5 %).

The microtiter plate test, which was thought to be the most sensitive, has been used to study bacterial biofilm formation. The ability of *P. mirabilis* and *P. vulgaris* to produce biofilm was investigated. 40 *Proteus* bacterial isolates (*P. mirabilis* and *P. vulgaris*) were examined, and it was discovered that all (100%) of the isolates formed a biofilm with the following ratios: 15% of the biofilm was weak, 77.5% was moderate, and 7.5% is strong.

Polymerase Chain Reaction has been used to investigate 3 genes for extended-spectrum β-lactamase (ESBLs) in *P. mirabilis* and *P. vulgaris* by using a specific primer for each gene of bla_{TEM}, bla_{SHV}, and bla_{CTX-M}. After performing the electrophoresis, the results showed that positive beta-lactamase PCR results contained 33 (82.5%) *SHV*, 38 (95%) *TEM*, and 37 (92.5%) *CTX-M* genes. It was found that all isolates of *P. mirabilis* were at a rate of 97-100 % identical to the sources of the isolates identified in the gene bank (NCBI), and the results showed that the highest matching rate of isolates was 100 % which originated in Iraq and India. This study revealed that *the* bla_{TEM} gene was the most frequent gene among these isolates, followed by *the* bla_{CTX-M} gene and then by *bla* *SHV*.

Because *P. mirabilis* bacteria were more prevalent than *P. vulgaris* in this study, eight *P. mirabilis* isolates were chosen for the sequencing study. The whole genomic DNA of eight isolates, PM1 to PM8, isolated from different geographic regions of Iraq, was extracted. The sequencing of *bla* $_{TEM}$, *bla* $_{SHV}$, and *bla* $_{CTX\text{-}M}$ genes was performed, and the sites of these genes on the genome of the isolates were determined. The phylogenetic tree analysis, using the MEGA X10.2.4 software program was achieved, and the matching of the current study isolates with NCBI-Gen bank global *P .mirabilis* strains, found that 4 isolates (PM1, PM2, PM3, and PM4) were related to India, two isolates (PM5 and PM8) were related to Iraq and one (PM6) isolate was related to Egypt. Therefore, additional research is required to create a genetic diversity map of *P. mirabilis* due to variable frequencies in the sequencing of *bla* $_{TEM}$, *bla* $_{CTX\text{-}M}$, and *bla* $_{SHV}$ genes of the isolates.

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List of abbreviations

Symbol	Description
AMP	Ampicillin
AZM	Azithromycin
BI	Bayesian inference
bp	Base pair
C30	Chloramphenicol
CIP10	Ciprofloxacin
CN10	Gentamicin
DHP-I	Dehydropeptidase I
E15	Sulfamethoxazole
ESBL	Extended-Spectrum B-Lactamases

HAUTIs	healthcare UTI_S
HCL	strong hydrochloric acid
IgA	Immunoglobulin A
IMI	Imipenem
IV	intravenously
KIA	Kligler iron agar
MCMC	Markov Chain Monte Carlo
MDR	Multi Drug Resistances
ME	minimal evolution
MHA	Muller Hinton agar
MIC	Minimum Inhibitory Concentration
ML	Maximum Likelihood
MP	Maximum Parsimony
MR	methyl-Fox Proscauer
MRK	Mannose-Resistant *Klebsiella*-like
MRP	Meropenem
NJ	Neighbor-Joining
PRL	Piperacillin
Pta	*Proteus* toxic agglutinin
SMX	Erythromycin
TCP	Tissue Culture Plates
TM5	Tobramycin
UTI	Urinary Tract Infection
VP	Voges proskaure

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Chapter One
Introduction

Introduction

Proteus spp. are motile, lactose-negative, urease-producing, Gram-negative, rod-shaped bacteria that can distinguish from ordinary enterobacterial bacilli into very elongated rods covered with millions of flagella, generating swarming colonies. They are part of the enterobacteriaceae family (Donnenberg *et al*., 2015).

Ten percent of urinary tract infections, cystitis, polio-nephritis, prostatitis, ulcer, eye, and intra-abdominal infections are caused by *Proteus spp*., an opportunistic member of the enterobacteriaceae family. The pathogens *P. vulgaris*, *P. mirabilis*, and *P. penneri* frequently infect immunocompromised people. Furthermore, alkalinization by *Proteus* members results in 15% of nephrolithiasis. The production of ESBLs by enterobacteriaceae is a widespread public health concern Peirano and Pitout, 2019; Ramadan *et al*.,2019). They create the hydrolytic enzymes needed to break down oxyimino-beta-lactam antibiotics. Several ESBL genes, including as bla_{TEM} and bla_{SHV}, contribute to the dissemination of isolates that are resistant to β-lactam antibiotics (Cullik *et al*.,2010). By gene mutation and horizontal gene transfer, the steady evolution of antibiotic resistant strains has caused the expression of resistance genes in antibiotic-sensitive bacterial strains, increasing the prevalence of MDRs globally(Algammal *et al*.,2020). According to several studies, MDR pathogens can come from a variety of sources, including people, animals, birds, seafood, and food(Makharita *et al*.,2020).

Many β-lactamases, which are responsible for resistance to β-lactam antibiotics in pathogenic bacteria, are inducible enzymes, and the mechanisms of induction have been studied by many investigators. However, studies on the kinetics of induced β-lactamase formation in Gram-negative bacteria have been more difficult than those on β-galactosidase formation in *Escherichia coli.* One reason for this is a lack of appropriate gratuitous inducers, such as isopropyl-thiogalactoside and methyl-l-thio-β-D-galactoside for β-galactosidase, which themselves are not substrates of the enzyme. Another difficulty is the growth inhibitory properties of the β-lactamase inducers;

several milligrams of an inducer antibiotic per milliliter are needed for maximum induction of β-lactamase in Gram-negative bacteria such as *Pseudomonas aeruginosa, Enterobacter cloacae, and Citrobacter freundii,* and growth is greatly impaired by these high concentrations of the antibiotic. The β-lactamase of *P. vulgaris*, however, can be induced by relatively low concentrations of β-lactam antibiotics and hence *P. vulgaris* is a suitable organism for kinetic analysis of induced β-lactamase synthesis in Gram-negative bacteria. It explains the kinetics of β-lactamase formation by clinical isolates of *P. vulgaris (*Minami *et al.*,1980), each with different β-lactamase activity. Plasmid-mediated extended-spectrum β-lactamases (ESBLs) capable of degrading the expanded-spectrum cephalosporins and monobactams are among the most important resistance determinants emerging worldwide in enterobacteriaceae. Strains producing ESBLs are resistant to the above-mentioned compounds and often exhibit a multidrug-resistant phenotype, including resistance to aminoglycosides and fluoroquinolones, leaving only a few reliable therapeutic options. Infections caused by *ESBL* producers are associated with increased morbidity, mortality, and healthcare-associated costs.

The three primary techniques for creating phylogenetic trees are character-based techniques, validation techniques, and distance matrix techniques (Hall *et al*.,2013). Although all mutations are assumed to be neutral by distance matrix methods, which accept a molecular clock, they provide a random clock-like rate (Wooding *et al*.,2004). Neighbour-joining (NJ) is the distance matrix technique that is most frequently utilized. "Jackknife estimate" and "bootstrapping" are examples of validation procedures. Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses are the character-based techniques that are most frequently utilized. One or more trees will be selected more strictly with the fewest alterations required to explain the character distribution seen in the data since maximum parsimony is an optimality criterion. According to (Kück *et al*.,2013), this strategy is sensitive to insufficient taxon sampling, allowing "long branch attraction," and is applicable to similar sequences or a collection of sequences. The nucleotide substitution tree model used by

the ML approach will select the tree that is "most likely to observe the data" out of all the trees in the specific dataset. A likelihood function is used in the character-based Bayesian inference (BI) approach to determine the posterior probability of trees. To offer a measure of precision, the Markov Chain Monte Carlo (MCMC) technique takes samples from a probability distribution. It is a measure of how strongly a topology or group of typologies are supported rather than how much variety there is in the data. Of course, the variety in the data has something to do with this. It is not, however, a straight forward correlation. All of these techniques only provide estimates of how a phylogenetic tree would appear for a certain collection of data. The majority of good techniques also show how variable these estimations are (Algammal *et al*.,2021).

Aim of Study

The aim of this study is to determine the incident of extended- spectrum β-lactamase (*bla* $_{TEM}$, *bla* $_{CTX-M}$ *and bla* $_{SHV}$) genes among the isolates of *P. vulgaris*, *P. mirabilis*, in addition to find the phylogenic relationships among these isolates which reflect the international source of these bacteria in Iraq.

Objectives of Study

1.Isolation and identification of *P. vulgaris* and *P. mirabilis* from urine specimens of patients suffering from urinary tract infections (UTIs).

2.Determination of the antibiotic susceptibility tests of the isolates against the common antibiotics.

3.Detection of *bla* $_{TEM}$, *bla* $_{CTX-M}$ *and bla* $_{SHV}$ genes using molecular methodology.

4.Comparative analysis of the a date of this study with the strains of the gene bank of NCBI to find the DNA variations .

Chapter Two

Literatures Review

Literatures Review

2.1 Urinary Tract Infection

UTIs are regarded as one of the most common causes of medical consultations on a global scale. Considered to be one of the world's leading causes of morbidity. There are two types of UTIs: difficult and simple. Infections linked to weakened immune systems and damaged urinary tracts make up the majority of complicated UTIs. Immunosuppression, urine retention caused by neurological impairment, renal transplantation, renal failure, pregnancy, or foreign substances such as biofilms, calculi, catheters, and other devices are common conditions associated with complex UTIs. The most frequent cause of subsequent bloodstream infections, catheter-associated UTIs (CAUTIs) are associated with higher rates of morbidity and mortality. The most common kind of UTIs called non-complicated UTIs, don't have a neurological, anatomical, or physiological basis and typically afflict women, kids, and elderly individuals. They are separated into lower tract UTIs and upper tract UTIs in the nephron (pyelonephritis) (cystitis) (Negut and Buiuc, 2008).

One of the most typical illnesses affecting women is a urinary tract infection. UTI is typically brought on by germs from the digestive tract and frequently occurs in conjunction with vaginal infections. Because of the architecture of the female lower urinary system and its closeness to the reproductive organs, women are far more susceptible to UTIs than males. Since the female urethra is comparatively short, less space is available for bacteria to enter. As vulvar vestibulitis and vaginitis are common, it also opens into the vulvar vestibule, a site that is very susceptible to infections. In this situation, the natural vaginal microbiota is frequently harmed by sexual activity as well as excessive use of intimate hygiene products. On the other

hand, the anus's proximity makes it easier for bacteria like *E. coli*, enterobacteriaceae, and *Streptococcus* species to colonize the reproductive system as well as the distal portions of the urinary tract. Other distinctive periods characterized by frequent urinary tract infections include pregnancy and the postpartum period. Although the infection initially appears to be unharmful, as the stage advances, the patient exhibits a variety of symptoms that, in extreme cases, can be fatal. According to studies, the most prevalent type of bacterial illness is urinary tract infection (Mohammed *et al* ., 2014).

2.2 Causes of UTI

Although viruses and other types of fungi can also cause infections in humans, bacteria are the main cause of these infections. However, it is thought that UTIs caused by viral or fungus infections are a rare occurrence (Mohammed *et al* ., 2014). In all population groups, enterobacteriaceae account for more than 95% of UTI cases, making them the most significant cause of UTIs. *Escherichia coli* is by far the most prevalent invader among these microorganisms, accounting for 90% of UTIs in outpatients and 50% of those in hospitalized patients. In catheterized individuals and those with anomalies of the urinary tract, *Proteus* species is a frequent source of urinary tract infections (UTI). It prefers the higher urinary tract, where it can seriously harm the kidneys. bacteremia, pyelonephritis acute, bladder or kidney stones, injury, or acute kidney failure. Urinary tract infections (UTI), which fall into two categories: hematogenous infections and ascending infections, are particularly essential for *Proteus* bacilli (Gupta *et al.*, 2001).

2.3 Epidemiology UTI

Proteus Spp. are widely distributed in the environment and can be detected in the human gastrointestinal tract (Armbruster *et al* ., 2012). UTIs are the most typical illnesses brought on by *Proteus species*. Before the commencement of bacteria, the

vaginal introitus has been observed to be colonized by *Proteus spp*. As a result, *Proteus spp*. causes urinary tract infections similar to *E. coli* by moving up from the rectum to the periurethral and bladder (Melzer *et al*., 2013).

Before being classified as group 2, *P. vulgaris* has been linked to UTIs, bloodstream infections, burn infections, and respiratory tract infections. Additionally, there has been one case study where *P. vulgaris* caused bacteremia and brain abscesses, with the digestive tract being thought to be the point of entrance. In the United States, gram-negative bacteremia occurs as a result of genitourinary tract infections in 35% of patients (Jamil *et al*., 2020).

2.3.1 Uncomplicated UTIs

The most frequent infections treated by outpatients in the United States are UTIs. The likelihood of developing a UTI rises with age, except for a peak in young women between the ages of 14 and 24. Approximately 20% of women over the age of 65 have the condition, compared to 11% of the general population. Around 10% of postmenopausal women report having a UTI in the previous year, and between 50% and 60% of adult women will experience at least one UTI in their lifetime. Etiology in older postmenopausal women varies depending on their health status, residential status (institutionalized or not), age, the presence of diabetes mellitus, history of/current catheterization, spinal cord dysfunction, and history of antibiotic use. Most UTIs in non-catheterized older adults are caused by a single bacterial species. However, in the presence of structural abnormalities and catheterization, it is not unusual to isolate more than one species in the urine culture. The increased use of catheters and instrumentation in these patients predisposes them to UTIs caused by Gram-negative rods such as *Proteus, Klebsiella, Serratia*, and *Pseudomonas*. In patients with diabetes mellitus, infections caused by *Klebsiella, Enterobacter, and Candida* are more common.

Flores - Mireles *et al*. (2015) reported that an uncomplicated UTI starts with contamination of the periurethral with bacteria from the patient's intestines (the ascending route) (step 1), which can then colonize the urethra and move to the bladder (step 2). This allows pili and adhesins bacteria to colonize the bladder and invade the surface cells of the host, inducing (step 3) Some bacteria can evade the immune system and increase their population by penetrating the host's cells or by changing their shape (step 5). Biofilm formation (step 6) encourages the production of toxins and protease enzymes from extracellular bacteria (step 4). Step 7: Bacterial injury to host cells encourages the release of vital resources from harmed cells, bacterial survival, ascent into the kidney, colonization of the kidney, and generation of bacterial toxins. Step 8: Without therapy, damage to host tissues (step 10) causes bacteria to pass through the cell wall, resulting in bacteremia (step 11). The same beginning stages are followed by urine pathogens that cause complicated urinary tract infections. The urethra-area colonization (step 1), invasion of the urethra, and invasion of the bladder are the ones that are described for simple infections (step 2). The kidney must be infected for bacteria to be similar to bladder catheterization (step 3), it builds up fibrinogen on the catheter, which produces an excellent environment for pathogens to bond with, infection causes neutrophil infiltration (step 4), and pathogens start to grow after binding fibrinogen-coated catheter (step 5). (step 6). Encourage epithelial cell deterioration (step 7). Kidney epithelial infection (steps 8, 9) promotes the formation of toxins and tissue harm (step 10).

2. Acute Wound

In the literature or clinical practice, there is no precise description of what constitutes a chronic wound. Because the healing process does not move forward, chronic wounds do not heal in the typical amount of time. A wound is deemed chronic, according to studies in the literature, after two to eight weeks of a drawn-out healing phase. Because of the development of biofilms in the wound environment,

chronic wound infections are challenging to treat. Among *Proteus* species, *P. mirabilis* is the most frequent etiological agent of infection. Among other virulence traits, it exhibits a potent capacity for biofilm formation. The biofilm restricts and delays the healing process in the majority (75. %) of chronic wounds. Although its presence does not equate to infection, it may be the cause of an infection. Antiseptic and antimicrobial-resistant bacteria become more prevalent and granulation is prolonged by biofilm (Kwiecińska-Piróg *et al* . 2020).

Accidental injuries and pathological wounds (post-operative wounds) are two different types of wounds. Regardless of the type of wound, infection is the association of microorganisms with host cells and their effort to expand and settle on them, harming the host's tissues (Mohammad *et al* ., 2013). Acute wounds are those that come from external injury to the skin for a brief duration, such as those from operations, bites, scratches, and bruising wounds, while chronic wounds are more common and last for00a00longer periods because they harm the integrity or cohesion of the dermis. Other skin tissues, acute and chronic wounds, and a variety of aerobic and anaerobic microbes, including bacteria, fungi, and parasites, can all cause inflammation (Bowler *et al* ., 2001). Therefore, although many of them are not appropriate for treating infected wounds, the use of broad-spectrum antibiotics is necessary for the successful treatment of infected wounds because failure to do so increases the resistance of bacterial strains that cause infection to those antibiotics (Adenike *et al*.,2012).

2.3.2 Medically Related UTIs

Infectious side effects after urological surgery Protocols are a big problem, especially in the context of rising antibiotic resistance. UTIs connected to healthcare (HAUTIs) are among all healthcare-associated illnesses, the most prevalent subtype. The substantial and ongoing Global Prevalence Study on the GPIU research Infections in urology seeks to learn more from an international viewpoint on HAUTIs. spanning

through (2003 and 2010) 19,756 individuals were evaluated, representing 9.4% of those who had a HAUTI diagnosis (70.4% of them were women).

2.4. Risk Factor of UTI

The principal anatomical risk factors for A UTI occurs when the urethra and anus are separated by less than 4.5 cm (Hooton *et al*., 1999). The majority of organisms that infect the urinary tract come from the digestive tract (Nielubowicz and Mobley, 2010; Epp and Laro-chelle, 2017).There are some common risk factors for UTIs that are different for men, women, and children, while other risk factors are universal.

Keeping Pee In Bad germs can accumulate in the bladder if don't use the restroom when need to or don't empty it when do. renal stones urinary system can become blocked by kidney stones, which will prevent the regular flow of urine. Diabetes can result in elevated blood and urine sugar levels. Higher urine sugar concentrations may encourage the development of bacteria. using a bladder or urinary catheter recently to save you from having to urinate on your own, these flexible tubes drain your bladder's urine into a bag. They are typically following some procedures. having previously experienced a UTI. Other elements that could raise the risk of UTIs include Sexual behavior, pregnancy and Age (older adults and young children are more likely to get UTIs), urinary tract structural issues, such as an enlarged prostate, and poor hygiene.

2.5 *Proteus spp.*

The human digestive system contains commensal enterobacterales called *Proteus spp*. *P. mirabilis* also frequently contributes to urinary tract infections (Girlich *et al* ., 2020). Rods that are gram-negative, of average size, not sporulated, and motile belong to the genus *Proteus*. One of the most

widespread *Proteus* species is *P. mirabilis*. In addition, it is a common environmental microbe that is found throughout nature (Algammal *et al*., 2021). The family enterobacteriaceae includes the genus *Proteus*, which was initially identified by Hauser in 1885. Along with the genera *Morganella* and *Providencia*, it belongs to the *Proteeae* tribe within this family (Rozalski and Staczek, 2011). The majority of other genera can be distinguished from *Proteus* species by their propensity to swarm through agar surfaces of solid media. Five species, including *P. mirabilis, P. vulgaris, P. penneri, P. hauseri, and P. myxofaciens*, as well as three unnamed *Proteus genomospecies*, make up the genus *Proteus* today. The only *Proteus* species that has no bearing on human pathogenicity is *P.myxofaciens*, which has been isolated from both living and dead larvae of the gypsy moth *Porteria* dispar (O'hara *et al*., 2000; Janda *et al*., 2006). *Proteus* microorganisms are abundant in the natural environment and can be found in contaminated soil, water, and manure. These bacteria participate in the breakdown of organic matter of animal origin due to their proteolytic activity, capacity to hydrolyze urea into ammonia and carbon dioxide, and ability to oxidatively deamine amino acids. Urinary tract infections (UTIs), both in ordinary hosts and in patients with indwelling catheters and anatomic or functional abnormalities of the urinary tract, are most frequently linked to *Proteus spp*.

Along with *E.coli* and *Klebsiella* species, of which *E. coli* is the main inhabitant, *Proteus* species are part of the natural flora in the human digestive tract (Struble *et al*., 2009). *P. mirabilis* may readily and quickly develop biofilms, which can cause foreign objects, like urinary tract catheters, to get contaminated. This is because of the flagella it has and the adhesins it produces. Its propensity to produce biofilms and the development of UTIs are related. Additionally, *Proteus* Spp. are frequently found in patient blood, more frequently in the context of a UTI. It's significant to note

that *Proteus* species have also been linked to several illnesses other than urinary tract infections, including primary bacteremias and, less frequently, wound infections and infections of the respiratory tract (Ioannou *et al* ., 2020).

A 90% of *Proteus* infections in people are caused by *P. mirabilis*, which is most frequently acquired from urine and wound infections. Unlike indole-positive species, *P. mirabilis* is not implicated in nosocomial infection. Humans seldom get *P. penneri* infections, which are limited to infections of the urine, and wounds in the belly, groin, neck, and ankle. UTI-causing *P. vulgaris* has a higher level of antibiotic resistance. The Proteus species can carry the genes encoding antibiotic resistance, making them particularly difficult to eliminate in patients with impaired immune systems, severe wounds, long-term catheterization, and complicated wounds (Ramalingam *et al*.,2021).

2.5.1Classification and environmental distribution of *Proteus Spp*.

