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Molecular Characterization of Some Exotoxins in Uropathogenic *Escherichia coli* and its Effect as Anticancer

A Thesis

Submitted to the Council of the College of Medicine, University of Babylon, as a Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in science / Medical Microbiology.

By

Tsahel Hamid Kadhum AL-Dulaimi

BSc. Microbiology\ College of Science for Women
University of Babylon (2006).
MSc. Microbiology \ College of Science for Women
University of Babylon (2012).

Supervised by

Prof.
Dr. Ilham Abbas Bunyan

Prof.
Dr. Thikra Adnan Jawad

2022 A.D

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(وَعَلَّمَكَ مَالَم تَكُن تَعلَمُ وَكَانَ فَضلُ اللهِ عَلَيكَ عَظِيمًا)

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Supervisor Certification

We certify that this thesis entitled "**Molecular Characterization of Some Exotoxins in Uropathogenic *Escherichia coli* and its Effect as Anticancer**" was prepared by**" Tsahel Hamid Kadhum AL-Dulaimi**" under our supervision at the college of Medicine, University of Babylon, as a partial requirement for the degree of Doctorate of Philosophy in Microbiology.

Professor

Dr. Ilham Abbas Bunyan

College of Medicine

University of Babylon

/ / 2022

Professor

Dr. Thikra Adnan Jawad

College of Science

University of Al-Qasim Green

/ / 2022

In view of the available recommendation, I forward this thesis for debate by the examining committee.

Professor

Dr. Hayam K. Al-Mosudi

Head of Department of Microbiology

College of Medicine

University of Babylon

/ / 2022

Dedication

I dedicate my true gratitude to the light of my eyes and the reason for my progress and my effort in life… My father

To the devoted woman who illuminated my life with her kindness and endless love… My Mother

To my faithful husband, who supports me in time of prosperity and adversity… My husband Mahmood

To my sweets, little angels… Mohammed and Lamar...

To those who supported me and stayed by my side… My sister and brothers

To the friends of high and beautiful wishes…

I dedicate the fruit of this humble effort...

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Thanks, and praise be to God who granted me this grace and gave me patience to accomplish this work.

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I am sincerely indebted and grateful to my family for supporting me to complete my thesis work.

Finally, my thanks also extend to everybody who helped me to complete this work.

Tsahel

Summary:

The study included (179) urine samples were collected from patients who were submitted at Al-Hilla General Teaching Hospital and Maternity and Children Hospital in Babylon province (Urinary Unit), for the period extending from March to December (2021). The collecting of samples were selected from both sexes with the different ages. Preliminary diagnostic results were positive for the bacterial culture 123/179 (86.71%) after it confirmed by using laboratory culture methods such as biochemical tests, VITEK 2 and the *16SrRNA*. *Escherichia coli* isolates were considered the highest incidence and the main causes of UTIs 56/123 (45.5%) in comparsion to other bacterial causes 67/123(55.5%) especially in the age group (22-31) by (43%), while the age group (52-61) were infected by (3.5%), the infection rate were higher in females than males (71.5%: 28.5%), respectively.

Antimicrobial susceptibility were examined by using VITEK 2 for checking the susceptibility for the bacterial isolates to the antibiotics. The results revealed that, bacterial isolates were highly sensitive to Imipenem, Meropenem (100%) and Amikacin (94.6%) while Gentamycin, Tobramycin, Ciprofloxacin and Minocycline recorded a lower sensitivity rates (69.6%, 62.5%, 55.4%, 51.7%) respectively. The results concluded variety of resistance was found in the isolates to Ticarcillin and Piperacillin (100%), and to Cefepime, Azetronam (94.6%) while the lowest resistance result were recorded with Trimethprime-Sulfament (64.3%).