Proteus is a nosocomial, opportunistic pathogen that is more prevalent in infections that are acquired in the community. Wound infections, respiratory tract infections, and both catheter-associated and community-acquired urinary tract infections have all been linked to *P. vulgaris* and *P. mirabilis* (UTI). There have likewise been reports of diarrhea in cats and dogs, as well as renal and urinary infections in companion animals. Several virulent factors help increase the pathogenicity of *Proteus spp*., and these virulent factors are controlled by virulent genes transcribed in operons (Pathirana *et al*., 2018). The Morganellaceae family includes the *Proteus* spp., They can be found in various hosts and are widely distributed to commensal gut bacteria. The current members of the genus are *P. mirabilis, P. penneri, P. hauseri, and unidentified genomospecies* 4, 5, and 6 are among the species.

Kingdom : Bacteria

Phylum : Proteobacteria

Class : Gamma Proteobacteria

Order: Enterobacteriales

Family: *Enterobacteriaceae*

Genus : *Proteus*

Species : *Proteus mirabilis* , *Proteus vulgaris*, *P. penneri, P. hauseri unidentified genomospecies* 4, 5, and 6 are among the species (Hauser, 1885).

These bacteria are separated from many scientific sources, including wounds and urine, and are regarded as opportunistic human pathogens. Numerous studies have shown their contributions to the pathogenesis of infections in people, demonstrating how the bacteria's virulence factors allow them to colonize and persist in various host organism habitats. However, it is known that *Proteus spp*. is a typical, widespread component of the human flora, and the gut acts as a reservoir for these proteolytic bacteria. *Proteus* Spp. have been linked to many different species in previous studies in hostile or commensal ways. Occasionally, researchers have also found cooperative relationships between *Proteus* species and their diverse animal hosts, which allow them to function as animal symbionts. The complex relationship between *Proteus* species and the animals that live in the same habitat as the bacterium is still unknown. There is currently little knowledge on the interactions between *Proteus spp*. bacteria and their natural settings (Wang *et al*., 2020).

2.5.2 Mode of transmission

Proteus can spread via a variety of suspect sources, including contaminated food, water, soil, healthcare workers' hands, patients' hands, equipment, and even intravenous solutions. Due to their widespread habitat, they have the potential to infect several anatomical areas throughout the body. They are frequently found in bodily fluids, ear and vaginal swabs, sputum, pus, wound infections, bronchoalveolar lavage, epidural ulcers, and long-term indwelling catheters (Ramalingam *et al*., 2021).

Human intestinal flora contains *Proteus spp*., which can spread infection after leaving the area. They can also spread accidentally through parenteral inoculation or infected catheters, particularly urinary catheters. However, the precise communication method is yet unknown (Abbott *et al*., 2007).

2.5.3 Pathogenicity of *Proteus spp*.

Proteus rods are opportunistic bacterial pathogens that, in the right circumstances, can lead to UTIs, which are frequently linked to more serious UTIs. They typically affect the upper urinary tract, which is a major location of infection. As a result, infections such as acute pyelonephritis, cystitis, and urolithiasis (stone development in the kidney or bladder) can occur. There have also been a few isolated reports of *Proteus spp*. bacteremia linked to UTIs. Other illnesses include rheumatoid arthritis, septicemia, wound infections, meningitis in newborns, and meningitis in adults *Kalra et al*. (2011). *Proteus* species endocarditis was reviewed by Bloch *et al*. (2010) along with *P. mirabilis and Pneumonia*.

It should be emphasized, nevertheless, that *Proteus* bacteria cause UTIs more frequently. Infections of this kind might be categorized as simple or complex. Simple infections affect people who are otherwise healthy, whereas complicated infections

typically affect people who have a urinary catheter in place, have structural or functional abnormalities in the urinary tract, are immunocompromised, are suffering from another illness, or have recently had surgery on the urogenital system. *E. coli* was discovered to be a frequent cause of simple illnesses. Gram-negative bacteria like *Proteus spp. , Providencia stuartii, Morganella morganii, E. coli, P. aeruginosa, K. pneumonia* as well as some Gram-positive bacteria, which are commonly the cause of complicated UTIs, may be polymicrobial. Hematogenous infections and ascending infections are both possible with *Proteus species*, however, the latter is more typical for these germs. a summary of important *P. mirabilis* virulence factors are involved in the colonization and obstruction of catheters, infection of the bladder and kidneys (cystitis and pyelonephritis), and the development of urinary stones. IgA, immunoglobulin A, MRK, mannose-resistant *Klebsiella*-like, MRP, mannose-resistant *Proteus-like*,(PMFs) *P. mirabilis* fimbriae, (Pta) *Proteus* toxin agglutinin, (ZapA) serralysin (Mohammed *et al*.,2016).

2.5.4 Virulence Factors

1. Motility

The primary virulence factor in *P. mirabilis* that affects the invasion and spread of infection in urinary tract segments is motility (Kuan *et al*., 2014). The infection starts in the periurethral area, invades the urethra, and then spreads to the bladder and other parts of the urinary tract (Hickling *et al.*,2017). Motility actively encourages engagement with these sites (Batirel *et al*.,2020). A peritrichous flagellated bacteria is *P. mirabilis* (Fattorini *et al*.,2020). This microbe has swarming motility, and when it swarms, the expression of virulence is amplified.

2. Quorum sensing

Cell-cell interaction is used by a variety of bacteria to regulate gene expression and sense population density (Stankowska *et al*.,2012). A luxS homolog found in *P. mirabilis* generates AI2 (Younis *et al*.,2016). Swimming or swarming motility, swarming cell differentiation, or the *in vitro* virulence is test are unaffected by this quorum sensing mechanism (Wang *et al*.,2006). However, the signaling molecule AI-2 generated by *P .mirabilis* can affect gene expression in animals that use it (Schneider *et al*.,2002).

3. Immune Evasion

Innate and adaptive immune responses will not occur in the presence of bacteria in the host (Norsworthy and Pearson,2017). *P. mirabilis* can evade detection in a variety of ways (Belas *et al*.,2004). The metalloproteinase (ZapA) is encoded by serum and secretory immunoglobulin A1 (IgA1), A2 (IgG), and *P. mirabilis* cleaves (Liu *et al.*,2015). The recovery of urinary bacteria, bladder bacteria, and kidney bacteria is drastically reduced as a result of the ZapA mutation (Arciola *et al*.,2018). It can change how flagellin and MR/P are expressed, deceiving the immune system (Zhang *et al*.,2015). As previously indicated, the neuropathogenesis of *P. mirabilis* is typical of stone formation (Fusco *et al*.,2017). This incident aids in the retention of urine, the development of a bacterial reservoir, the prevention of washout, and immune system evasion (Habibi *et al*.,2015).

4. Adhesion

Fimbriae are responsible for *P. mirabilis* bacterial adherence, which is a crucial stage in colonization and infection development (Hasan *et al*., 2020). The following are the five types of common fimbriae that are linked to infection:-

a. Uroepithelial cell adhesion (UCA/NAF) fimbriae:

These fimbriae have the shape of long, flexible rods and play a crucial role in the uroepithelial cell's ability to adhere to one another as well as in the colonization of the urinary system (Jiang *et al*.,2018). This uca operon contains *PMI0532–PMI0536* genes from the HI43201 genomic sequence (Chahales and Thanassi, 2017).

b. *P. mirabilis* P-like pili (PMP) fimbriae:

P. mirabilis strain was originally discovered in PMP fimbriae in a dog with a urinary illness (Debnath *et al.,*2018). PMP is present in the human *P. mirabilis* uropathogenic strain *HI4320* (Sun *et al*.,2019). There are nine genes in the human genome structure (PMI2216–PMI2224) (Baldo and Rocha, 2014). To confirm PMP fimbriae's involvement in *P. mirabilis* pathogenesis, more research is necessary (Tsai *et al*.,2017).

c. Mannose-resistant / *Proteus*-like (MR/P) fimbriae:

This type of fimbria, which was built along the usher-chaperone pathway, is the most suited form in *P. mirabilis*. The genes *mrpABCDEFGHJ* and *mrpl* make up the clustered gene *MR/P*. (Aljanaby and Aljanaby, 2018). The smaller subunits of the major fimbrial structural subunit code for *mrPBEFG*, the chaperone for *mrpD*, the usher for *mrpC*, the protein for *mrpH* for the fimbria pilin, and the protein for *mrpJ* for the repressive flagellar regulon transcription. From on to off (*mrp* operon transcription) or from off to on (halt *mrp* operon transcriptions), *mrpI* encodes the invertible component of the recombinase. The formation of these fimbriae was consistent with pyelonephritis (Majeed and Aljanaby, 2019).

d. Ambient-temperature fimbriae (ATF)

The ATF fimbriae are crucial to *P. mirabilis* ambient way of life (Hasan *et al* ., 2020). Only at 23˚C do these fimbriae manifest themselves most optimally (Roh *et al*

.,2010). The *P. mirabilis* colonization host's fimbriae are not necessary for the ATF mutant strain or the wild strain that causes urinary tract infection (Simms and Mobley *et al*., 2008).

e. *Proteus spp*. fimbriae (PMF)

Five functional genes, *pmfACDEF,* are responsible for the genetic organization of PMF fimbriae (Bode *et al*., 2016). The role of PMF fimbriae was previously shown to be introduced but not in the kidneys, in the bacterial cells' colonization of the lower urinary tract. More recent studies have shown that this role is essential for the colonization of bacterial cells in the bladder and kidneys (Sarshar *et al*., 2020).

2.6 Toxins and Enzymes

a. *Proteus* toxic agglutinin (Pta)

Proteus toxic agglutinin, a protein with The outer membrane autotransporter, which regulates cell aggregation, has a catalytic domain that can lyse kidney and bladder cells (Gupta *et al*.,2019). Reduced pathology and a severe colonization deficiency in the kidneys, spleen, and urine were both present in the *P. mirabilis* negative Pta gene (Engel *et al*., 2007).

b. Hemolysin:

A poison called hemolysin enters the eukaryotic cell's target membrane, creating holes that lead to ion efflux and ultimately cell disintegration (Cabezas *et al*., 2017). Hemolysin encourages the propagation of bacterial infections in the kidney and causes ascending UTIs to progress to pyelonephritis (Chan *et al*.,2019). *P. mirabilis* hemolysin genes (*hpmA* and *hpmB*) are a two-part secretion (Dal Peraro and Van Der Goot, 2016). *HpmA* is reported to be located outside the membranes involved in the *HpmA* secretion cycle in the periplasm, where *HpmB* binds and activates it. Human renal proximal tubular epithelial cells generated by *HpmA* display cytotoxicity

(Etxaniz *et al*.,2020). The *hpmA* mutant *P. mirabilis* infection and the wild-type strain have the identical potential for colonization (Hertle *et al*.,2005). Due to the presence of specific virulence factors, this hemolysin is probably not as present during *in vivo* infection or masks its role (Ostolaza *et al*.,2019).

c. Urease:

Urease is essential to the pathophysiology of *P. mirabilis*, which promotes the production of kidney and bladder stones and/or inhibits the urinary tract (Flannery *et al*.,2009). For, the pathogenesis of *P. mirabilis*, urease is crucial (Ranjbar *et al*.,2015). This enzyme causes the urinary tract to encrust or get blocked as well as stones to form in the kidney and bladder (Armbruster *et al*.,2017). The multimeric nickel metalloenzyme causes the pH to rise, hydrolyzes urea into ammonia and dioxide, and precipitates multifunctional urine ions that lead to the formation of stones, is codified by the cluster of urease genes (*ureRDABCEFG*) (Alamuri *et al*.,2009). This pH adjustment is necessary for *P. mirabilis* catheter colonization because it promotes bacterial adherence and the formation of biofilm incrustation (Carlini and Polacco, 2008). *P. mirabilis* infection causes stone formation, which has several benefits including evading the host immune system, ureter blockage, ammonia exposure to the host cells, and obvious tissue damage (Konieczna *et al*.,2012). These findings point to a nutrient-rich, protective habitat for the bacterium (Rutherford *et al*.,2014).

2.7 β-lactamases (Extended-Spectrum β-Lactamase)

Multiple medication resistance can be caused by resident gene mutations or resistance agents on chromosomes. It might, however, grow by acquiring resistance genes via horizontal transfer. These resistance genes cause the issue of rapid dissemination and treatment failure since they are commonly found on plasmids, transposons, and integrons. Most *P. mirabilis* isolates were previously vulnerable to common antibiotic classes, however, recent research from many nations has shown

that *P. mirabilis* isolates are becoming more resistant to antibiotics. The synthesis of several classes of ESBLs has been documented in the β-lactam resistance patterns of *P. mirabilis* isolates. Class 1 integrons, which are frequently seen in clinical isolates and are strongly related to antibiotic resistance, have received a great deal of attention. The second important kind of integrons derived from clinical isolates continues to include class 2 integrons. In the nonreplicative transposon, class 2 integrons are typically introduced. The spread of ESBLs signals a serious threat to control nosocomial infectious illnesses, complicating the selection of appropriate antibiotic treatments. Investigations have been made into the incidence of integrons and described gene cassettes in Gram-negative bacteria with integron-associated multidrug resistance. However, *P. mirabilis* rarely discusses it. One of the last-resort medications for treating infections brought on by MDR Gram-negative bacteria is colistin. Although this bacterium is naturally resistant to colistin, it is frequently ignored and not tested for plasmid-mediated colistin-resistant (*mcr*) genes. However, this bacterium can act as a reservoir to pass these genes to bacteria that are colistin-susceptible. The genotypic diversity of various bacterial species has been evaluated using a variety of molecular techniques, including pulse-field gel electrophoresis, ribotyping, and repetitive extragenic palindromic PCR (Rep-PCR). It has been demonstrated that rep-based fingerprinting is a quick and accurate method for identifying various Enterobacteriaceae populations. Variations in conserved intergenic palindromic DNA sequences are used in rep-fingerprinting for PCR amplification and isolate characterization. These DNA elements serve as amplification targets to create different bands and are stable, noncoding intergenic repetitive sequences that are dispersed throughout the genome (Mirzaei *et al*., 2021).

Gram-negative bacteria with ESBL resistance genes have been examined in Saudi Arabia, with the majority of research coming from the country's central and eastern regions. Data on the distribution of *ESBL* resistance genes and their resistance profile among enterobacteriaceae and *A. baumannii* are still scarce, despite the spread

of antibiotic resistance among bacterial pathogens in the southern region. 98.1 % of the *Acinetobacter* species collected from patients in intensive care units had MDR characteristics, according to a prior study conducted at the Aseer Central Hospital, a regional hospital in the south. A large distribution of class D carbapenemase-encoding genes was also discovered in A by another study carried out in the same hospital. A recent study found that patients at the King Abdullah Hospital, a referral facility in Bisha in the country's southern area, had a high prevalence of MDR Gram-negative bacteria and a rate of ESBL production of 27% (Ibrahim *et al* ., 2021).

2.7.1 *CTX-M* gene

The ESBLs which can hydrolyze expanded-spectrum cephalosporins (such cefotaxime, ceftriaxone, cefepime, or ceftazidime), as well as monobactams (such as aztreonam), pose a public health risk (Coque *et al*., 2008a; Pitout and Laupland, 2008). The primary *TEM*, *SHV*, *CTX-M*, *VEB*, and *GES* enzymes are class A ESBLs. The *CTX-M* family is one among them that has had the most variations described in recent years (123 variants until 2011, last accession December 5, 2011).

In the late 1980s, enterobacterial strains isolated in Europe were identified as containing the first *CTX-M*-type enzyme of clinical origin, *CTX-M-1* (Bonnet *et al*., 2004). Because *CTX-Ms* are becoming more well-known, their explosive global spread has been dubbed the "*CTX-M* pandemic" (Cantón and Coque, 2006). Although numerous updates on *CTX-M β-lactamases* have been published (Bonnet, 2004; Livermore, 2007; Rossolini, 2008; Hawkey and Jones, 2009; Naseer and Sundsfjord, 2011), new information regarding the dispersion and clonality of *CTX-M* producing isolates, molecular epidemiology, protein plasticity, evolution and origin of the $bla_{CTX\text{-}M}$.

The source of the genes producing *CTX-M*-type β-lactamases is known, unlike the majority of other acquired ESBLs. The chromosomal genes found in members of the genus *Kluyvera*, which contains several environmental species with negligible to

no pathogenic activity toward humans, are the origins of *CTX-M* determinants. In strains of *Kluyvera ascorbate*, precursors of genes encoding enzymes of the *CTX-M-1* and *CTX-M-2* groups have been found (Bonnet *et al*., 2004), whereas strains of *Kluyvera georgiana* have precursors of genes encoding enzymes of the *CTX-M-8* and *CTX-M-9* group (Rossolini *et al*.,2008). It is unknown what the sources of the genes that code for the *CTX-M-25* and *CTX-M-45* subgroups were, however it is most likely other members of the genus *Kluyvera*.

2.7.2 *TEM* gene

The *TEM* β-lactamases are among the most thoroughly researched enzymes involved in antibiotic resistance. They have a high frequency in hospitals and clinics all over the world and work by hydrolyzing the β-lactamases ring of penicillins, cephalosporins, and related antibiotics

Penicillin-resistant bacteria produced *TEM*-1, the first *TEM* allele to be isolated, in 1963 (Datta & Kontomichalou, 1965). The human bacterial flora was quickly colonized by various species of *TEM*-1, but no reports of *TEM* variations with different kinetic properties surfaced for the following 20 years. But newer *TEM* variations started to arise about the time a new wave of innovative β-lactams antibiotics hit the market at the start of the 1980s. One to three amino acid changes were frequently present in these variations, which led to an expansion of the resistance spectrum to include one or more of the novel β-lactams. Variants of the *TEM* alleles that are currently known provide resistance to the majority of the novel β-lactams that have been developed over the previous three decades. The organic development of *TEM*-1 demonstrates the significance of anticipating the evolution of antibiotic resistance in addition to the creation of new antibiotics (Salverda *et al*., 2010).

2.7.3 *SHV* gene

In *E. coli*, the first *blaSHV-1* gene was discovered in the 1970s (Pitton *et al* ., 1972). According to Matthew *et al*. (1979) Clinical isolates may contain SHV-type ESBLs more commonly than any other kind of ESBL. The term SHV stands for (sulfhydryl variable). This label was chosen since it was believed that p-chloromercuribenzoate's inhibition of *SHV* activity was substrate-related and changeable depending on the substrate used for the test (Bali *et al*., 2010).

Extended-spectrum *SHV* β-lactamases are members of functional group 2be, albeit they were most recently classified as members of serine β-lactamase subclass A1, clustering with the clinically significant *TEM* and *CTX-M* enzymes (Bush *et al*., 2013; Philippon *et al*., 2016). Two subdomains make up *SHV* ESBLs: they consist of an antiparallel five-strand sheet bordered by helices, and an all-helical subdomain (Matagne *et al*., 1998). The active site is situated inside the cleft made by the subdomains, just like in *TEM* β-lactamases, and it contains the Ser70 residue that mediates the nucleophilic attack on the carbonyl group of the β-lactam ring. Numerous conserved structural and functional amino acid motifs have been found close to this serine residue(Jelsch *et al*., 1993).

Acinetobacter baumannii is one of the most potent associations outside of enterobacteriaceae, contributing to the alarming expansion of ESBL-producing strains, especially in clinical epidemics (Blackwell *et al*., 2016). This phenomenon appears to be caused by plasmid transfer from nosocomial enterobacteriaceae that encode *SHVs*, as was seen for *SHV-12* in the Netherlands (Naiemi *et al*., 2005) or *SHV-5* in the USA, where this variant is the most common ESBL gene in enterobacteriaceae (Naas *et al*., 2007).

2.9 Phylogenetic Tree

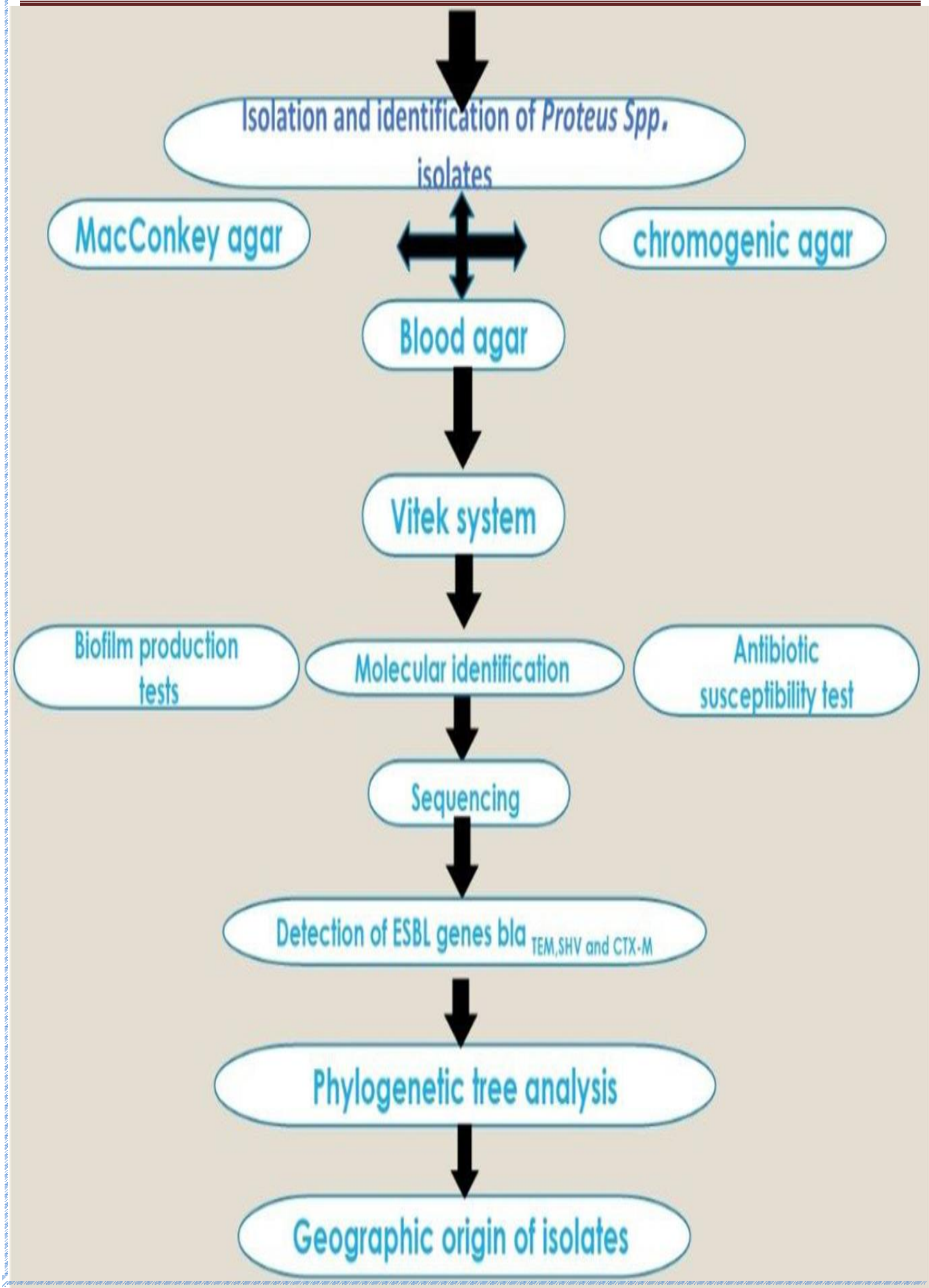
A phylogenetic tree, also known as an evolutionary tree, is a diagram that shows how different taxa have evolved over time. The evolutionary relationships between taxa are denoted by the phrases evolutionary tree, phylogenetic tree, and cladogram, which are sometimes used interchangeably. Although there are minute distinctions, the terms dendrogram and cladogram are frequently used interchangeably. Despite the fact that the context—that is, the depiction of the evolutionary connections of taxa—remains the same, it is crucial to be aware that the vocabulary used in the literature is not always consistent (Hall *et al*.,2013).

The "comparative technique" is frequently used to describe the usage of phylogenetic trees, which have established themselves as a standard tool in the study of adaptation. In order to evaluate whether a given "adaptation" has various origins or is associated with other features or environmental factors, it is first essential to demonstrate that it is distributed as an apomorphy within the group in question. While several statistical methods have been proposed for these investigations, they all start with the premise that multiple distinct character origins that are connected with historical or environmental circumstances are proof of adaptation. In fact, some researchers contend that only in a historical context—that is, based on explicit phylogenetic trees—can adaptation be discussed. There's no question that more research in this field will lead to more accurate statistical tests for adaptation based on character distributions on phylogenetic trees(Dees *et al*.,2016).

Chapter Three

Materials and Methods

Study design



3. Materials and Methods

3.1 Materials

3.1.1. Laboratory Equipment and Instrument

The list of laboratory tools and supplies used in this investigation found in Table 3-1.

Table 3-1: Laboratory Equipment or Instrument and Supplies

Equipment or Instrument	Company (Country)
Autoclave	Webco (Germany)
Benson burner	Memmert (Germany)
Centrifuge	MSE(England)
Cooled centrifuge	Germany(Hitachi)
Cooling incubator	Memmert (Germany)
DensiChek™	BioMérieux (France)
Disposable petri dishes	Al-Hani company (Lebanon)
DNA Nano drop	Pioneer (Korea)
Electrical oven	DLTG(China)
Electrophoresis gel system	Optima (Japan)
ELIZA(enzyme linked immunosorbent assay)	HumaReader(German)
Eppendorf's micro-centrifuge	Hettich(Germany)
Gel documentation system	Electroform
Hood	Bio LAB(Korea)
Hot plate & magnetic stirrer	Stuart(UK)
Laminar flow clean bench	Labtech (Korea)
Light microscope	Kruss(Germany)
Micropipette 0.5-10,1-20µl, 5-50	CYAN (Belgium)

µl,10-200 µl,100-1000µl	VWR(USA)
Micropipettes	Brand-w (Germany)
Microtiter plates 96 well	AFMA (Jordan)
PCR thermal cycler	ESCO (Singapore)
pH meter	Hanna (U.S.A.)
Plastic test tubes	VWR(USA)
Refrigerator	Concord (Lebanon)
Sensitive Balance	Denver (USA)
Spectrophotometer	Cecel
Sterilized cotton swabs	Sterellin Ltd. (UK)
UV- Transilluminator	Optima(Japan)
VITEK 2 Compact system	BioMérieux (France)
Volumetric flasks	Jlassco (India)
Vortex	Griffin (England)
Water bath	Memmert (Germany)
Water distiller	GFL(Germany)

3.1.2. Biological and Chemical Materials

The biological and chemical materials used in this study were listed in Table 3-2.

Table 3-2: Biological and Chemical Materials

Material	**Company (country)**
Absolute ethanol (99%)	Merk (England)
Acetone	BDH (England)
Agar- Agar	Oxoid(England)
Crystal violet stain	Syrbio/Switzerland
Deionized sterile D.W.	Bioneer(Korea)
DNA ladder marker (100-1500) bp	Bioneer (Korea)

Ethanol 99%	Merck (India)
Ethidium bromide	Promega/USA
Glucose	Sigma (England)
Glycerol (C3H8O3)	Himedia (Switzerland)
H_2SO_4	BDH (England)
Hydrogen peroxide (H_2O_2) 3%	Merck (England)
KOH	BDH (England)
Kovac's reagent	HIMEDIA (India)
Loading dye	Promega/USA
NaOH	BDH (England)
Normal Saline solution	S.D.I (Iraq)
Oxidase discs	Mast (England)
Peptone water	HIMEDIA (India)
Phosphate buffered saline	Schuchard (German)
Tris-Borate EDTA buffer (TBE)	Promega (USA)
Urea Solution	SD-Fine (India)

3.1.3 Commercial Kits

The components of the commercial kits used in present study are listed in Table 3-3.

Table 3-3: Components of the Commercial kits used in this study

Type of Kit	**Company(Origin)**
DNA extraction Kit	DSBIO
DNA ladder	Promega -USA
Green master mix	Macrogen /Korea
Primers	Macrogen /Korea

3.1.4 Culture Media

The preparation of the culture media in the current investigation followed the manufacturer's instructions. The media were autoclaved at 121°C for 15 minutes, and then stored at 4°C pending use. The media used in this investigation are displayed in Table 3–4.

Table 3-4: Culture media

Media	Company(Origin)
Blood base agar	Oxoid (England)
Brain-Heart infusion broth	Himedia (India)
Kligler Iron agar	Oxoid (England)
MacConkey agar	Oxoid (England)
Muller-Hinton agar	Oxoid (England)
Nutrient agar	Oxoid (England)
Peptone water	Oxoid (England)
Simmon's citrate agar	Oxoid (England)
Skim milk agar	Himedia (India)
Tryptic Soy broth	Oxoid (England)
Urea agar base	Oxoid (England)
UTI chromogenic agar	CHROMagar™ (France)

3.1.5 Antibiotic Disks

The antibiotic disks used in this study are listed in Table 3-5.

Table 3-5: Antibiotic Disks, Symbol, and Organism Producer (CLSI,2022).

Antibiotic Discs	Symbol	Disc potency (µg)	Organism Producer
Ampicillin	AMP	10	*Penicillium chrysogenum* Semi-synthetic antibiotic
Ciprofloxacin	CIP	10	*Streptomyces*

Gentamycin	CN	10	*Actinomycetes* (micromonospora pupurea)
Imipenem	IMI	10	*S.cattleya*
Meropenem	MRP	10	Semisynthetic
Piperacillin	PRL	100	Semisynthetic
Erythromycin	E15	15	Saccharapolyspora erthraea (*Streptomyces*)
Sulfamethoxazole	SMX	100	Synthetic antibiotics
Tobramycin	TM5	5	*S. tenebrarius*
Azithromycin	AZM	30	Semisynthetic

3.1.6 Polymer Chain Reaction (PCR) Mixture

PCR reaction mixture used in the study were listed in Table 3-6.

Table 3-6:- PCR Reaction Mixture

Contents	**Volume**
Master Mix	12µl
Template DNA	3 µl
Forward primer (10 pmol/µl)	2 µl
Reverse primer (10 pmol/µl)	2 µl
Nuclease free water	6 µl
Total volume	25 µl

3.1.7. Go Taq G2 Green Master Mix Materials

The Master Mix Materials used in the study are listed in Table 3-7.

Table 3-7:- contents of master mix

No.	Material
1.	DNA polymerase enzyme (Taq)
2.	dNTPs (400µm dATP, 400µm d GTP, 400µm dCTP, 400µm
3.	$MgCl_2$ (3mM)
4.	Reaction buffer (pH 8.3)

3.1.8. Commercial Primers

The commercial primers used in present study are listed in Table 3-8.

Table 3-8:- Commercial primers used in this study

Primer	Sequence (5----------›3)		Amplicon size (bp)	Conditions (D,A and E)	Cycle No.	References
TEM	F	GAGTATTCAACATT CCGTGTC	861	94˚C/30 sec 57˚C/1 min 72˚C/2 min	35	(Bokaeian *et aI.*, 2015)
	R	TAATCAGTGAGGCACCTATCTC				
SHV	F	AAGATCCACTATCGCCAGCAG	231	94˚C/30sec 64˚C/1 min 72˚C/1 min	35	
	R	ATTCAGTTCCGTTTCCCAGCGG				
CTX-M	F	GACGATGTCACTGGCTGAGC	499	94˚C/30 sec 57˚C/1 min 72˚C/1 min	35	
	R	AGCCGCCGACGCTAATACA				

3.2 Methods

3.2.1 Sterilization

The culture media (prepared and synthetic) used in this study was sterilized with an oxidizer device at a temperature of 121 and a pressure of 15 pounds/ing² for a period of 15 minutes according to the manufacturer's instructions. Then the media was left to cool to 45°C and then placed in the incubator at 37°C for an hour to ensure

that there was no contamination and moisture removal, then kept at 4°C in the refrigerator until use.

As for the glassware, it was sterilized in an electric oven at 160-180 °C for two hours. The solutions that are affected and destroyed by high heat were sterilized by microfiltration units with a diameter of 0.22 micrometers (Greenwood *et al*., 2007).

3.2.2. Preparation of Solutions and Reagents

3.2.2.1 Normal Saline Solution:-

Ready to use, sterile normal saline (NS) was used for the preparation of culture suspension.

3.2.2.2 Oxidase reagent

Tetramethyl para phenylene diaminedihydro chloride, 1 gm, and 100 ml of distilled water were combined to create the solution, which was then freshly made in a dark bottle (Forbes *et al*., 2007).

3.2.2.3 Catalase reagent

In a dark bottle, hydrogen peroxide (3%) was made from stock solution (Forbes *et al*., 2007).

3.2.2.4 Kovac´s Reagent

This reagent was made by combining 10g of P-dimethyl amino benzaldehyde with 150 ml of isoamyl alcohol, adding 50 ml of strong hydrochloric acid (HCL) gradually, making sure to gently shake the mixture before use. This chemical is employed to identify indole synthesis (Collee *et al*., 1996).

3.2.2.5 Methyl Red Reagent

This reagent was made by combining 200 ml of distilled water with 300 ml of ethanol after 0.1g of methyl red had been dissolved. This chemical is utilized to identify bacterial fermentation of glucose (Collee *et al*., 1996).

3.2.2.6 Voges-Proskauer (VP) Reagent

Solution I:- To make this solution, 40 g of KOH were dissolved in 100 ml of distilled water to get the final concentration of 40%. This is displayed (VP1).

Solution II :- 5 g of α-naphthol were dissolved in 100 ml of 100% pure ethanol to create this solution. This is pictured (VP2). They are used to check whether bacteria have the capacity to generate acetone (Baron *et al*., 1994).

3.2.2.7 Kliglers Iron agar(KIA)

By streaking the surface of the slant and inoculating the media with the tested bacterium, the media was exposed to the bacteria for 24 hours at 37 degrees Celsius. A favorable result due to sugar fermentation with or without gas production at the butt of the slant is shown by the media turning yellow (MacFaddin, 2000).

3.2.2.8. Gram stains solutions (Jawetz *et al*., 2019).

1. Primary stain: 2 gm Crystal violet, 20ml 95% ethyl alcohol, 0.8gm ammonium oxalate and100 ml distilled water.
2. Stain fixative agent: 2 gm potassium iodide, 1gm iodine crystals and 100 ml distilled water.
3. Decolorize: 70% ethyl alcohol+30% acetone.
4. Counter stain: 4.0 gm safranin, 200 ml 95% ethanol and 800 ml distilled water.

3.2.2.9. McFarland standard solution

The solution was prepared according to what was mentioned in (Forbes *et al*.,2007) and it consists of two solutions: -

- Solution A: (1.175) g of aqueous barium chloride ($BaCl_2$. $2H_2O$) was dissolved in 100 ml of sterile distilled water.
- Solution B: 1 ml of concentrated sulfuric acid (H_2SO_4) was added to 100 ml of solution A to (99.5) ml of solution B. The resulting solution

was mixed well and distributed in an amount of 5 ml in test tubes, tightly closed to prevent evaporation and kept in the dark. At 25°C until use (taking into account mixing the contents of the tube before use). The solution was used for comparison to obtain an approximate number of bacterial cells of 1.5 * 10^8 colony forming units.

3.2.2.10. Ethidium bromide solution

It was prepared by dissolving 0.05g of ethidium bromide in 10ml distilled water and stored in a dark reagent bottle (Sabnis, 2010).

3.2.2.11. Preparation of 1X TBE Buffer

The concentrated 10X TBE buffer was diluted to create the 1X TBE buffer. Agar was dissolved in this solution and utilized in the electrophoresis process 46. To create the final concentration of 1X TBE, 100ml of 10X TBE were added to 900ml of sterile distal water (Green and Sambrook, 2012).

3.2.2.12. 6X DNA Loading buffer blue

It comes in a ready-to-use, pre-mixed form with 100 mM Tris-HCl (pH 8.0), a combination of dyes (Xylene Cyanol and Bromophenol Blue), 30% glycerol, and 100 mM EDTA (pH 8.0). x6 loading Buffer DNA sample preparation for loading on agarose and polyacrylamide gels is done in Blue. The glycerol content makes the sample more dense and guarantees that a layer of DNA forms at the bottom of the well. The ability of EDTA to inhibit metal-dependent nucleases is well established. Monitoring the progress of electrophoresis is possible with tracking dyes. To examine the DNA after extraction, only 1µl of loading dye was combined with 5 µl of material (Green and Sambrook, 2012).

3.2.3. Culture Preparation

Following the manufacturer's instructions, culture medium were created and autoclaved at 121°C for 15 minutes to sterilize them:-

3.2.3.1 Nutrient Agar Medium

The manufacturer's recommended technique was used to create the nutrient agar media. When necessary, it is utilized to culture and activate bacterial isolates.

3.2.3.2 MacConkey Agar Medium

The MacConkey agar medium was made using the procedure advised by the manufacturer. was a selective and differential media. It was used in the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria (Jung and Hoilat, 2020).

3.2.3.3 UTI Chromogenic Agar Medium

Suspend In 1000 ml of pure distilled water, 32.45 grams To completely dissolve the medium, heat it to boiling. Use an autoclave to sterilize items for 15 minutes at 15 Ibs of pressure (121°C). to 45–50 °C. Stir thoroughly, then transfer to sterile Petri dishes. This medium, which is a differential agar, allows for the presumptive identification of the main pathogens responsible for urinary tract infections. A complex combination of partially digested proteins makes up peptone (Himedia).

3.2.3.4 Brain Heart Infusion Medium

52 grams of brain heart infusion agar or 37 grams of brain heart broth are suspended in a liter of autoclaved (15 minutes at 121 °C) medium for enrichment. If the broth is employed, it is used to cultivate and keep the bacterial isolates supplemented with 15% glycerol.

3.2.3.5. Blood Agar Medium

40 grams of blood agar base were dissolved in 1000 milliliters of distal water to create the blood agar medium. It underwent a 15-minute autoclave at 121 °C, followed by a 50 °C cooling process. There was also five percent fresh human blood added. This medium was employed to grow bacterial isolates and test the bacteria's capacity to hemolyze blood cells. Is an enriched, bacterial growth medium, isolation, identification and determine the type of hemolysis (Niederstebruch *et al*., 2017).

3.2.3.6. Peptone Water Medium

To make this medium, 8g of peptone were dissolved in 1000 ml of distilled water, then the mixture was divided among test tubes and autoclaved. It was utilized to demonstrate how bacteria may break down the amino acid tryptophan to produce indole (Colwell, 1996)..

3.2.3.7. Motility Medium

To make this medium, 4 g of agar agar was mixed with 100 ml of nutritional broth, and then 1000 ml of distilled water was added to finish. It was then autoclaved for 15 minutes at 121°C to disinfect it. It was handed out in tubes. This medium was used to measure the motility of microorganisms (MacFaddin, 2000).

3.2.3.8. Urea Agar Media

This medium was made by mixing 100 ml of urea agar base sterilized by autoclaving at 121 ˚C for 15 minutes, chilled at 50 ˚C, and 15 ml of urea solution sterilized by filtering (0.22 m in diameter). After bringing the pH level to 7.1, the medium was dispersed into clean test tubes and allowed to harden into a slant shape. Using this medium, the capacity of bacteria to manufacture the urease enzyme was examined (MacFaddin, 2000).

3.2.3.9. Muller-Hinton Agar Medium

Suspended 38 grams in 1000 ml distilled water, heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes, it is used in anti-microbial susceptibility testing (MacFaddin, 2000).

3.2.3.10. Simmons' Citrate Medium

It was used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium salts as its only nitrogen source (Forbes *et al*., 2007).

3.2.3.11. Tryptic Soy Broth

This medium was made by combining 30 g of dehydrated medium with 1 L of deionized water, then sterilizing it in an autoclave for 15 minutes at 121°C. During the culture of the bacterial isolates, this medium served as an enrichment medium. (MacFaddin, 2000).

3.2.4. Collection of Samples

The samples were obtained properly to prevent any potential contamination (Collee *et al*., 1996). Patients with UTIs were typically the ones who provided urine samples. Midstream urine samples were taken in sterilized screw-cap containers, inoculated into culture media, and then incubated aerobically for 24 hours at 37°C. Patients' urine samples totaling 210 were collected. These patients were admitted to Babylonian hospitals (Al-Hillah Teaching Hospital, Mirgian Teaching Hospital, and Imam Al-Sadiq Teaching Hospital). The collection of these samples took place between September 2021 and July 2022.

3.2.5. Preservation of Bacterial Isolates

The bacteria were kept on nutrition and brain heart infusion agar slants at 4˚C for a month before being cultured on new culture media. Also, the bacterial isolates

were kept for a long time in BHI broth that had been treated with 15% glycerol, then they were kept at -20˚C for 6–8 months (Collee *et al*., 1996).

3.2.6 Laboratory Diagnosis

3.2.6.1. Tests for Identifying Bacteria

First diagnoses of bacterial isolates thought to be members of the *Proteus* genus were made based on the features of their culture, such as growth on various culture media, color of expanding colonies, diameter of colonies, colony height, colony edge, and others. The colonies on the medium of the MacConkey agars are pale in color, not fermented for lactose, and have an unpleasant smell that is comparable to the smell of rotting fish. The species belonging to the genus *Porteus* appear on the surface of chromogenic agars brown in color, and swarming motility appears on the media of the blood agars.

3.2.6.2. Microscopical Analysis and Colonial Morphology

To see the shape of the cells, their arrangement, and their interaction with the gram stain (positive or negative), which the cells appear as in the form of gram-negative rods, smears from bacterial isolates were made, stained with a gram stain, and then studied under a light microscope (Levinson *et al.*, 2012).

3.2.6.3. Biochemical Tests

The *P. vulgaris* and *P. mirabilis* isolates from other samples were distinguished using the following biochemical assays. According to (MacFaddin, 2000), the following tests were performed: the catalase test, the oxidase test, the Indol test, the methyl-red test, the Vogues Proskauer test, the citrate utilization test, the motility test, and the Kligler's Iron Agar test.

3.2.6.3.1. Oxidase Test

This reagent was ready-made as filter paper discs called oxidase discs that contained tetramethyl-paraphenylene diamine dihydrochloride. They were kept in a dark, cool environment. As the color of the smear changed to purple, the oxidase test was positive. A small fraction of the bacterial colonies were dispersed on the disc with a wooden stick. Tetramethyl-p-phenylene diaminedihydrochloride, a redox dye, and a specific bacterial oxidase that would catalyze the flow of electrons between electron donors in bacteria, are required for the test (Forbes *et al* .,2007).

3.2.6.3.2. Catalase Test

A sterile wooden chopstick was used to transfer one of the colonies on the MacConkey agar medium to a clean, dry glass slide, where a drop of hydrogen peroxide (3%) was then added. The increase of air bubbles implies a favorable test outcome (Forbes *et al*., 2007).