The ability of bacteria in producing of biofilm were detected at 50(89.3%) and according to that isolates that produce biofilm subdivided in to different

groups, strong biofilm forming by 25(50%), moderate biofilm forming by 19(38%) and weak biofilm forming 6(12%). In addition, antimicrobial Amikacin and Ciprofloxacin were checked against the isolates forming biofilm by using Micro titer plate method. So that, Amikacin inhibit producing biofilm begin with concentration of (64 µg/ml) and reached to the higher inhibition rate at concentration (1024 µg/ml) whereas, the highest inhibition rate for Ciprofloxacin were at concentration (128 µg/ml) reaching to the highest inhibition rate at concentration (1024 µg/ml).

Genetic study was applied in detection of toxin genes alpha hemolysin, secreted autotransporter and cyto-lethal distending (*hlyA, sat* and *cdtB*) by using PCR after DNA was extracted from the bacterial isolates that diagnosed as *Escherichia coli* by *16SrRNA*. Alpha hemolysin gene were detected in 21/56 (37.5%) from isolates whereas secreted autotransporter gene detected in 35/56(62.5%) from the bacterial isolates. However, no one of isolates contain cyto-lethal distending B gene. All isolates were detected recorded in the gene bank with the accretion number OL539543.1, OL539542.1, ON112284, ON112285, ON112286, ON112287, ON112288, ON112289, ON112290, ON112291, ON112292, ON112293, ON777812, ON777813, ON777814, ON777815 and ON777816. Also, The results also showed that there were no significant differences between the presence of α hemolysin and secreted autotransporter genes with the ability of bacteria to form or not form biofilms at ($P<0.162$) and ($P<0.114$) respectively .

Bacterial growth revealed the ability of bacteria in producing α hemolysin by using blood agar and the toxin were extracted after culturing of bacteria on Luria broth medium then the toxin purified by gel filtration method using

Summary

Sephadox G200 with the activity (5 u\ ml), regarding to that, the toxin were collected and experimentally examined on normal and cancer cell lines alone and with Ciprofloxacin, the results showed the ability of toxin and Ciprofloxacin to inhibit of the normal and cancer cells at higher rate of concentrations (500&1000µg\ml). Also, the toxin and Ciprofloxacin were found to have a lowest effect against prostate cells in the experiment.

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List of Abbreviations

Abbreviation	Full term
ABU	Asymptomatic bacteriuria
BHI	Brain heart infusion
ESBL	Extended Spectrum beta lactamase
CDT	Cytolethal distending toxin
cdtB	Cytolethal distending B gene
CFU	colony forming units
DMSO	Dimethyl sulfoxide
DNA	Deoxyribase nucleic acid
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunoabsorbance assay
EMB	Eosin Methylene Blue Media
ESBL	Extended Spectrum Beta-Lactamase
ExPEC	Extra-intestinal Pathogenic *E. coli*
HEp-2	Human liver cell line
HK-2	Human bladder cell line
HlyA	α-hemolysin toxin
hlyA gene	α-hemolysin gene
IBCs	Intracellular Bacterial comunities
LT	Heat labile-toxin
LPS	Lipopolysaccharide
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
MtP	Microtiter Plate method
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium

	bromide
OD	Optical density
PAI	Pathogenicity Islands
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
QIRs	Quiescent internal reservoirs
RPMI	Roswell Park Memorial Institute 1640 Medium
RTX family	Repeats in Toxin family
SAT	Secreted autotransporter toxin
sat	Secreted autotransporter gene
ST	Heat stable-toxin
STx	Shiga-toxin
µg/mL	Microgram/milliliter
UPEC	Uropathogenic *E. coli*
UTIs	Urinary tract infections

Chapter One

Introduction

and Literatures Review

1.1. Introduction

Urinary tract infections (UTIs) represents one of the major nosocomial infections and one of the major bacterial infections affecting 150 million people all over the world each year (Washino *et al.,* 2020).The disease is widespread and affect a large proportion of the human population which is more commonly in females. The incidence of infection in females is typically associated with muscles of the lower pelvic with such dysfunction occurs during menopause time due to a shorter urethra in female than male, also, UTIs are more common during pregnancy due to a variety of changes happening to the female body and especially the urinary tract area. It depend on anatomical factors, the integrity and susceptibility of host defense mechanisms, the virulence of the infecting organisms, the family history, obesity, diabetes and sex intercourse (Tandogdu and Wagenlehner, 2016).