3.2.6.3.3. Indole Test

The bacterial isolates to be tested were added to the peptone broth, and the tubes were then incubated at 37°C for 24 hours before five drops of the Kovacs reagent were added and the tubes were gently shaken (Forbes *et al*., 2007).

3.2.6.3.4. Methyl Red Test

Bacterial isolates were placed inoculated into tubes with methyl-Fox Proscauer MR-VP medium. The tubes were then incubated at 37 °C for 48 hours, following which 5 drops of the methyl red reagent were added to each tube, and the tubes were gently shaken. The medium's transformation to red is an advantageous outcome (Forbes *et al*., 2007).

3.2.6.3.5. Voges-Proskauer (VP) Test

This test was used to look into the bacteria's capacity to make acetone. Bacterial isolates were introduced to tubes containing MR-VP medium before the addition of 6 drops of reagent VP1 and 2 drops of reagent VP2. The tubes were then incubated at 37 °C for 48 hours. The medium's transformation from yellow to pink signifies a positive test result (Forbes *et al*., 2007).

3.2.6.3.6. Citrate Utilization Test

Simmon's citrate agar medium was inoculated with bacterial isolates using the stripping method, and the tubes were then incubated at 37°C for 24 hours to show growth proof of the medium's positive test color, which changed from green to blue (Forbes *et al*., 2007).

3.2.6.3.7. Urease Test

This test was performed to look into the bacteria's capacity to manufacture the urease enzyme. vaccinated The tubes were incubated at 37 °C for 24 hours while using the planning approach to isolate bacteria from the urea agar medium's inclined surface. A positive test is indicated by a yellowish medium that turns pink (Forbes *et al*., 2007).

3.2.6.3.8. Kligler Iron Agar (KIA)

Inoculate the bottom of the tube butt with bacterial isolate by stabbing method and the surface. The color of the medium at the bottom of the tube and the color of the medium tilted from red to yellow indicates the fermentation of the sugar lactose and glucose. The color of the medium at the bottom of the tube changed from red only to the production of hydrogen sulfide H_2S and the accumulation of gas in The bottom of the tube was below the planting medium or slanted by the planning method, then the tubes were incubated at a temperature of 37 °C for a period of 24-48 hours. A change to a yellow color indicates the fermentation of glucose sugar only,

and a black color deposition at the bottom of the tube indicates the formation of air pockets, evidence of gas production (Macfaddin, 2000).

3.2.6.4. Detection of biofilm production

To detect the formation of biofilms, tissue culture plates with 96 wells and trypticase soy broth (Himedia) were employed. Semi-quantitative assessments of biofilm growth were made using. *P. mirabilis* and *P. vulgaris* isolates were grown in separate wells of 96-well plates at 37˚C in trypticase soy broth medium supplemented with 1g of glucose. After the 24-hour growth period, the plates were thoroughly washed three times with ordinary saline to remove any free-floating bacteria. The plates were stained for 15 minutes at room temperature with 100 ml of a 0.1% (w/v) crystal violet solution, and then washed with normal saline. The crystal violet solution was then extracted from the biofilm using 150 µl of 95 percent ethanol and acetone [8:2 (v/v)] to remove it from the wells. The plates were measured at 630 nm by a microplate reader, which produced the final values of mild, moderate, and strong. The results were interpreted as follows: if ODc < OD < 2 * ODc had weak bacterial adhesion, 2 * ODc < ODc < 4 * ODc had moderate bacterial adhesion, and if 4 * ODc < OD, ODc had high bacterial adhesion (Hemati *et al*., 2016).

3.2.6.5. Antibiotic Sensitivity Test

Disk diffusion was used to test the in vitro susceptibility of *Proteus spp.* isolates to 11 antimicrobial agents in accordance with the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2016). A sterile cotton swab was used to disseminate the activated isolates on Muller Hinton agar (MHA) for 18 hours at 37°C. The growth was then corrected to 0.5 McFarland's standard (1.5108 CFU/mL). Antibiotic disks were placed on MHA and carefully pressed down to make sure they completely contacted the agar that had been injected with bacteria. After 24 hours of incubation at 37°C, the diameter of the inhibition zone in millimeters (mm)

was measured. Results were interpreted as either sensitive or resistant, per the (CLSI, 2022).

3.2.7 VITEK System Bacterium Identification

In VITEK system, biochemical techniques are also used to identify bacteria (Pincus *et al* .,1998).

A. Suspension Planning

A suitable number of colonies of a pure culture are transferred using a sterile swab or applicator stick, and the microbe is then suspended in 3.0 ml of sterile saline (aqueous 0.45 percent to 0.50 percent NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear glass (polystyrene) test tube. A turbidity meter known as the Densichek is used to measure the suspension's turbidity, which is adjusted in accordance with the McFarland turbidity range for gram negatives (0.50-0.63).

B. Inoculation

Identification cards are injected with a turbidometrically regulated solution of pure colonies in saline. To evaluate the growth and vitality of the suspension, these cards have one negative control cell and many distinct biochemical broths in reaction cells. Using an integrated vacuum equipment (cassette), microbe suspensions are injected onto identification cards. The identification card is inserted into the nearby slot while the transfer tube is inserted into the relevant suspension tube. A test tube with the microorganism suspension is placed onto a particular rack. Manually inserting the filled cassette into a vacuum chamber station. The organism suspension is driven through the transfer tube and into the station's micro-channels once the vacuum is applied and the air is turned back on. Conventional oxidase, coagulase, and catalase tests, as well as the identification cards are injected with a turbidometrically regulated solution of pure colonies in saline. To evaluate the growth and vitality of the suspension, these cards have one negative control cell and many distinct biochemical broths in reaction cells. Before inoculating the cards,

microorganism suspensions are applied to the cards and the results of a gram stain are required.

C. Card Sealing and Incubation

Inoculated cards are loaded into the carousel incubator after passing through a device that seals the card and cuts off the transfer tube. At 35.5 + 1.0˚C, all card kinds are incubated online. Every 15 minutes, one card is taken from the carousel incubator, carried to the optical system for reaction readings, and then put back until the next read time. Over the course of the entire incubation, data are gathered every 15 minutes. Period Depending on the growth, incubation times range from two to fifteen hours.the organism's rate. The computer with VITEK programming determines if by determining each well's positivity or negativity using an laser scanner The reactions are monitored when the incubation time is over and the identification is printed.

3.2.8 Genotyping assays

3.2.8.1 DNA Extraction

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company (DSBIO)

1. Before beginning DNA extraction, the culture was activated by inoculating it into 10 ml of nutritional agar medium and allowing it to sit at 37 °C for an overnight period.

2. A 1.5 ml microcentrifuge tube should contain 0.5-2 ml of an overnight culture. To pellet the cells, centrifuge at 12,000 rpm for 2 minutes. Take away the supernatant.

3. To the pellet, add 200 µl of Solution DS was added To produce a homogenous solution, it is imperative that the sample and Solution DS are completely mixed right away by vortexing or pipetting.

4. 20 µl of Proteinase K and 220 µl of Solution MS were added. Combine completely by vortexing. A homogeneous solution can be obtained after 10 minutes of incubation at 65°C. The water beads should spiral downward against the tube wall.

5. To the sample, 220 µl of ethanol (96–100%) was added, and thoroughly mix by vortexing. It's crucial to completely combine the sample and ethanol. There might be precipitation. Put the mixture from step 4 in a 2 ml collection tube (supplied) and pipet it into the spin column. 1 minute of centrifuging at 12,000 rpm. Delete the flow-through. Add 500 µl Wash Buffer PS, and centrifuge for 1 min at 12,000 rpm. Discard flow-through.

6. 500 µl of Wash Buffer PE was added and centrifuged at 12,000 rpm for 1 minute after adding. Toss out the flow-through. Once more go through step 6. Centrifuge the column membrane for three minutes at 12,000 rpm. Throw away the collecting and flow tubes.

7. Pipette 30-100 µl of Eluent Buffer AE (Prewarm to 65°C) was added straight onto the membrane while the spin column is within a clean 1.5 ml microcentrifuge tube (not included). After two minutes of incubation at room temperature, elute the sample by centrifuging it at 12,000 rpm for two minutes. DNA that has been purified is in the tube. Maintain the DNA at -20 °C.

3.2.9. Detection of Some Virulence Factors Genes by PCR

The following procedures have been used to create the PCR amplification mixture in accordance with the manufacturer's instructions.

3.2.9.1. Estimation of DNA concentration

The DNA concentration of specimens was estimated using nanodrop by putting 1µl of the extracted DNA in the instrument to detect the concentration in ng/µm and purity detected by noticing the ratio of O.D. 260/280 to check the contamination of

DNA specimens with protein, the accepted 260/280 ratio of pure DNA was between 1.8 (Shim *et al*., 2010).

3.2.9.2. Primer Pairs Preparation

All primer pairs used in this study were dissolved using sterile ddH_2O. Stock solution (100 p mol/µl) was prepared by adding ddH_2O to the vial containing lyophilized primer while working stock of 10 p mol/µl was made by mixing 10 µl of the stock primer and 90 µl of ddH_2O.

3.2.9.3. Reaction Mixture

Amplification of DNA was carried out in a final volume of 20 µl reaction mixture as mentioned in Table 3-6 .

3.2.9.4. PCR protocols for detection of gene technique

Detection of *bla* $_{TEM}$*, bla* $_{CTX-M}$*, and bla* $_{SHV}$ genes were carried out by using a 12 µl master mix of Gold conventional PCR (Bioneer, South Korea) including 3 µl DNA, 2 µl from each primer forward and reverse, and the volume was completed up to 6 µl with free nucleases deionized water according to the instructions of the company and reaction buffer mixed.

3.2.9.5. PCR program thermal controller

PCR cycling thermal program parameters used in this reaction for detection of *Proteus Spp*. specific, *bla* $_{TEM}$*, bla* $_{CTX-M}$, and *bla* $_{SHV}$ genes were shown in Table 3-9**.**

Table 3-9 : PCR thermal cycling program for *Proteus Spp.* specific gene *SHV*, *TEM* and *CTX-M* genes

No.	Step	Temperature(°C)	Time	No. of cycle
1.	Initial Denaturation	95	5 min	1
2.	Denaturation Annealing Extension	94 57 72	30 sec 1 min 1 min	35

3.	Final Extension	72	5 min	1

***PCR thermal cycling program 64 °C for *SHV* annealing**

3.2.9.6. Detection of Amplified Products by Agarose Gel Electrophoresis

One gram of agarose powder was dissolved in 100 milliliters of 1X TBE buffer to produce agarose gel. 10 ml of TBE buffer was combined with 90 ml of cold water to produce a melting agarose gel. Thereafter, this gel was melted in a microwave until the mixture became clear. Depending on how the agarose gel will be used, different amounts of agarose can be dissolved. Following extraction, 0.7 percent of agarose gel is used to visualize the DNA, and 1.5 percent to 2 percent of agarose sheet is used to visualize the PCR result (amplicon). Ethidium bromide, which serves as an alternative to Simply Safe, had a stock solution concentration of 10 mg/ml. Just 5 µl of the ethidium bromide stock solution was added to 100 ml of melting agarose gel to get the final concentration of 0.5 mg/ml (Green and Sambrook, 2012). The comb was properly positioned, the agarose was put into the gel tray with the ends covered, and it was then allowed to dry. A second well of the gel is used to load 5 µl of the amplified DNA (the product of the PCR procedure), and a first well is used to load 5 µl of the DNA marker. The run was carried out in line with the gel percentage and gel size, and the electrodes were securely fastened. For genomic DNA, agarose gel electrophoresis takes 45 minutes; for PCR product, it takes an hour.

3.2.10. DNA sequencing analysis

DNA sequencing was done to examine the genetic diversity of *P. mirabilis* and *P. vulgaris* isolates' $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{TEM} genes. DHL shipped the PCR products in an ice bag to the Macrongen company in Korea. Using NCBI BLAST analysis, the homology sequence identity and mutation analysis were performed. *P. mirabilis* isolates from the current study had their $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{TEM} genes registered in the NCBI-Gen Bank database with accession numbers.

3.2.11. Phylogenetic analysis

Using the MEGA4 software program (Tamura *et al*., 2007) and the neighbor-joining phylogeny tree, the phylogenetic tree was created using the neighbor approach (Saitou and Nei, 1987). The maximum composite likelihood approach (Tamura *et al*., 2004) is used to calculate the evolutionary distances, and the bootstrap-like 1000 data set method was used to assess the stability of the trees (Felsenstein *et al.*, 1985).

3.3. Statistical Analysis

The data analysis was done using SPSS 23, and p-values under 0.05 were considered significant. The categorical variables' frequencies and percentages are described using descriptive statistics. The UTI patient's odds ratios for *Proteus mirabilis* and *P. vulgaris* that produce ESBL were assessed using binary logistic regression analysis. To examine the correlation between isolates with antimicrobial resistance and the presence of the genes $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{TEM}, the Chi-square test was used (Grewal *et al*., 2017).

Chapter Four

Results and Discussion

4. Results and Discussion

4.1 Isolation of *P. mirabilis* and *P. vulgaris* :-

A total of 210 urine specimens obtained from patients (of both gender) with UTIs, Only 30 isolates (14 %) were identified as *P. mirabilis* and only 10 isolates identified as *P. vulgaris* whereas the rest 170 isolates (81%) belonged to other type of bacteria as shown in table 4-1.

Table 4-1:- Percent of *Proteus Spp.* Isolate

Name of Isolates	No. of Isolates	Isolates %
Proteus mirabilis	30	14
P. vulgaris	10	5
E. coli	100	48
Klebsiella	30	14
Other bacteria	40	19
Total	210	100

According to data of Table 4-1, *P. vulgaris* (5%) *P. mirabilis* (14%), and other bacteria (81%) were the most frequently identified bacteria from urine samples of catheterized and non-catheterized patients. This conclusion may be the result of the fact that other bacteria, such *E. coli*, account for more than 75% of community-acquired UTIs across all age groups, while *S. saprophyticus* only accounts for 10%. *E. coli* infections occur in roughly 50% of hospitalized patients. About 40% of the bacteria are gram-negative species like *Klebsiella, Proteus, Enterobacter, and Serratia*, and the rest are gram-positive bacteria like *E. faecalis, S. saprophyticus*, and *S. aureus* (Stephen and Maxine, 2009; Stewart *et al*., 2010).

The findings of this study are equivalent to those obtained experimentally by AL- Jumaa *et al*. (2011), who separated *Proteus spp*. from urine samples at a rate of (33.33 %), and by AL- Ta'ee, (2002), who was

successful in isolating *Proteus* from urine sample at a rate of (33.33 %) (38 %). *Proteu*s has also been isolated from individuals with urinary tract infections by Laftaa (2001) at a rate of (37.33%), although AL-Grawy (1999) only isolated *Proteus* from urine at a rate of (6%).

The results of biochemical tests for the diagnosis of *P. mirabilis* and *P. vulgaris* and shown in Table 4-2 that all bacterial isolates were negative for the tests oxidase, production of Indole , Voges Proskauer and positive for catalase, methyl red and urease tests, while the results were different for the citrate consumption test, and on kliger iron agar medium, it was fermented for glucose sugar and produced hydrogen sulfide gas H_2S. These characteristics are identical to the diagnostic results of the genus *Proteus*, according to Holt *et al*. (1994).

Table 4-2:- Biochemical test of *Proteus Spp.*

Biochemichal test		***P. mirabilis***	***P. vulgaris***
Oxidaise		-	-
catalse		+	+
Indole		-	+
Methl- red		+	+
Voges proskaure		-	-
Citrate		variable	-
Urease		+	+
KIA	k/A		
	H2S	+	+
	Co2	+	+

KIA=Kligler iron agar , K= alkaline slant (red), A= acid butt (yellow)

4.1.1 VITEK-2 system GN-card

Diagnosis was confirmed for all clinical bacterial isolates by using the VITEK-2 kit as a confirmatory test, and the accuracy of the test ranged

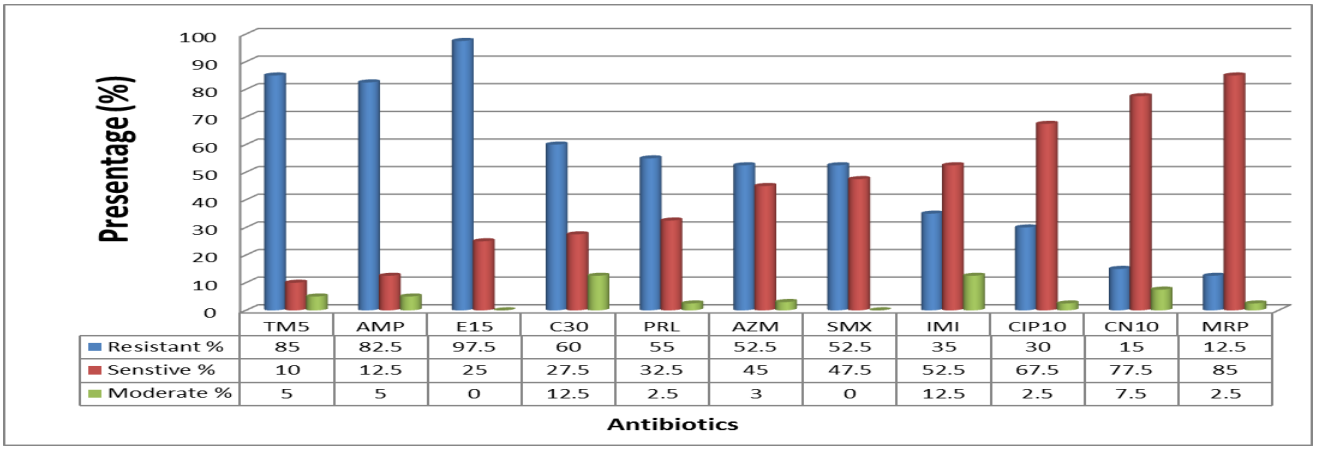
between 96-99%. The results showed (10) isolates belonging to the *P .vulgaris* variety and (30) isolates belonging to the variety *P. mirabilis* from clinical isolates.

4.2 Susceptibility for Antibiotic

The sensitivity of 40 bacterial isolates to eleven different common antibiotics was tested. The majority of isolates exhibited antibiotic resistance, particularly β-lactamase antibiotics. It was found that most of the bacterial isolates possess multiple resistance to the tested antibiotics. The results presented in Figure 4-1 showed that the most effective antibacterial against *P.mirabilis* and *P. vulgaris* isolates

are erythromycin with high resistance (97.5 %), followed by Tobramycin 5 with (85 %), ampicillin with (82.5 %), chloramphenicol (60 %), piperacillin (55 %) and sulfamethoxazole, azithromycin with resistance (52.5 %), while other antibiotics showed lower resistance and high sensitive such as meropenem is showed high sensitivity to *P. mirabilis* and *P. vulgaris* isolates

with (85 %), gentamicin (77.5 %), tobramycin 30 and ciprofloxacin (67.5 %), and imipenem (52.5 %) sensitivity.

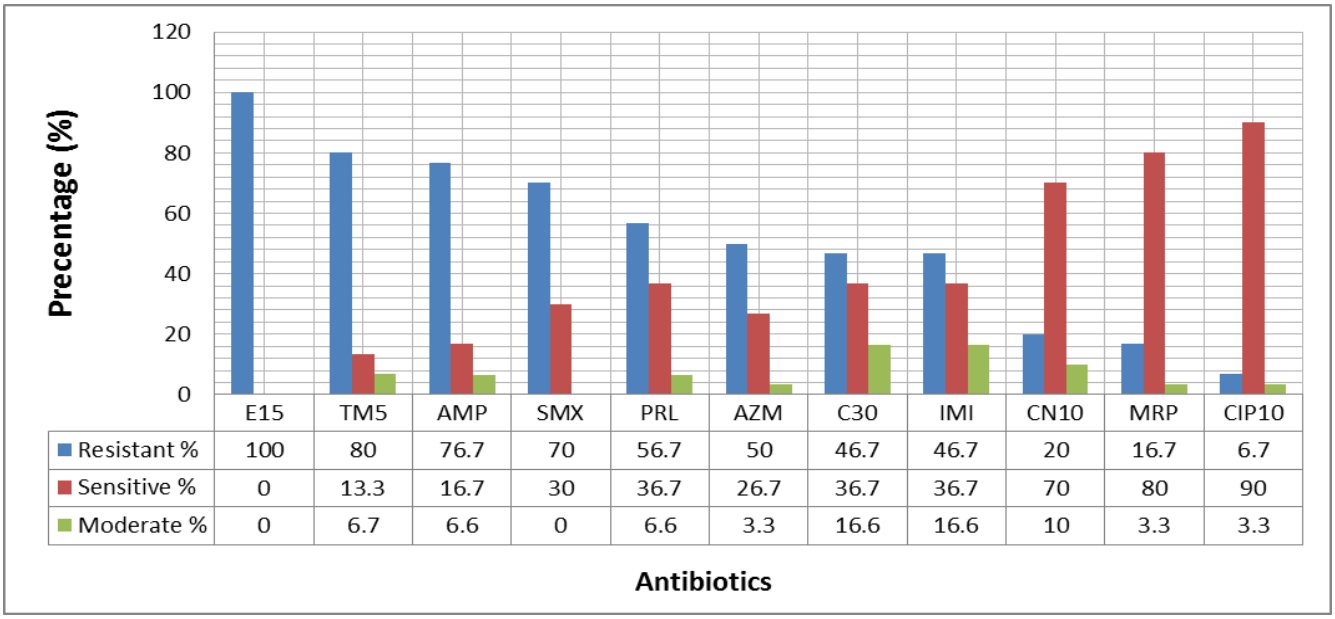


	TM5	AMP	E15	C30	PRL	AZM	SMX	IMI	CIP10	CN10	MRP
Resistant %	85	82.5	97.5	60	55	52.5	52.5	35	30	15	12.5
Senstive %	10	12.5	25	27.5	32.5	45	47.5	52.5	67.5	77.5	85
Moderate %	5	5	0	12.5	2.5	3	0	12.5	2.5	7.5	2.5

Figure 4-1: Antibiotic susceptibility of *P. mirabilis* and *P. vulgaris*

Aberrations : PRL; Piperacillin , E15; Erythromycin , AZM ; Azithromycin , IMI ; Imipenem, C30 ; Chloramphenicol , TM5 ; Tobramycin , AMP ; Ampicillin , CN10 ; Gentamicin , MRP ; Meropenem , SMX ; Sulfamethoxazole , CIP10 ; Ciprofloxacin

The 30 isolates of *P. mirabilis* are tested on 11 antibiotics and showed the results found in Figure 4-2 erythromycin, with a resistance rate of 100%, followed by tobromycin 5 (80%), ampicillin 76.7%, sulfamethoxazole (70%), piperacillin (56.7%), azithromycin (50%), chloramphenicol (46.7%), imipenem (46.7%), gentamicin (20%), meropenem (16.7%),and ciprofloxacin (6.7%). It was found that most of the bacterial isolates possess multiple resistance to the tested antibiotics**.**



	E15	TM5	AMP	SMX	PRL	AZM	C30	IMI	CN10	MRP	CIP10
Resistant %	100	80	76.7	70	56.7	50	46.7	46.7	20	16.7	6.7
Sensitive %	0	13.3	16.7	30	36.7	26.7	36.7	36.7	70	80	90
Moderate %	0	6.7	6.6	0	6.6	3.3	16.6	16.6	10	3.3	3.3

Figure 4-2: Antibiotic Susceptibility of *P. mirabilis.*

Aberrations :-PRL; Piperacillin , E15; Erythromycin , AZM ; Azithromycin , IMI ; Imipenem, C30 ; Chloramphenicol , TM5 ; Tobramycin , AMP ; Ampicillin , CN10 ; Gentamicin , MRP ; Meropenem , SMX ; Sulfamethoxazole , CIP10 ; Ciprofloxacin

These results are almost in agreement with Ahmed, (2019) what was stated that the rate of resistance of clinical isolates of *P. mirabilis* to ampicillin antagonists (88.3%),piperacillin(72.7%),clindamycin (66.7%),amoxicillin/clavulanicacid (66.2%), and trimethoprim/sulfamethoxazole (50%).

The result of gentamycin is according to Bourély, (2019). The level of resistance to gentamicin was higher for *P. mirabilis* (10.3% (8.5–12.3), and the Cantón, (2019). amikacin (96.% to 100 % susceptibility range), ertapenem (84.2 % to 100%), and imipenem were the most effective antibiotics in enterobacterales for imipenem (70.3-

100 %). Ciprofloxacin shown less action when *K. pneumoniae* and *E. coli* resistance percentages were near to 40% and more than 25%, respectively. Piperacillin-tazobactam susceptibility ranged from 66.6% to 100% , and amoxicillin-clavulanic acid susceptibility ranged from 58.3 % to 81.5%, with regard to the interactions of penicillins with beta-lactamase inhibitors.

It is noted from the results presented in the Figure 4-2 that the most efficient antibiotics against isolates of the bacteria *P. mirabilis* urinary tract infection are the two antibiotics tobromycin 3 (3.3%), ciprofloxacin (6.7%) and meropenem (16.7%) , while all isolates were resistant to sulfamethoxazole (100%).

This study agrees with Ramachandran *et al*. (2022) in all strains of *P. mirabilis, K. pneumoniae,* and *K. oxytoca* were ampicillin resistant. but contradict this study when showed *P. mirabilis* 100 % resistance to ciprofloxacin**.**

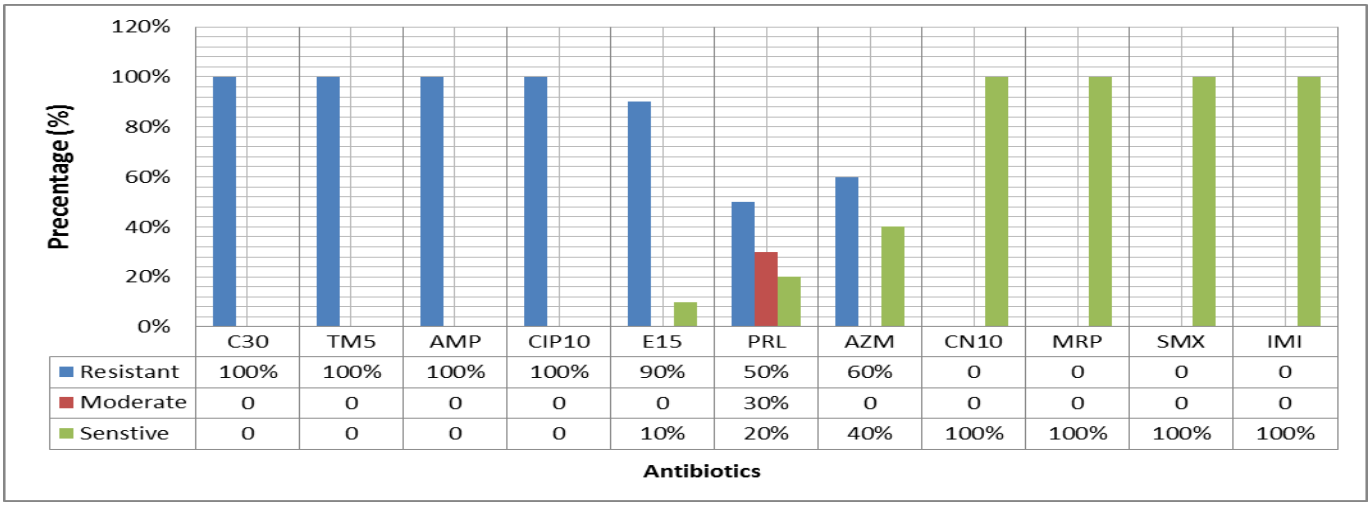
Rodulfo *et al*.(2021) *P. mirabilis* isolates exhibited high resistance to gentamicin, netilmicin, tobramycin, ciprofloxacin, sulfamethoxazole/trimethoprim, imipenem, ampicillin, cephalothin, and amoxicillin/ clavulanic acid**.**

The results of the current study contradict Hassen *et al*. (2008) who stated that the rate of susceptibility of *P. mirabilis* bacteria isolated from urine, bacteremia, burn wounds (73.6%) gentamicin (75.9%) norfloxacin (79 %) ciprofloxacin.

The results of the current study agree with Al-Bassam and Al-Kazaz (2013), who reported that the rate of resistance of clinical isolates of *P. mirabilis* to antibiotic piperacillin (40%). The current study differs from them in the rate of bacterial resistance to gentamicin (50 %), ciprofloxacin (40%), and imipenem (15%). These researchers indicated that the rate of isolates *P. mirabilis* urinary was resistant to imipenem (3%), ceftazidime (50 %), and meropenem (3%). and gentamicin and amikacin (10-20%), (3%) , cefotaxime and ciprofloxacin (50%).

Whereas ten isolates (PV31 to PV 40) belonging to *P. vulgaris* are tested against 12 common antibiotics, as shown in Figure 4-3. The majority of isolates exhibited antibiotic resistance, particularly β-lactam antibiotics. All 10 isolates of *the P. vulgaris* test were resistant to ampicillin, chloramphenicol, ciprofloxacin, and tobramycin have the greatest rate of resistance (100%) whereas piperacillin, azithromycin, tobramycin 30, and erythromycin and have fairly high rates of resistance 5/10 (50%), 6/10 (60%), 8/10 (80%) and 9/10 (90%), respectively. The isolates, however, were all completely susceptible to sulfamethoxazole, gentamycin, meropenem, and imipenem. In a study performed by Lazm *et al*. (2018), *Proteus* isolates were shown to be 93.3% and 80% resistant to amoxicillin and penicillin, respectively**.**

The types of antibiotics and their frequency of usage in the various patients from whom the samples were taken may be the cause of the diversity in the resistance pattern of *P. vulgaris* isolates. In a different investigation, ciprofloxacin sensitivity was discovered in all *Proteus* isolates (Fam *et al*., 2013). However, research by Daini *et al*. (2008) revealed that *P. vulgaris* was resistant to ciprofloxacin**.**



	C30	TM5	AMP	CIP10	E15	PRL	AZM	CN10	MRP	SMX	IMI
Resistant	100%	100%	100%	100%	90%	50%	60%	0	0	0	0
Moderate	0	0	0	0	0	30%	0	0	0	0	0
Senstive	0	0	0	0	10%	20%	40%	100%	100%	100%	100%

Figure 4-3: Antibiotic Susceptibility of *P. vulgaris*

Abbreviations: PRL; Piperacillin , E15; Erythromycin , AZM ; Azithromycin , IMI ; Imipenem , C30 ; Chloramphenicol , TM5 ; Tobramycin , AMP ; Ampicillin , CN10 ; Gentamicin , MRP ; Meropenem , SMX ; Sulfamethoxazole , CIP10 ; Ciprofloxacin.

Since the majority of the isolates were resistant to three antimicrobial drugs or more, it has been determined that they were multidrug resistant. Ampicillin, amoxicillin, and cephalothin were thought to be the most frequently prescribed antibiotics in hospitals even before the results of urine analyses, and they were also the most readily available on the market without a prescription and very inexpensive in terms of price. This may be why multidrug-resistant strains have emerged that are resistant to most of the antimicrobial agents tested. A general increase in the emergence of resistant bacteria has been caused by the widespread usage and more frequently the misuse of antimicrobial medications (Manikandan *et al.*, 2011).

This study similarity with Bilal, *et al*. (2019). The percentage of resistance of *P. vulgaris* to several antibiotics was 94% for chloramphenicol, tigecycline, and ampicillin, 88% for cefotaxime, 76% for ciprofloxacin and nitrofurantoin, and 50% for amikacin (59%) according to research on the organism's antimicrobial susceptibility. These findings were in line with those of other researchers, such as those of Feglo *et al*. (2010) who found that (84.6%) of *Proteus* isolates recovered from various clinical samples are characterized by multidrug-resistant phenotype and Al-Jumaa *et al*. (2011) who discovered that (80%) of *Proteus* isolates were resistant to Amoxicillin and (93.3%) of them were resistant to penicillin.

Ciprofloxacin resistance can result from any of the three quinolone resistance mechanisms: mutations that change the drug's targets, mutations that lower drug accumulation, and plasmid-mediated *qnr* genes that shield cells from the poisonous effects of quinolones. These genes primarily exist in enterobacteriaceae, and they have an impact on how quinolone resistance develops and spreads (Hooper, 2003; Fonseca *et al*., 2008). Fluoroquinolones, a class of potent antibacterial drugs, include the ciprofloxacin family and are used to treat a variety of nosocomial and community-acquired diseases.

All *P. vulgaris* isolates were susceptible to imipenem and meropenem, which are antibiotics that work against gram-negative bacteria (Htoutou *et al*., 2011). The common β-lactam antibiotics (amoxicillin, cefaclor, ampicillin, vancomycin, and amoxicillin/clavulanic) exhibited high resistance from all bacterial isolates, while imipenem and ciprofloxacin exhibited modest resistance. The identical plasmid pattern was found after plasmid analysis of six multidrug-resistant and two susceptible bacterial isolates. This demonstrated that the resistance phenomena among the isolated opportunistic bacteria are not caused by the R-factor according to Alharbi *et al*. (2014).

This study agreed with Kwiecinska *et al.* (2016) in terms of *P. vulgaris* results but contradicts it in terms of *P. mirabilis* results. All isolates under investigation were responsive to cephalosporins, imipenem, ciprofloxacin, piperacillin with tazobactam, and norfloxacin. 5 % of *P. vulgaris* strains exhibited piperacillin resistance. Amoxicillin and clavulanic acid resistance were present in five *Proteus spp*. strains (12.5%), including four (20.0%) of *P. mirabilis* and one (5.0%) of *P. vulgaris* strains. *P. mirabilis* had one amikacin-resistant strain (5.0%). Trimethoprim-sulfamethoxazole was ineffective against five *P. mirabilis* (25.0%) and one *P. vulgaris* (5.0%) strains. Ciprofloxacin was effective against all *P. mirabilis* and *P. vulgaris* strains that were examined. Three (15.0%) of the 20 *P. mirabilis* strains were, however, categorized in the intermediate category. Urine was used to isolate strains of ciprofloxacin intermediates.

Since indiscriminate use of antibiotics creates a selection pressure that increases the prevalence of resistant bacteria, *Proteus spp.* high. levels of antibiotic resistance may be an indication of the degrees of resistance among the enterobacteriaceae and may be *Salmonellae* (Barrow and Felthan,2003). The characterization of several plasmid-mediated *TEM*-type lactamases in *Proteus* is proof of the great diversity of β-lactamases produced by this

species and of its potential function as a plasmid reservoir for β-lactamases (Bonnet *et al*., 1999).

4.3 Multi Drugs Resistance (MDR)

The term "multi-resistance" (MDR) has no one set definition, but different definitions have been given to it (Falagas *et al*., 2006). An isolate is said to be multi-antibiotic resistant if it is at least resistant to three antibiotics, preferably from two different classes, or if it is resistant to more than one antibiotic. Two isolates were resistant to three antibiotics, four isolates were resistant to four antibiotics, fourteen isolates were resistant to six medications, and one isolate was resistant to nine antibiotics, according to Table 4-3.

Table 4-3 Frequency of multidrug-resistant isolates of *Proteus Spp.*

Resistant isolate No.	Classes of antibiotics
3	SMX / AZM / C30 / TM5 / AMP / CIP/ E15
3	PRL / SMX / AZM / C30 / TM5 / AMP / CIP / E15.
2	PRL / SMX / C30 / TM5 / AMP / CIP / E15.
4	SMX / C30 / TM5 / AMP / CIP / E15.
2	PRL / SMX / C30 / TM5 / AMP / E15.
1	AZM / C30 / AMP / E15.
1	AZM / IMI / AMP / CIP / E15.
1	PRL / TM5 / IMI / AMP / E15.
1	PRL / AZM / SMX / IMI / AMP / MRP / CIP / E15.
1	PRL / AZM / C30 / TM5 / AMP / CN10 / MRP / E15.
1	PRL / AZM / TM5 / IMI / AMP / E15.
1	PRL /SMX /TM5 / IMI / AMP / E15.
1	PRL / C30 / TM5 / IMI /AMP/ MRP / E15.
1	PRL / SMX / AZM / E15.
1	PRL / SMX / C30 / TM5 / CN10 / E15.
1	PRL /SMX / AMP / E15.
1	PRL / C30 / TM5 / CN10/ E15.
1	PRL / SMX / AZM / TM5 / IMI / E15.
1	PRL / SMX / AZM / TM5 / IMI / AMP / E15.

1	PRL / SMX / AZM / C30 / TM5 / IMI / MRP / E15.
1	PRL / TM5 / E15.
1	SMX / TM5 / IMI / AMP / E15.
1	SMX / C30 / TM5 / IMI / E15.
1	SMX / AZM / TM5 / AMP /CN10 /E15.
1	SMX / TM5/ AMP / CN10 / E15.
1	SMX / TM5 / E15.

Abbreviations: PRL; Piperacillin , E15; Erythromycin , AZM ; Azithromycin , IMI ; Imipenem, C30 ; Chloramphenicol , TM5 ; Tobramycin , AMP ; Ampicillin , CN10 ; Gentamicin , MRP ; Meropenem , SMX ; Sulfamethoxazole , CIP10 ; Ciprofloxacin.

Erythromycin (99.9%) and tobramycin (98%) were both found to have multi-drug resistance, and these results were similar to those of Algammal *et al*., 2021, who reported 100% resistance to both erythromycin and penicillin-G, and Pattanayak *et al*., 2018, who reported 100% resistance to both erythromycin and tetracycline. Wong *et al*. (2013), Salih *et al.* (2019), Dadheech *et al*. (2015)

4.4 Detection of Biofilm Formation by *Proteus Spp*.

The ability of *P. mirabilis* and *P. vulgaris* to produce biofilm was investigation in our study. Where 40 isolates of *Proteus* bacteria (*P. mirabilis* and *P. vulgaris*) were studied, and the result was that all (100 %) isolates formed a biofilm with the following ratios: 15 % is weakly information , 77.5 % is moderate and 7.5 % is strong information of biofilm as illustrated in Figure 4-4 A.

This results similar with Qaddoorri and HA-Neddawi (2015) where all isolates tested positively (100%) for biofilm formation.in Pelling and Jones (2019) all of the *Proteus* isolates (100%) vigorously developed the biofilm, while enterococcus species (92%), *Pseudomonas* species (79%), and *Staphylococcus* species (72%), were also capable of doing so.

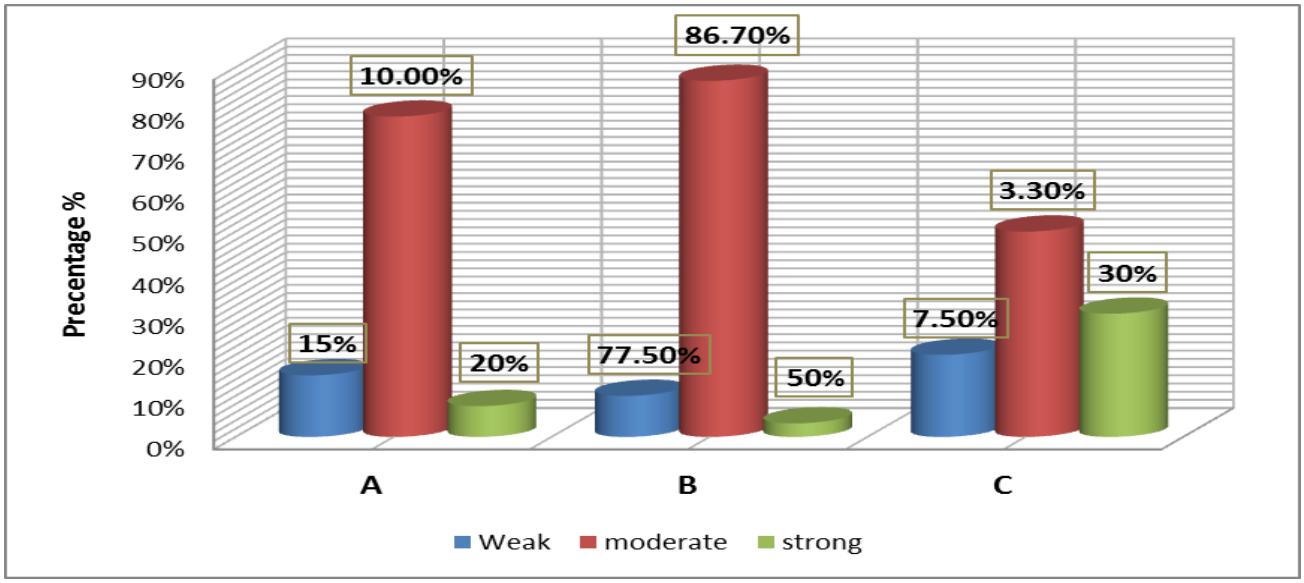


Figure 4-4: Percentage of biofilm formation by *P. mirabilis* and *P. vulgaris* (A), by *P. mirabilis* (B) and by *P. vulgaris* (C)

in this work , the capacity of *P. mirabilis* to generate biofilm was examind . where 30 isolate were studied , and the result was that all (100%) isolate formed a biofilm with the following ratios :- 77.5 % is weakly information , 86.7 % is moderate and 5% is strong information of biofilm as illustrated in figure 4-4 B.

The disparity in results between studies may result from several circumstances, like as the various nations from which the specimens were gathered, the quantity and kind of clinical specimens used to get the isolates, as well as variations in the isolates' capacity to form biofilm. A crucial and significant function may also be played by the initial amount of adhering cells and the variations in the Quality and output of each isolate's autoinducers (quorum-detecting signaling molecules) (Haji *et al.*, 2018).

A varied activity in biofilm development was reported in the current investigation, and this finding agrees with the findings of Halstead *et al*. (2015).

The literature describes the Tissue Culture Plates (TCP) assay as an easy and quick way to measure the production of biofilms by various bacterial strains. A common dye called crystal violet is known to bind to negatively charged compounds on cell surfaces, including nucleic acids and polysaccharides, and as a result, it provides a general assessment of the entire biofilm. For quickly gaining access to a variety of gram-positive and gram-negative bacteria for cell adhesion and biofilm

development, it has been employed as a standard approach (Al-Dahmoshi *et al.*, 2013).

Both Biofilm Producers and Non-Producers had high levels of resistance to sulfamethoxazole and moderate levels of imipenem resistance, piperacillin, erythromycin, azithromycin, imipenem, tobramycin, chloramphenicol, tobramycin, ampicillin, gentamicin, meropenem, sulfamethoxazole, ciprofloxacin were discovered to be immune to non-producers out of the 12 antibiotics. *P. mirabilis* has recently raised concerns about the production of biofilms. In a recent investigation, *P. mirabilis* isolates found in urine samples produced more biofilm than those found in various catheter segments. This study found a significant prevalence of *P. mirabilis*, which is consistent with research on catheterized patient urine. When considered as a whole, our results show that more research on *P. mirabilis* biofilm development is required to comprehend the illness process and create fresh preventive and therapeutic approaches.