In clinical practice, UTIs are among the most common bacterial infectious diseases encountered. Since normal bacterial flora enters the urinary tract where it ascends through the urethra from the vagina, intestines and perineum, it possesses many virulence factors that enable adherence to the perineum and urethra then migrate to the bladder and invade the uretherlial lining. Also, UTIs resulted from successful colonization of the urinary tract by pathogenic microbes, commonly caused by *Escherichia coli* which accounts for 90% of community acquired, 50% of hospital acquired and 80% of uncomplicated UTIs cases worldwide (Korbel *et al*., 2017).

Escherichia coli infection usually originated from intestinal normal flora or from fecal colonizes at periurethral area which resulting in UTIs known as Uropathogenic *E. coli* (UPEC). Moreover, the bacteria present in water or

food considered as a stool contamination which causes disease in the alimentary tract and urinary tract infection (Al. Dulaimi, 2016).

Escherichia coli species includes many strains that have virulence factors which possess pathological effects, the pathogenicity of UPEC is due to the possession of several virulence factors such as adhesions, resistant plasmids, biofilm formation, capsular antigen, lipopolysaccharides (LPS) and toxins. The distribution of these virulence genes in the genome of *E. coli* is very important in determination criteria which play roles in the disease progression (Brons *et al.*, 2020).

Uropathogenic *E. coli* (UPEC) is a substantial agent of UTIs alongside some other infections which possess several adherence factors that lead to increase the ability to invade urinary tract epithelial cells. These virulence factor enable the bacteria to penetrate of the host tissues which encounter as a determinants of bacterial pathogenesis and increase urinary tract infection susceptibility in contrast, the bladder epithelium contains many host defenses such as urine flow and secretion of antimicrobial substances that eliminate the presence of bacteria (Nojoomi and Ghasemian, 2019).

Toxins are proteins or substances produced by some strains of *E. coli* that plays a vital role in causing UTIs. These toxins can modify cell signal pathways and modulate the host's inflammatory response as Cytotoxic necrotizing factor 1 (Cnf1), Secreted autotransporter toxin (Sat), cytolysin A, α-hemolysin (HlyA) and Vacuolating autotransporter toxin (Vat) (Soltani *et al*., 2018).

Hemolysins are lipoproteins that cause pores to form on cells' surfaces that it attaches to causing them to release ATP and leading to their deaths, it

is associated with *E. coli* that cause severe UTIs and causes hemoglobin releasing into the environment which is then metabolized by the bacteria when it attaches to red blood cells (Parvez and Rahman, 2018; Ahmed, 2021).

Another important toxin, Secreted autotransporter toxin (Sat) is a class-1 Serine Protease Autotransporter (SPATE) which is have a passenger domain generate of a 107-kDa protein that expressed significantly more often by *E. coli* strains associated with the clinical symptoms of acute pyelonephritis than by fecal strains and presenting cytotoxic activity in renal and bladder cells (Vieira *et al*., 2020).

The Cytolethal distending toxin (CDT) toxin is an AB_2-type toxin produced by several pathogenic gram-negative bacteria which causes DNA damage and cell cycle arrest. The catalytic subunit CdtB is highly conserved between species and causes single and double-strand DNA breaks via DNase I-like activity, it increases bacterial gut colonization, promotes pro-inflammatory responses and dysregulates the immune response (McCoy *et al*., 2021).

The aim of the study:

The present study aimed for:

Detection of toxin genes (alpha Hemolysin toxin, Cytolethal distending toxin and Secreted autotransporter toxin), phylogenetic study and study of the cytopathic effects of alpha hemolysin on normal and cancer cell line.