Table 4-4:- The grade and percentage of biofilm formation by *P. mirabilis* isolates

Biofilm formation Grade and MDR	Isolate No.	%
Weak	3	10
Moderate	26	86.7
strong	1	3.3
Total	30	100

This study found that the resistance to several antibiotics, including piperacillin/tazobactam, cefoperazone, ceftriaxone, meropenem, and imipenem, was significantly higher among biofilm producers than non-producers, indicating that producers of biofilms were more resistant to antibiotics than non-producers. Though comparable investigations have been carried out for other infections such as Uropathogenic *E. coli*, there is currently little research that indicates a relationship between *P. mirabilis* biofilm development and medication resistance(Tajbakhsh *et al*.,2016). The discrepancies in antibiotic resistance and biofilm development in

diverse bacteria are probably caused by various resistance mechanisms. Additionally, we discovered a substantial correlation between the *P. mirabilis* isolates capacity to form biofilms and their resistance to sulfamethoxazole, piperacillin/tazobactam. To better understand the illness process and create efficient treatments for mammals infected with *P. mirabilis* that is resistant to antibiotics, additional research on the biofilm development by *P. mirabilis* is required.

Traditional antibiotic therapy makes it difficult to get rid of biofilm bacteria for two main reasons. Slime, another name for the polymeric aggregation known as biofilm polysaccharide, is often made up of proteins and polysaccharides (Hall-Stoodley *et al*., 2004).

While *P. vulgaris* isolates were all biofilm-forming, where 5 isolates (PM1, PM3, PM6, PM9, and PM10) were moderate, 3 isolates(PM2, PM5, and PM8) strong, and 2 isolates (PM4 and PM7)are weakly biofilm-forming, as shown in the following Figure 4-4C.

The outcomes from this study indicate that bacteria will attach strongly to the infection site if there is a strong or moderate biofilm present. However, poor positive results could indicate that the bacteria are under stress or that their growth is insufficient, which would result in a weak or nonexistent biofilm. Additionally, the variations in isolate's capacity to build biofilm were discovered. The primary number of adhering cells and the variations in the quantity and quality of autoinducers (quorum sensing signaling molecules) produced from each isolate may also be crucial and significant factors (Maleki *et al*., 2018). Under certain circumstances, biofilm development helps bacteria survive in a variety of hostile environments and is crucial to the persistence of infection (Hall-Stoodley *et al*., 2004). Costerton *et al*., (2001) One of the most remarkable and clinically significant characteristics of microbial biofilms is their markedly increased antibiotic resistance in comparison to their free-floating counterparts, which poses substantial challenges in the treatment of biofilm-associated infections. The Minimum Inhibitory Concentration (MIC) of antibiotics for biofilms maybe 1000 times higher than that for planktonic bacteria, according to

research (Hiby *et al*., 2010).

This necessitates research into bacterial biofilms. *P. mirabilis*, one of the *Proteus* strains, has been shown to generate biofilms on catheter materials (Sabbuba , 2002; Liaw *et al*., 2003), albeit the gene necessary for biofilm development has not yet been discovered. According to Pratt and Kolter (1998), gene products crucial for the establishment of biofilms are likewise crucial for disease. Although it could not be proven, there was evidence linking *P. vulgaris* to the illness. It has been documented that *Proteus spp*. causes crocodile septicemia (Novak and Seigel, 1986). These findings show how dangerous *Proteus spp.* is to the health of Chinese alligators. Not all isolates in the current investigation had 100% biofilm formation or 100% multidrug resistance, as indicated in Table 4-5, which compares the percentages between biofilm formation and antibiotic resistance in Table 4-6.

Table 4-5:- The grade and percentage of biofilm formation by *P. vulgaris* isolates

Biofilm formation Grade and MDR	**Isolate No.**	**%**
Weak	2	20
Moderate	5	50
strong	3	30
Total	10	100

In natural environments, biofilms are the most common type of microbial life. The first two phases in the formation of a microorganism's biofilm are adhesion to an abiotic or biotic surface and the construction of a structure to promote adhesion. Extracellular polymers, which are produced by microorganisms within the growing biofilm, are known to shield bacteria from environmental changes, assist in delivering nutrients and removing metabolic waste, and cluster cells close together to promote cell-to-cell contact. Multi-species biofilms, such as coexisting bacteria and fungi, are possible (Potera *et al.*, 1999). Both *P. vulgaris* and *P. mirabilis* can be found in the natural environment and in the small intestines of both humans and animals. Both *P. vulgaris* and *P. mirabilis* are capable of growing as biofilms on the surfaces of many

different items, including human insertion devices. Immunosuppressed patients frequently get *Proteus* infections, particularly urinary tract infections.

This study agrees with Kwiecinska *et al*. (2016). A biofilm was created by every examined strain. The range of *P. mirabilis* strains observed absorbance values was 0.656–2.319, whereas *the P. vulgaris* range was 0.708–1.800. based on the requirements.

High resistance to all antibiotics was observed in Table 4-6, where there was no discernible correlation between the grade of biofilm formation and antibiotic resistance. This could be a justification or proof that once a biofilm forms in any grade, it is adequate to withstand many lifelong antibiotics. This was what set *P. mirabilis* and *P. vulgaris* apart and contributed to their multi-drug resistance.

Table 4-6: Correlation between biofilm grade and antibiotic resistance.

antibiotic	Biofilm						P. value
	weak		moderate		strong		
	N0.	%	No.	%	No.	%	
PRL	2	9	17	77.2	14	0	0.076
SMX	2	9.6	18	85.7	1	4.7	0.063
AZM	2	13.3	13	86.7	0	0	0.107
IMI	1	7.2	13	92.8	0	0	0.117
C30	4	16.7	17	70.8	3	12.5	0.051
TM5	4	12.1	26	78.8	3	9.1	0.072
AMP	5	16	24	75	3	9	0.067
CN10	1	16.7	4	66.7	1	16.6	0.003
MRP	1	20	4	80	0	0	0.014
E15	12.9	13	31	79.5	3	7.6	0.011

CIP10	**3**	**25**	**6**	**50**	**3**	**25**	**0.015**

Abbreviations :- PRL; Piperacillin , E15; Erythromycin , AZM ; Azithromycin , IMI ; Imipenem , C30 ; Chloramphenicol , TM5 ; Tobramycin , AMP ; Ampicillin , CN10 ; Gentamicin , MRP ; Meropenem , SMX ; Sulfamethoxazole , CIP10 ; Ciprofloxacin

4.5 Detection of β-Lactamase Genes of *Proteus Spp.* Isolates

As shown in Table 4-7, The isolates of *P. mirabilis* and *P. vulgaris* that had positive beta-lactamase PCR results contained 33 (82.5%) *SHV*, 38 (95%) *TEM*, and 37 (92.5%) *CTX-M* genes (Table4-7 and 4-8). From PM1, except for PM 2, PM8, PM 19, and PM25 to PM30, and from PV31, except for PV32 to PV39, 33 isolates(82.5%) passed the *bla TEM*, *SHV*, and *CTX-M* genes. The genes *bla TEM* and *CTX-M* were found in 4 (10%) isolates (PM2, PM15, PM25, and PV40), *blaTEM* was found in just one (2.5%) specimen (PM 2), and these genes were absent in two (5%) isolates (PM8 and PM19).

Table 4-7: Distribution of gene group of *P. mirabilis* and *P. vulgaris* isolates.

Isolate No.	***TEM* gene**	***SHV* gene**	***CTX-M* gene**	**Genotype**
PM1	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM2	+Ve	-Ve	+Ve	*TEM* and *CTX-M*
PM 3	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 4	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 5	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 6	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 7	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 8	-Ve	-Ve	-Ve	None
PM 9	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 10	+Ve	+Ve	+Ve	TEM,SHV and *CTX-M*
PM 11	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 12	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 13	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 14	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*

PM 15	+Ve	-Ve	+Ve	*TEM* and *CTX-M*
PM 16	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 17	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 18	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 19	-Ve	-Ve	-Ve	None
PM 20	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 21	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 22	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 23	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 24	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 25	+Ve	-Ve	+Ve	*TEM* and *CTX-M*
PM 26	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 27	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 28	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 29	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PM 30	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PV 31	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PV 32	+Ve	-Ve	-Ve	*TEM*
PV 33	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PV 34	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PV 35	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PV 36	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PV 37	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PV 38	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PV 39	+Ve	+Ve	+Ve	*TEM*,*SHV* and *CTX-M*
PV 40	+Ve	-Ve	+Ve	*TEM* and *CTX-M*

Abbreviation: +ve: gene possessing., -ve: gene lacking

When ESBLs type β-lactamases were produced using antibiotic resistance, the identification of the antibiotic-resistant isolates that carried the bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes revealed an association between antibiotic resistance and positive molecular detections for the bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes in *P. mirabilis* and *P. vulgaris* isolates. The isolates of *P. mirabilis* and *P. vulgaris* that tested the most drug-resistant are listed in Table 4-8.

Table 4-8: Distribution of bla_{SHV}, bla_{TEM} and bla_{CTX-M} Genes of *P. mirabilis* and *P. vulgaris*

Gene	No.	%
TEM,SHV and *CTX-M*	33	82.5 %
TEM and *CTX-M*	4	10 %
TEM	1	2.5 %
None	2	5 %

In the isolates (*bla TEM, SHV, and CTX-M*) the three resistance genes implemented strong resistance to three antibiotics was revealed, while the *bla TEM and CTX-M* genes appear excellent resistant to four antibiotics. this result is shown in Table 4-9.

Table 4-9: Pattern of highest resistance isolates of *P. mirabilis* and *P. vulgaris* isolate to the different type of antibiotcs among these β-lactamases genes

Antibiotic	*TEM,SHV* and *CTX-M*		*TEM* and *CTX-M*		*TEM*		None	
	No.	%	No.	%	No.	%	No.	%
PRL	17	51.5	4	100	1	1	0	0
E15	26	78.8	4	100	0	0	0	0
AZM	19	57.6	1	25	0	0	1	50
IMI	13	39.4	1	25	0	0	0	0
C30	18	54.5	4	100	1	100	1	50
TM5	29	87.9	4	100	1	100	0	0
AMP	28	84.8	3	75	1	100	1	50
CN10	5	15	1	33	0	0	0	0
MRP	5	15	0	0	0	0	0	0
SMX	25	75.8	3	75	0	0	2	100
CIP10	10	30	1	25	1	100	0	0

Abbreviations: PRL; Piperacillin , E15; Erythromycin , AZM ; Azithromycin , IMI ; Imipenem , C30 ; Chloramphenicol , TM5 ; Tobramycin , AMP ; Ampicillin , CN10 ; Gentamicin , MRP ; Meropenem , SMX ; Sulfamethoxazole , CIP10 ; Ciprofloxacin

The occurrence of the β-lactamase gene is extremely significant and is a cause for health concern. By destroying the β-lactam ring in medicines, this enzyme helps bacteria develop resistance to common β-lactam ring antibiotics. According to a study (Bradford *et al*., 2001), bla_{TEM}, bla_{SHV}, and bla_{CTX-M} were related to six common β-

lactamase resistance genes in *Proteus* species (*bla*$_{TEM-1}$, *bla*$_{CMY}$, *bla*$_{CMY2}$, *bla*$_{SHV}$, *bla*$_{OXA}$, and *bla*$_{CTX}$).

Large-scale use of the diverse class of antibiotics known as β-lactams. Concerns regarding our reliance on β-lactam medications and the rise of pan-resistant organisms are raised by the expanding range of β-lactamases produced by isolates of the family enterobacteriaceae (Qin *et al*., 2008). *Proteus* possesses an inherent resistance to cephalosporin and ampicillin because of an extended spectrum of β-lactamase (Coque *et al*., 2008). Chromosome-encoded class C beta-lactamases may be expressed to cause resistance to expanded spectrum cephalosporins (Fam *et al*., 2013). The global occurrence of antibiotic-resistant infections is mostly caused by horizontal gene transfer, which is mediated by R plasmids, transposons, and integrons. Previous studies have shown that potential ESBL species including *K. pneumoniae* and *E. coli* have a high propensity to carry and transfer *bla* genes, which poses a threat to human health (Bailey *et al*., 2011).

Because the genes are frequently present on mobile elements like transposons and integrons, a transfer may take place by conjugation (Tissera and Mae Lee, 2013). Several of these species might be pathogenic strains with the ability to spread illness and have deadly consequences. For instance, nosocomial infections have been linked to the bla$_{CTX-M}$ and bla$_{TEM}$ genes in opportunistically pathogenic enterobacteriaceae (Zhang *et al*., 2008).

The treatment is rendered ineffective by *P. mirabilis* multidrug resistance, and novel, forward-thinking methods are required to combat the problem. Gene networks are used to examine the antimicrobial resistance genes of *P. mirabilis*, enriched pathways, and gene ontology terms to comprehend the molecular basis. The *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla* $_{CTX-M}$ genes could be used as potential therapeutic targets because they are important in controlling essential bacterial processes. The pharmacological targets mentioned in this study's findings will help researchers create new defenses against the

multidrug-resistant *P. mirabilis* (Shaaban *et al.*, 2020). Diabetes patients' feet, the urinary tract, the respiratory tract, burns, wounds, and many other illnesses are all caused by *P. mirabilis*. Because of its strong antimicrobial resistance, this infection requires new therapeutic approaches to be defeated. One method for treating multidrug-resistant (MDR) *P. mirabilis* infections, particularly biofilm-based illnesses, is the use of bacteriophages (Gomaa *et al*.,2019). Highly resistant infections may be released as a result of several events, including the misuse of antibiotics and the dissemination of clonally resistant germs. Among the drug resistance mechanisms, ESBLs are crucial in the development of resistance to widely used antibiotics like penicillin and cephalosporins. ESBL genes can further contribute to an increase in drug resistance, including in MDR isolates, as a result of the extensive dissemination of pathogens in the community through plasmids and integrons. When compared to Khalilzadegan and colleagues' findings that *CTX-M* and *TEM* include the majority of ESBL genes, our studies revealed that $bla_{CTX\text{-}M\text{-}15}$ (58.3%) was the most often found gene.

Analyzing the epidemiological data from previous years may reveal a dramatic rise in antibiotic resistance. Overall, the data show that bacteria in the enterobacteriaceae family are continuously developing resistance to both β-lactams and other classes of antibiotics (Johnson and Stell,2000; Wiegand,2007). Studies have demonstrated that the genes responsible for the production of *CTX-M* and *TEM* β-lactamases are more prevalent among tested strains than genes encoding *SHV*-type β-lactamases with no detection report. In this regard, the results of our study are in line with those of previous studies Fursova, *et al*., (2015).

As shown in Table 4-7 and 4-8, 38 isolate is positive to bla_{TEM}, 37 isolate with $bla_{CTX\text{-}M}$, 33 isolate with bla_{SHV}, one isolate with bla_{TEM} and 2 isolate with none one to $bla_{TEM,CTX\text{-}M,\ and\ SHV}$ genes.

Globally, the emergence and spread of β-lactam resistance in nosocomial enterobacteriaceae become a severe issue. Particularly concerning is the rise of resistance to carbapenems and third- and fourth-generation cephalosporins. Different molecular tactics are used by gram-negative bacteria to build resistance to these antibiotics: Extending the range of already extensively distributed plasmid-encoded -lactamases by substituting amino acids results in the production of extended-spectrum β-lactamases (ESBL) according to the original definition; acquiring ESBL-encoding genes from environmental bacteria.

The resistance typically depends on the expression of genes from the bla_{TEM},bla_{SHV}, and bla_{CTX-M} gene families, among others. Large groups of enzymes with evolutionary affinity, *bla TEM* β-lactamases, *SHV* β-lactamases, and *CTX-M* β-lactamases, are produced by the genes bla_{TEM}, bla_{SHV}, and bla_{CTX-M}, respectively. Since the discovery of the first *TEM-1* β-lactamase, 185 novel β-lactamases of the TEM family have been reported globally, whereas 93 variations are responsible for the creation of ESBLs. Of the 177 different *SHV* family enzyme types, 45 have been identified as extended-spectrum β-lactamases. More than sixty enzymes make up the *CTX-M* family.

It is well known that transferable elements like plasmids or transposons may harbor *bla* genes encoding antibiotic resistance. The horizontal spread of antibiotic resistance among bacterial strains may be facilitated by this location of *bla* genes as shown in Table 4-9.

4.5.1 DNA Amplification of bla_{CTX-M} Gene

The DNA amplification was accomplished by the Thermo-cycler apparatus under the optimal conditions using specific primers as mentioned in the Tables 3-8. The result of the PCR reaction revealed the presence of a single band (499 bp) of the target sequence for bla_{CTX-M1} gene *P. mirabilis* and *P. vulgaris* as in Figure 4-5 and Figure 4-6 respectively.

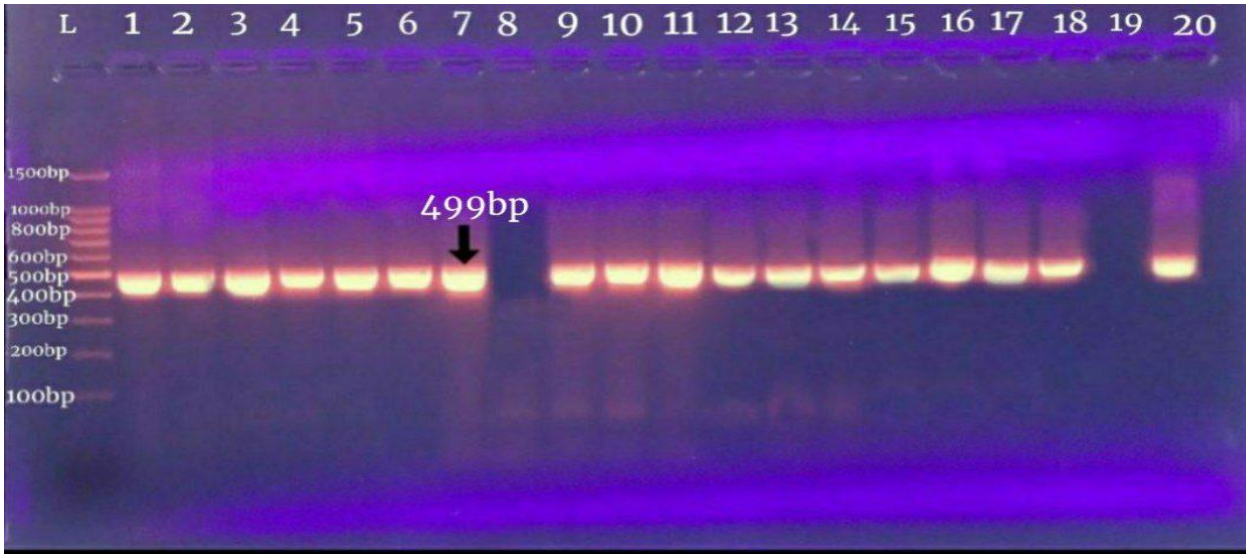


Figure 4-5*:* Agarose gel electrophoresis of PCR products of *bla*$_{CTX-M1}$ gene of *P. mirabilis* isolates. The products were separated in 1% agarose gel at 7.5 V\Cm for 80 min. Lane L: DNA ladder (1500 bp), Lanes 1-20: the isolates PM1 to PM 20, represent the positive results (499 bp), except PM8 and PM19 isolates that represent the negative results .

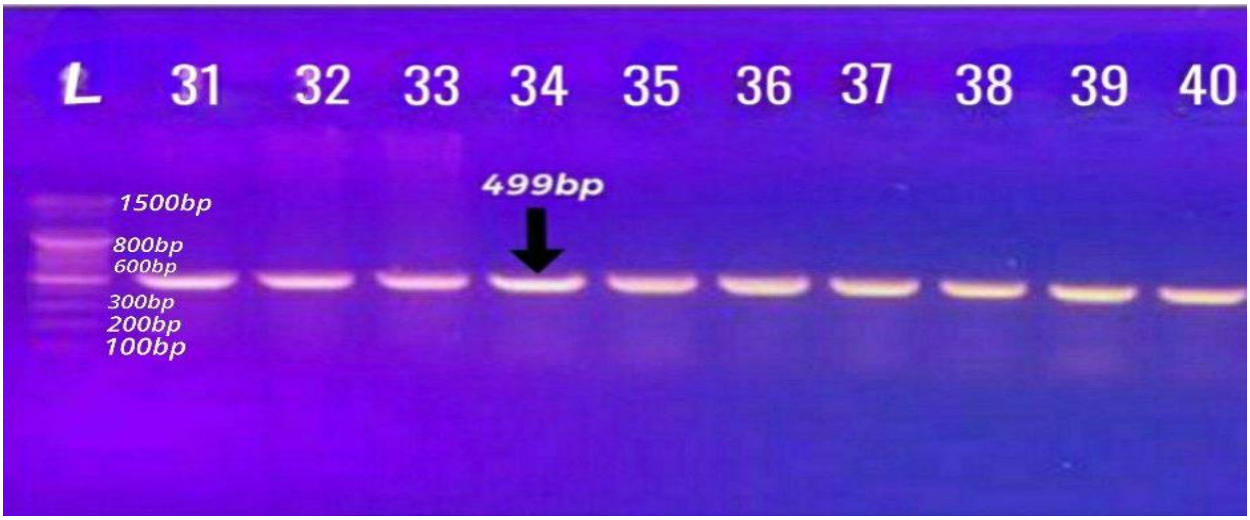


Figure 4-6*:* Agarose gel electrophoresis of PCR products of *bla*$_{CTX-M1}$ gene of *P. vulgaris* isolates. The products were separated in 1% agarose gel at 7.5 V\Cm for 80 min. Lane L: DNA ladder (1500 bp), Lanes 31-40: the isolates PV31 to PV40 represent the positive results (499 bp) .

The class A extended-spectrum β-lactamases (ESBLs) known as *CTX-M*-type enzymes are quickly gaining popularity among enterobacteriaceae in all parts of the world. Serine β-lactamases, or ESBLs, are categorized as class A by Ambler's molecular and structural classification system**.**

The majority of extended-spectrum β-lactamases (ESBLs) generated by enterobacteriaceae members are now *CTX-M* enzymes (Canton and Coque,2006).

Since 1995 (Ishii *et al*., 1995), when Toho-1, now known as *CTX-M-44*, was discovered in Japan, ESBLs frequently contain *CTX-M* enzymes. All 71 clinical isolates of the *P. mirabilis* strain that produced *CTX-M* and were gathered in Japan between 2001 and 2003 had $bla_{CTX\text{-}M\text{-}2}$ (Shibata *et al.*, 2006). In Japan, 54 healthcare facilities participated in statewide monitoring research in 2006 that found that 28 of 74 *P. mirabilis* clinical isolates (37.2%) produced ESBLs and that all but one of them had $bla_{CTX\text{-}M\text{-}2}$ (Kanayama *et al*., 2010). In this research, the clonality of *P. mirabilis* isolates that produce *CTX-M-2* in Japan was not examined. To clarify the genetic processes involved in the acquisition of $bla_{CTX\text{-}M\text{-}2}$ in this organism, we investigated the geographic distribution and genetic environment of the $bla_{CTX\text{-}M\text{-}2}$ gene of *P. mirabilis* isolates in Japan in this work.

In Musa *et al*.,(2019) 55% of the ESBL producers, the *TEM* gene was found on its own, and in 35% in conjunction with the *CTX-M* gene. The *CTX-M* gene appears to be becoming more prevalent in *Proteus* species. *Proteus* species exclusively had the *TEM* gene in several studies done in India (Fazeli, 2014; Miriagou *et al.*2010). In Italy, only the *TEM* gene was present in 44% of the *Proteus* that produced ESBLs (Endimiani *et al*.,2005). Every ESBL-producing *Proteus* present in Iraq carried this gene (*Hindi et al.*,2014). In recent investigations conducted in India, 1.8% of strains carried the *TEM* gene, 52.9% of both genes, and 35.3% of ESBL-producing *Proteus* carried the *CTX-M* gene alone (Caubey *et al*.,2018). When the dominant gene spreads, the *CTX-M* gene seems to first develop in conjunction with the *TEM* gene before dislodging the others. It appears that the selection pressure brought on by improper usage of antibiotics has fostered the expansion of ESBLs among enterobacteriaceae.

4.5.2 DNA Amplification of bla_{SHV} Gene

The DNA amplification was accomplished by the Thermo-cycler apparatus under the optimal conditions using specific primers as mentioned in Tables 3-8. The result of the PCR reaction revealed that the presence of a single band (231 bp) of the target sequence for bla_{SHV} gene *P. mirabilis* and *P. vulgaris* as in Figures 4-7 and 4-8 respectively.

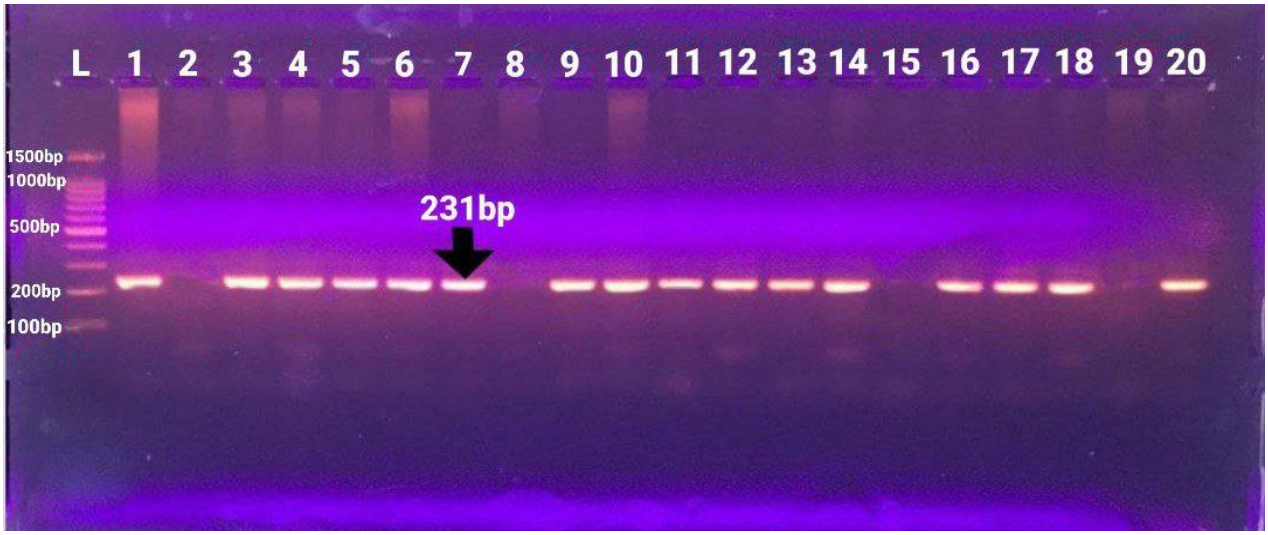


Figure 4-7*:* Agarose gel electrophoresis of PCR products of bla_{SHV} gene of *P. mirabilis* isolates. The products were separated in 1% agarose gel at 7.5 V\Cm for 80 min. Lane L: DNA ladder (1500 bp), Lanes 1-20: the isolates PM1 to PM20, except PM2,PM8, PM15 and PM19 isolates, represent the negative results (231 bp) .

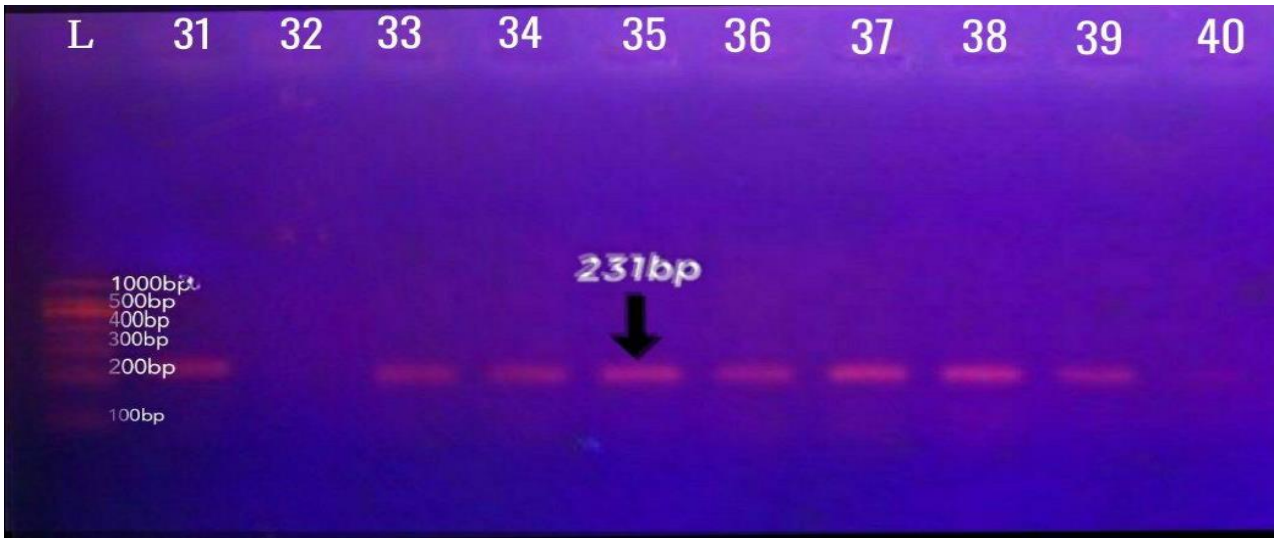


Figure 4-8*:* Agarose gel electrophoresis of PCR products of bla_{SHV} gene of *P. vulgaris* isolates. The products were separated in 1% agarose gel at 7.5 V\Cm for 80 min. Lane L: DNA ladder (1500 bp), Lanes 31-40: the isolates PV31 to PV 40, except the isolates PV32 and PV40, represent the negative results(231 bp) .

Other Enterobacteriaceae-related bacterial species may have plasmids with transposable genes expressing *SHV-1*. The mobility of this family of enzymes is demonstrated by the presence of extended-spectrum β-lactamases of the *SHV* family on self-transmissible plasmids.

The other beta-lactamase is known as *SHV* because of its sulfhydryl variable active site. Bacteria that produce ESBLs are becoming more widely known. They are responsible for a variety of clinical illnesses, such as intra-abdominal infections, cholangitis, and urinary tract infections.

Proteus spp. which produces ESBLs is becoming more common everywhere, especially in the United States, Asia, and Europe (Uzunović *et al*.,2016). The prevalence of bla_{TEM} and bla_{SHV} was 60% and 23.3%, respectively, in Malaki and Khademian (2022). The geographic distribution, kind of organisms, and source of infections may all play a role in these variations in bla_{TEM} and bla_{SHV} distribution. In *P. mirabilis* isolates from Iraq, bla_{TEM} was present 60% of the time (Fattah Hamid *et al*.,2020), but in China, it was present 52% of the time (Li and wang, 2022). All *Proteus spp*. isolates in Argentina have the bla_{TEM} gene, according to research on enterobacteria resistance to β-lactam/β-lactamase inhibitors (Conza *et al*.,2014). The bla_{TEM} rate among *P. mirabilis* isolates in India was 81.9% (Chinnam *et al*.,2021), whereas it was 35% (Algammal *et al*.,2021) in Egypt. Malekjamshidi *et al*. (2010) calculated that 83% of ESBL-positive *P. mirabilis* specimens in Tehran, Iran, have bla_{TEM}. Additional research revealed varying rates between 8.3% and 91% (Lev and Fursova,2018).

4.5.3 DNA Amplification of bla_{TEM} Gene

The DNA amplification was accomplished by the Thermo-cycler apparatus under the optimal conditions using specific primers as mentioned in Tables 3-8. The result of the PCR reaction revealed the presence of a single band (861 bp) of the target

sequence for the *bla*$_{TEM}$ gene of *P. mirabilis* and *P. vulgaris* as in Figures 4-9 and 4-10 respectively.

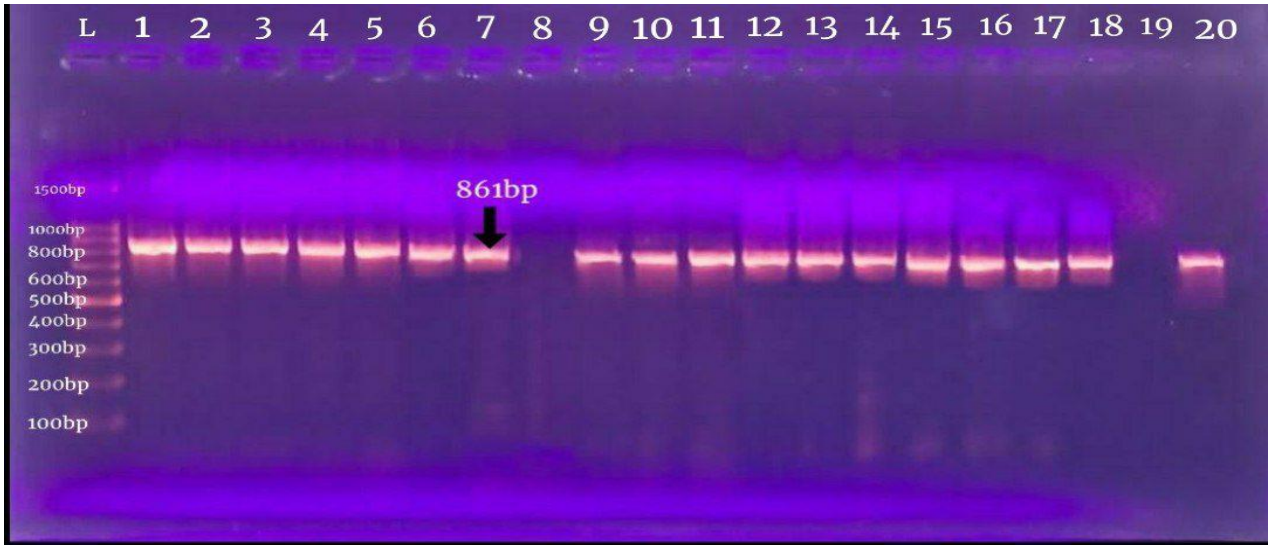


Figure 4-9*:* Agarose gel electrophoresis of PCR products of *bla*$_{TEM}$ gene of *P. mirabilis* isolates. The products were separated in 1% agarose gel at 7.5 V\Cm for 80 min. Lane L: DNA ladder (1500 bp), Lanes 1-20: the isolates PM1 to PM 20, except the isolates PM8 and PM19, represent the negative results (861 bp) .

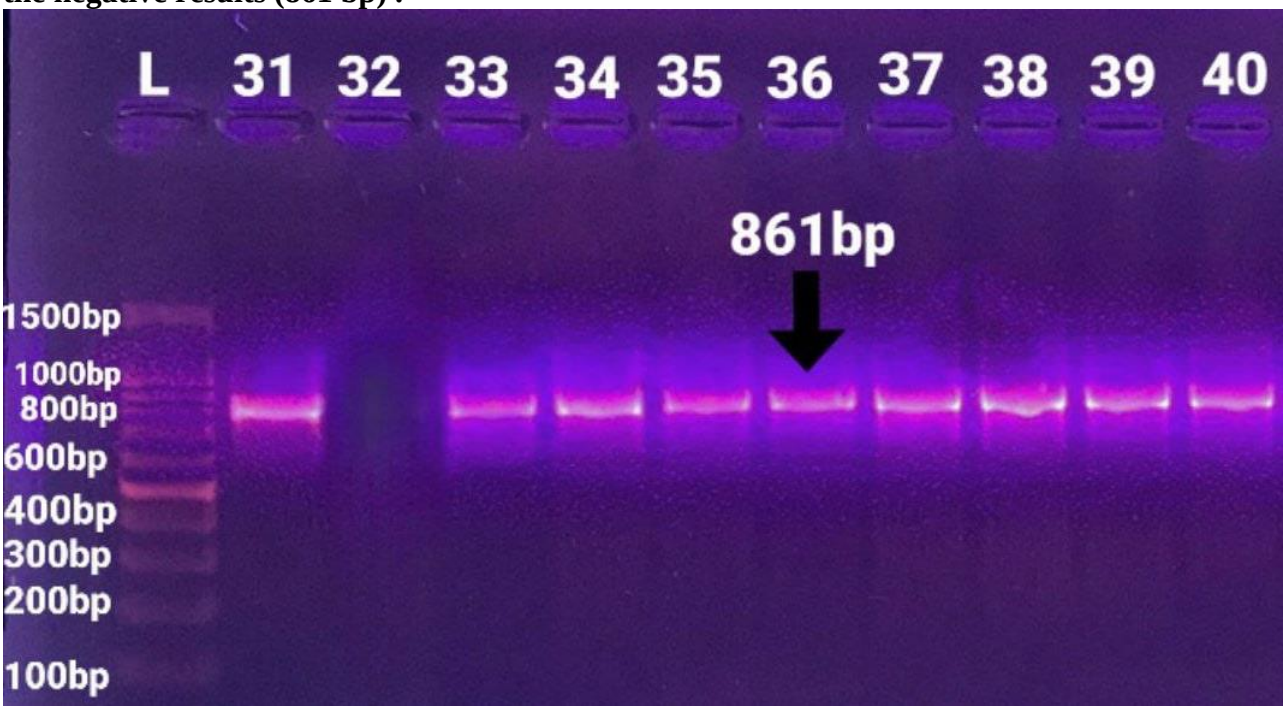


Figure 4-10:- Agarose gel electrophoresis of PCR products of *bla*$_{TEM}$ gene of *P. mirabilis* isolates. The products were separated in 1% agarose gel at 7.5 V\Cm for 80 min. Lane L: DNA ladder (1500 bp), Lanes 31-40: the isolates PV31 to PV40, except the isolates PV32, represent the negative results (861 bp)

Their capacity to hydrolyze broad spectrum β-lactamases antibiotics and inhibition by β-lactamases inhibitors, particularly clavulanate, define them biochemically.

Gram-negative bacteria that produce ESBLs are an increasing problem that spread across the entire nation. The *CTX-M* gene type predominated among 84% of ESBL producers, although the *TEM* gene was also on the rise. Due to the synthesis of the *TEM* and *CTX-M* genes, *P. mirabilis* BDUMS1 (*KY617768*) and *E. coli* BDUMS3 (*KY617770*) in particular demonstrated a significant prevalence rate of resistance. The findings led to the conclusion that UTIs are more likely to increase antibiotic resistance (Rajivgandhi *et al*.,2018).

Extended-spectrum beta-lactamase (ESBL)-producing *Proteus Spp.* is one particular AMR issue that has spread globally and affects both humans and animals. Due primarily to the synthesis of *CTX-M, TEM,* and *SHV* β-lactamases, which are encoded by the bla_{CTX-M}, bla_{SHV}, and bla_{TEM} genes, respectively, these bacteria are resistant to penicillins, cephalosporins, and aztreonam. These genes may be expressed chromosomally or via plasmids. In both animals and people, *CTX-M*-enzymes has emerged as the most prevalent form of ESBL among these three. They are not very closely related to *TEM or SHV* β-lactamases, and their name *CTX* indicates their strong hydrolytic activity against cefotaxime. Bilal and Anjum,(2019) all representative *P. vulgaris* isolates tested positive for the bla_{TEM} gene (100%), but only 50% of the isolates tested positive for the *qnr* gene. These findings were consistent with those of other studies that indicated a greater frequency of the bla_{TEM} gene in *Proteus* isolates (Dallenne *et al*., 2010). Furthermore, identical results for the bla_{TEM} gene were also reported by Tissera and Mae Lee, (2013). Extended-spectrum beta-lactamase is a mediator of *Proteus* improved resistance to beta-lactam medicines. According to Fam *et al*., (2013), integrons, transposons, and horizontal gene transfer may be to blame for this rise in antibiotic resistance.

4.6 DNA Sequencing and Phylogenetic Analysis

4.6.1 DNA Sequencing

To obtain a trimmed sequence, each data sequence was trimmed from beginning to end, according to normal waves. When compared to NCBI- Blast, this sequence has a high level of identity to other global sequence data. The waves produced by scanning the sequences indicate the strong and weak regions of the sequences, which are then trimmed, resulting in increased identity with global sequences at NCBI-Blasting.

Because *P. mirabilis* bacteria were more prevalent than *P. vulgaris* in this study, eight *P. mirabilis* isolates were chosen for DNA sequencing analysis.

The results of nucleotide sets are checked and confirmed by using (NCBI) – Basic Local Alignment Search Tool (BLAST analysis)- nucleotide blast-Search a nucleotide database using a nucleotide query online, which was a perfect program and gave the exact results of identity percentage with other world strains. Sequence alignment must be performed by using bla_{CTX-M1},bla_{SHV}, and bla_{TEM} gene of *P. mirabilis* sequences database information recorded in GenBank to find identity and similarity score degrees of gene and compared with our local isolates.

The results of sequence alignment of the eight local isolates showed identity ranging from 97% to 100% (Figure 4-11 , 4-12 and Table 4-10), good query cover, and max score with other world strains of *P. mirabilis*.

Table 4-10 : Alignment results of eight local *P. mirabilis* isolates with reference isolates retired from NCBI

Local Isolate	Reference of the isolate with highest percentage similarity(%)			
	Gene	Accession No.	Similarity (%)	Country
P. mirabilis PM.1	bla_{CTX-M1}	JQ235796.1	100	India
P. mirabilis	bla_{CTX-M1}	JQ235796.1	100	India

PM.2				
P. mirabilis PM.3	bla_{CTX-M1}	JN019836.1	99	India
P. mirabilis PM.4	bla_{SHV}	JQ235833.1	98	India
P. mirabilis PM.5	bla_{TEM}	LC613166.1	100	Iraq
P. mirabilis PM.6	bla_{TEM}	KY640468.1	97	Egypt
P. mirabilis PM.7	bla_{TEM}	LC613166.1	98	Iraq
P. mirabilis PM.8	bla_{TEM}	LC613166.1	97	Iraq

4.6.2 Phylogenetic Analysis of Local and World Strains

The phylogenetic tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The dataset was cleansed of positions with gaps or missing data (Complete deletion option). MEGA X 10.2.4 is used to perform phylogenetic analysis.

There were 7 global taxa about *the* bla_{CTX-M1} gene of *P. mirabilis* that were downloaded from NCBI and submitted with 3 local sequences to Mega X 10.2.4 software to obtain Figure 4-11.

There were 9 global taxa about the bla_{TEM} gene of *P .mirabilis* were downloaded from NCBI and submitted with 4 local sequences to Mega X 10.2.4 software to obtain Figure 4-12.

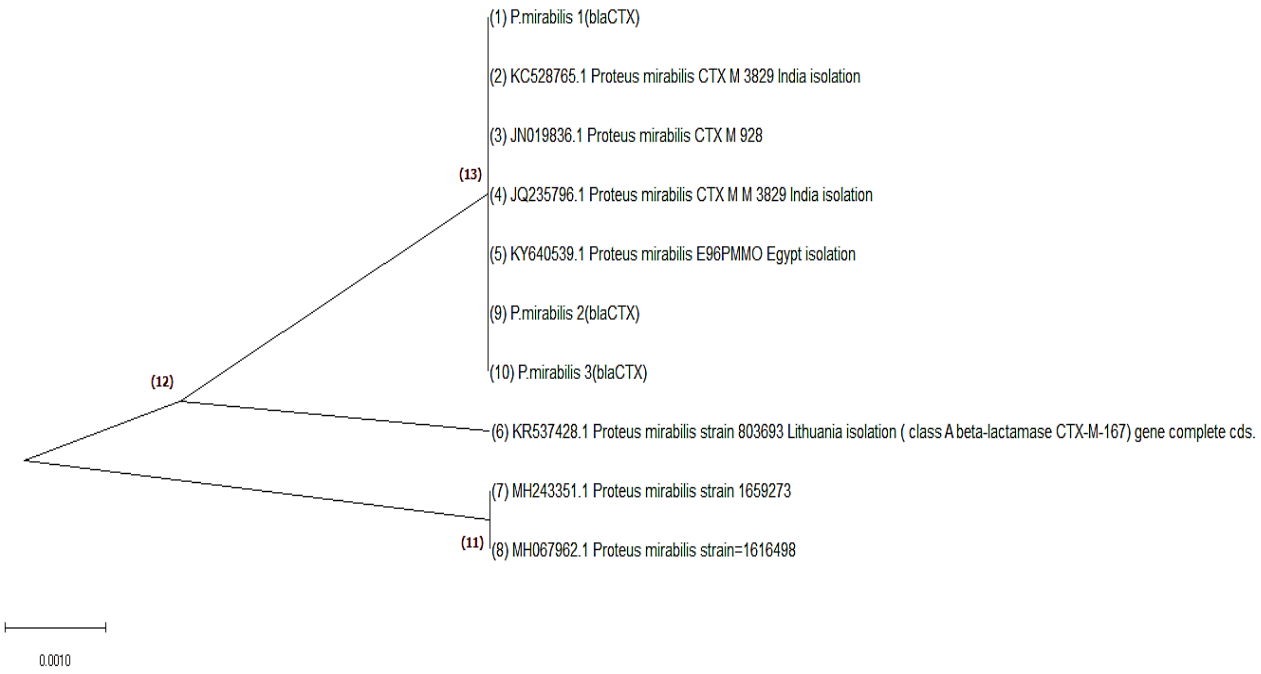


Figure 4-11 : Phylogenic tree of bla$_{CTX\text{-}M1}$ gene partial sequences of local and global sequences using neighbor joining bootstrap 500 tree figure. Evolutionary relationships of 10 taxa. PM 1 to 3 represent local isolates.

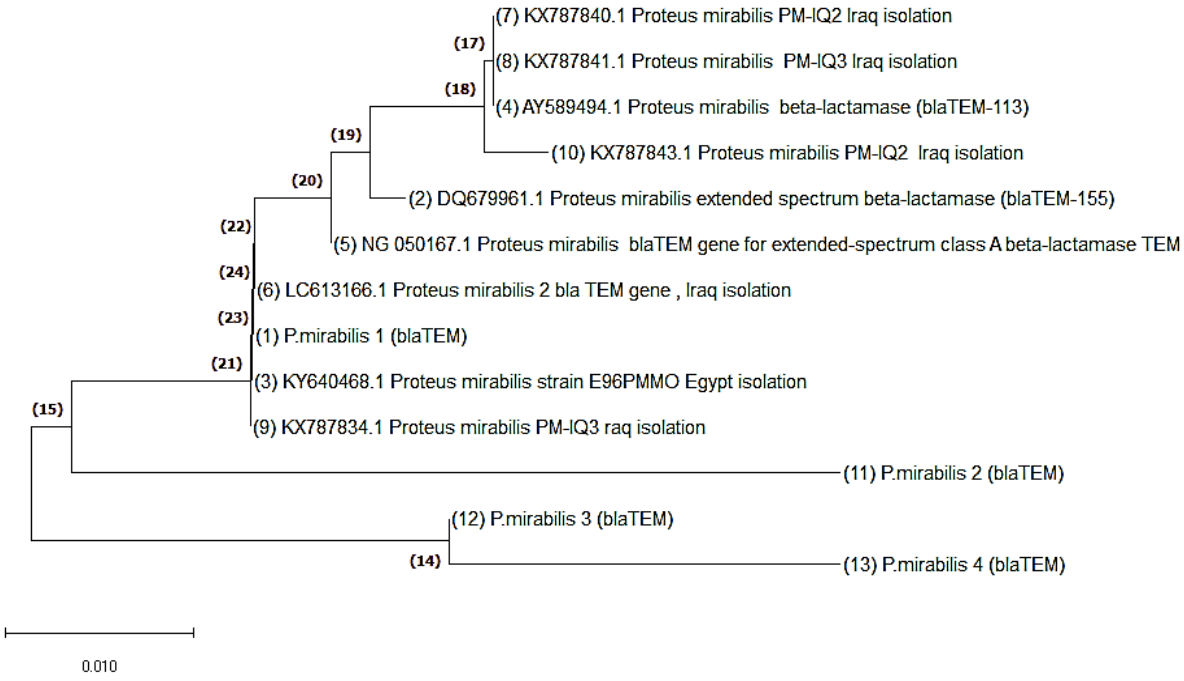


Figure 4-12 : Phylogenic tree of bla$_{TEM}$ gene partial sequences of local and global sequences using neighbor joining bootstrap 500 tree figure. Evolutionary relationships of 13 taxa. PM 1 to 4 represent local isolates.

The Molecular Evolutionary Genetics Analysis (MEGA) program is a desktop software that allows you to compare homologous gene sequences from various species or multigene families, with a focus on inferring evolutionary links and patterns of DNA and protein evolution. MEGA features several useful tools for assembling sequence data sets from files or web-based repositories, as well as tools for visualizing the results in the form of interactive phylogenetic trees and evolutionary distance matrices (Kumar *et al*., 2008). The first stage in the analysis was to align all of the sequences from three genes in this study with other worldwide references using MEGA X 10.2.4 's (Clustal W) program step. This program was shown to have a high degree of similarity with all world sequences, including the sequences used in this study. These (Clustal W) results were significant since they were directly utilized in the phylogenetic tree design.

The Neighbor-Joining (NJ) approach, which is a simplified version of the minimal evolution (ME) method, is used in this study to determine the close relationship between world and local sequences. Because it does not need the assumption of a constant rate of evolution, the NJ method yields an unrooted tree. An out-group taxon is needed to find the root (Saitou and Nei, 1987; Rzhetsky and Nei, 1992).

In $bla_{CTX\text{-}M1}$ gene phylogeny Figure 4-11, we submitted 10 sequences, 3 sequences belong to local sequences and 10 sequences belong to global sequences obtained by download from NCBI they submitted to a MEGA X 10.2.4 software program for obtaining phylogenic relationship among local and global sequences, after submitting these sequences to MEGA X 10.2.4 at the first time we found alignment by Clustal W, then use NJ method at bootstrap 1000, the local sequences of *P. mirabilis* PM1, PM 2, and PM 3 form clusters with four global sequences. the *P. mirabilis* PM 1 was near to the sequence KC528756.1 *P. mirabilis CTX-M* 3829 (India isolation) while the local sequences of *P. mirabilis* PM 2 and PM 3 was near the sequence E96PMMO (Egypt isolation).

In the phylogeny of the bla_{TEM} gene Figure 4-12, the local sequences *P. mirabilis* PM 2, PM 3, and PM 4 were closely related to each other's and all three isolates were near the sequence KX787834.1 *P. mirabilis* PM-IQ3 which isolate in Iraq. In contrast, the local sequences *P. mirabilis* PM1are near the sequence LC613166.1 which is also isolated in Iraq. thus, the Phylogenic relationship among local and world strains provides high information about the origin and genetic evolution of local isolates.

Multiple sequence analysis is used in such biological studies to extract important phylogenetic and evolutionary information using different scoring matrices (BLOSUM62 for BLAST, BLOSUM50 for SEARCH, and FASTA) (Alsayed *et al*.,2014).

Conclusions and Recommendations

Conclusions

1. It has been established that all *P. mirabilis* and *P. vulgaris* isolates are capable of possessing a wide range of virulence genes, including as urease, adhesion factors, and swarming activity.
2. While other medicines like meropenem and imipenem exhibited low resistance and great sensitivity to *P. mirabilis* and *P. vulgaris* isolates, all isolates are resistant to erythromycins, tobramycin 5, ampicillin, chloramphenicol, piperacillin and sulfamethoxazole, and azithromycin.
3. *P. mirabilis* and *P. vulgaris* clinical isolates all shown various degrees of biofilm formation capacity.
4. Most of the examined *P. mirabilis* and *P. vulgaris* isolates had extended spectrum β-lactamase genes (*bla* $_{TEM}$, *bla* $_{CTX-M}$, and *bla* $_{SHV}$) present.

Recommendations

1. Clinical microbiology can be enhanced by more precisely identifying poorly described, seldom isolated, or biochemically aberrant strains of bacteria using sequencing, VITEK, or on a molecular basis as opposed to phenotypic basis.
2. Conducting epidemiological studies on the causes of the spread of antibiotic resistance by detecting the genes responsible for the characteristic of resistance, which are often carried on plasmids and transposons.
3. To reduce the possibility of microorganisms developing drug resistance, new antibiotics should be used very selectively and for brief periods of time.
4. using samples from the environment and animal diseases to conduct significant research on other *Proteus* species.

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Appendix

Appendix 1:- Biofilm Forming Capacity of *P. mirabilis* and *P. vulgaris*

Isolate	OD	Grade
PM1	0.119	Moderate
PM 2	0.115	Moderate
PM 3	0.106	Moderate
PM 4	0.105	Moderate
PM 5	0.103	Moderate
PM 6	0.246	Strong
PM 7	0.104	Moderate
PM 8	0.088	Moderate
PM 9	0.091	Moderate
PM 10	0.097	Moderate
PM 11	0.085	Moderate
PM 12	0.109	Moderate
PM 13	0.138	Moderate
PM 14	0.111	Moderate
PM 15	0.095	Moderate
PM 16	0.076	Weak
PM 17	0.105	Moderate
PM 18	0.100	Moderate
PM 19	0.094	Moderate
PM 20	0.118	Moderate
PM 21	0.107	Moderate
PM 22	0.124	Moderate
PM 23	0.112	Moderate
PM 24	0.081	Weak
PM 25	0.113	Moderate
PM 26	0.116	Moderate
PM 27	0.115	Moderate
PM 28	0.108	Moderate
PM 29	0.081	Weak
PM 30	0.094	Moderate
PV 31	0.365	Moderate
PV 32	0.440	Strong
PV 33	0.345	Moderate
PV 34	0.210	Weak
PV 35	0.521	Strong
PV 36	0.399	Moderate
PV 37	0.200	Weak
PV 38	0.447	Strong
PV 39	0.250	Moderate
PV 40	0.240	Moderate

Appendix 2:- Phenotypic of Antibiotic Susceptibility of *P. mirabilis* and *P. vulgaris* isolates

Isolate	PRL	SMX	AZM	C30	TM5	IMI	AMP	CN10	MRP	CIP	E15
PM1	S	R	R	R	R	I	R	S	S	S	R
PM 2	R	R	R	R	R	R	R	S	S	S	R
PM 3	R	R	R	I	R	R	R	I	S	S	R
PM 4	R	R	R	S	R	R	S	S	S	S	R
PM 5	S	R	S	S	R	S	S	S	S	S	R
PM 6	I	R	S	I	R	I	R	R	S	S	R
PM 7	S	R	R	S	R	S	R	R	S	S	R
PM 8	S	S	R	R	S	S	R	S	S	S	R
PM 9	R	S	S	S	R	S	S	S	S	S	R
PM 10	I	R	R	R	R	S	R	S	S	S	R
PM 11	R	R	R	I	R	I	R	I	S	S	R
PM 12	S	R	S	R	R	R	R	S	S	S	R
PM 13	R	S	S	R	R	S	S	R	S	S	R
PM 14	R	R	S	S	S	S	R	S	S	S	R
PM 15	R	R	S	R	R	S	S	R	S	S	R
PM 16	R	R	S	R	R	S	R	S	S	S	R
PM 17	S	R	R	R	R	R	R	R	R	S	R
PM 18	R	R	R	S	S	S	I	S	S	S	R
PM 19	S	R	S	R	R	I	R	S	S	S	R
PM 20	R	S	S	R	R	R	R	S	R	S	R
PM 21	S	S	R	S	R	R	R	S	R	I	R
PM 22	R	R	I	I	R	R	R	S	S	S	R
PM 23	R	S	R	S	R	R	R	S	S	S	R
PM 24	R	S	R	R	R	S	R	R	R	S	R
PM 25	R	R	S	R	R	I	R	I	I	S	R
PM 26	R	R	R	I	I	R	R	S	R	R	R
PM 27	R	S	S	S	R	R	R	S	S	S	R
PM 28	S	R	S	R	R	R	I	S	S	S	R
PM 29	S	S	R	S	S	R	R	S	S	R	R
PM 30	S	R	S	S	R	R	R	S	S	S	R
PV 31	R	R	R	R	R	S	R	S	S	R	R
PV 32	R	R	R	R	R	S	R	S	S	R	R
PV 33	I	R	R	R	R	S	R	S	S	R	R
PV 34	S	R	S	R	R	S	R	S	S	R	R
PV 35	R	R	S	R	R	S	R	S	S	R	R
PV 36	I	R	S	R	R	S	R	S	S	R	R
PV 37	R	R	S	R	R	S	R	S	S	R	R
PV 38	R	R	R	R	R	S	R	S	S	R	R
PV 39	S	R	R	R	R	S	R	S	S	R	R
PV 40	I	R	R	R	R	S	R	S	S	R	S

Aberrations : PRL; Piperacillin , E15; Erythromycin , AZM ; Azithromycin , IMI ;Imipenem ,TOB30 ; Tobramycin , C30 ; Chloramphenicol , TM5 ; Tobramycin , AMP ; Ampicillin , CN10 ; Gentamicin , MRP ; Meropenem , SMX ; Sulfamethoxazole , CIP10 ; Ciprofloxacin

الخلاصة

Proteus هي بكتيريا متحركة ، سلبية اللاكتوز ، منتجة لليورياز ، سالبة الجرام ، على شكل قضيب يمكن أن تميز عن العصيات المعوية العادية إلى قضبان ممدودة للغاية مغطاة بملايين الأسواط ، مما يؤدي إلى تجمع مستعمرات. هم جزء من عائلة enterobacteriaceae.

في هذه الدراسة تم عزل وتحديد *P. vulgaris* و *P. mirabilis* من عينات بول المرضى الذين يعانون من التهابات المسالك البولية (UTIs) .تم أخذ مائتين وعشره عينة ادرار من مرضى في مستشفى الإمام الصادق التعليمي ، ومستشفى الحلة التعليمي ، ومستشفى المرجان التعليمي في بابل. تم جمع عينات هذه الدراسة بين سبتمبر ٢٠٢١ ويوليو ٢٠٢٢.

اشتملت الدراسة الحالية على ٤٠ عزلة من *Proteus mirabilis* و *P. vulgaris* معزولة من ٢١٠ عينة بول سريرية (سميت PM 1 إلى PV40). تم التعرف على هذه العزلات اعتمادًا على الصفات المورفولوجية للمستعمرات والخلايا ، والاختبار البيوكيميائي ، و VITEK 2 المضغوط. إنه يزدهر على أجار الدم ، وآجار MacConkey ، ووسائط أجار الكروموجينيك في المسالك البولية. إنه يتحرك وينتج طبقة رقيقة من دوائر متحدة المركز تحاكي التموجات الناتجة عن رمي صخرة في بحيرة بسبب نمو حشد على أجار الدم. ومع ذلك ، في أجار MacConkey ، لا يتجمع وينتج مستعمرات ناعمة أو خفيفة أو عديمة اللون.

تم إجراء اختبار الحساسية للمضادات الحيوية ضد ١١ نوعًا من المضادات الحيوية ، باستخدام طريقة انتشار القرص وفقًا لمعهد معايير البكتيريا المسببة للأمراض في المختبر السريري ، CLSI-2022. أظهرت غالبية العزلات مقاومة للمضادات الحيوية وخاصة المضادات الحيوية β- lactamase. وجد أن معظم العزلات البكتيرية تمتلك مقاومة متعددة للمضادات الحيوية المختبرة. أظهرت النتائج أن أكثر مضادات الجراثيم فعالية ضد عزلات *P. mirabilis* و *P. vulgaris* كانت الاريثروميسين بمقاومة عالية (٩٧.٥٪). يليه توبراميسين ٥ بنسبة (٨٥٪) ، أمبيسلين مع (٨٢.٥٪) ، كلورامفينيكول (٦٠٪) ، بيبراسيلين (٥٥٪) ، سلفاميثوكسازول ، أزيثروميسين مع مقاومة (٥٢.٥٪) ، بينما أظهرت مضادات حيوية أخرى مقاومة أقل وحساسية عالية مثل الميروبينيم والإيميبينيم أظهرت مقاومة منخفضة لعزلات *P. mirabilis* و *P. vulgaris* مع (٣٥٪) ، سيبروفلوكساسين (٣٠٪) ، جنتاميسين (١٥٪). و imipenem (١٢.٥ ٪).

تم استخدام اختبار طريقة زراعة الانسجة ، والذي كان يعتقد أنه الأكثر حساسية ، لدراسة تكوين البكتيريا الحيوية. تمت دراسة قدرة *P. mirabilis* و *P. vulgaris* على إنتاج الغشاء

الحيوي الرقيق. ٤٠ عزلة بكتيرية متقلبة (*P. mirabilis* و *P. vulgaris*) تم فحصها واكتشف أن جميع العزلات (١٠٠٪) شكلت غشاء حيوي بالنسب التالية: ١٥٪ من البيوفيلم ضعيف ، ٧٧.٥٪ متوسط ، و ٧.٥٪ قوي.

تم استخدام تفاعل البوليميراز المتسلسل لفحص ٣ جينات للطيف الممتد من lactamase –β ($ESBL_S$) في *P. mirabilis* و *P. vulgaris* باستخدام مادة أولية محددة لكل جين من *bla* $_{TEM}$، *bla* $_{SHV}$ و *bla* $_{ctx-m}$. بعد إجراء الرحلان الكهربائي ، أظهرت النتائج في *CTX-M* أن النتائج الإيجابية لتفاعل تفاعل البوليميراز المتسلسل بيتا لاكتاماز تحتوي على ٣٣ *SHV*(٨٢.٥٪)، ٣٨ (٩٥%) *TEM* و ٣٧ (٩٢.٥٪) جينات *CTX-M*. وجد أن جميع عزلات *P. mirabilis* كانت متطابقة بنسبة ٩٧-١٠٠٪ مع مصادر العزلات المحددة في بنك الجينات (NCBI) ، وأظهرت النتائج أن أعلى نسبة مطابقة للعزلات كانت ١٠٠٪. نشأت في العراق والهند. أظهرت هذه الدراسة أن جين *bla* $_{TEM}$ هو الجين الأكثر شيوعًا بين هذه العزلات ، يليه جين *bla* $_{CTX-M}$ ثم *bla* $_{SHV}$.

نظرًا لأن بكتيريا *P. mirabilis* كانت أكثر انتشارًا من *P. vulgaris* في هذه الدراسة ، فقد تم اختيار ثمانية عزلات من *P. mirabilis* لدراسة التسلسل الجيني.الحمض النووي الجينومي الكامل لثمانية عزلات PMI إلى PM8. المعزولة من مناطق جغرافية مختلفة من العراق. تم إجراء تسلسل جينات *bla* $_{TEM}$ و *bla* $_{SHV}$ و *bla* $_{ctx-m}$ ، وتم تحديد مواقع هذه الجينات على جينوم العزلات. و تم إجراء تحليل شجرة النشوء والتطور باستخدام برنامج MEGA X10.2.4 ، وتعزل الدراسة الحالية مع NCBI سلالات البنك العالمي *P. mirabilis* ، وجدت أن ٤ عزلات (PM1 و PM2 و PM3 و PM4) كانت مرتبطة بالهند ، وعزلتان (PM5 و PM8) كانت مرتبطة بالعراق وعزلة واحدة (PM6) كانت مرتبطة بمصر. لذلك ، يلزم إجراء بحث إضافي لإنشاء خريطة التنوع الجيني لعزلات *P. mirabilis* و *P. vulgaris* بسبب الترددات المتغيرة في تسلسل جينات *bla* $_{TEM}$ و *bla* $_{ctx-m}$ و *bla* $_{SHV}$ للعزلات.

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التوصيف الجزيئي لجينات *bla* $_{CTX-M}$ *bla* $_{TEM}$ و*bla* $_{SHV}$ في عزلات انواع المتقلبات

رسالة

مقدم الى مجلس كلية العلوم-جامعة بابل كجزء من متطلبات نيل درجة الماجستير في العلوم/علوم الحياة

من قبل
اسراء علي حسن جواد

بكلوريوس علوم حياة-التقانة الاحيائية

جامعة بابل (٢٠١٦-٢٠١٧)

اشراف

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