The objectives of the study:

1. Isolation and identification of *Escherichia coli* by cultural characteristics, VITEK 2 compact and confirmation by *16SrRNA*.

2. Using VITEK 2 compact to detect the antibiotic susceptibility.
3. Biofilm determination by micro titer plate method and detection the biofilm inhibition by Amikacin and Ciprofloxacin.
4. Specific primers used for toxin genes detection (alpha Hemolysin toxin, Cytolethal distending toxin and Secreted autotransporter toxin) and using phylogenetic tools.
5. Extraction and Partial purification of alpha hemolysin toxin by gel filtration and study the effects of (HlyA) on Kidney (normal cell), Colon cancer and Prostate cancer cell line.

1.2 Literature Review:

1.2.1 Urinary tract infections:

Urinary tract infections (UTIs) are the most common type of human disease which is usually resulting from a bacterial attack on the urinary tract (UT) that includes both the upper and lower parts. The disease typically resulting from bacteria, viruses and fungi might be causal, UTIs are mostly caused by bacteria and it may progress to pyelonephritis and blood infection (Tessema *et al*., 2020)

According to the site of infection, UTIs are grouped into pyelonephritis (spreads to the upper urinary tract) and cystitis (infection of the lower urinary tract). Uropathogenic *Escherichea coli* colonizes the bladder by surface structural components and through type 1fimbriae which binds to urothelial cells integrin and uroplakins receptors that allowing *E. coli* to invade and colonize bladder leading to form intracellular bacterial communities (IBCs) (Yang *et al*., 2022). Cystitis occurs in both females and males, the majority of females will develop UTIs in their lifetime, so are 14-times more common in females than males by virtue of the shortened urethra (Kurdi, 2020).

The kidney stones can lead to urinary tract obstruction causing acute pyelonephritis, when microbes rise to the kidneys or also be obtained through the spread of blood leading to infect the kidneys and increases the risk of developing a blood-borne infection (Al-Tamemi, 2021).

According to risk factors the UTIs are grouped to the complicated or uncomplicated, the uncomplicated UTIs is classified as a UTIs without

structural or functional abnormalities of the urinary tract and without relevant comorbidities which includes both cystitis and pyelonephritis, (Dason *et al*., 2011; Venkatesan *et al*., 2020). UTIs can also be complicated by several risk factors that can lead to treatment failure, repeat infections or significant morbidity and mortality with a poor outcome. A complicated UTIs is any urinary tract infection therefore, all UTIs in immunocompromised patients, males and those associated with fevers, stones, sepsis, urinary obstruction, catheters or involving the kidneys are considered complicated infection. It tends to be caused by a much wider range of organisms which is significant because multidrug resistance is increasing and therefore specific antibiotic regimens will vary (Sabih and Leslie, 2022).

Asymptomatic bacteriuria (ABU) is the presence of bacteria in the properly collected urine of patients that have no sign of infection but significant numbers of microbes live in the urinary tract. It is very common in clinical practice while few infants and toddlers have asymptomatic bacteriuria. The incidence increases with age which is up to 15% or greater in females and males age 65 to 80 years and as high as 40% to 50% after age 80 (Givler and Givler, 2022).

Esherichea coli that causes ABU is differed in virulence properties that are less expression of virulence factors compared to those causing symptomatic infection, so have less hemolysins, losing a portion of the genome involved in the production of type 1 fimbriae and inactivation of genes encoding P fimbriae lead to the formation the strains can colonize the urinary tract without inducing inflammation, also the mucosa remains inert

even in the case when the urine contains a substantial number of bacteria (Terillizi *et al*., 2017).

Recurrent UTIs are symptomatic UTIs that follow recovery of an earlier infection after appropriate treatment or can come from an unrelated another infection more likely in an immune-compromised host or the reappearance of latent initial infection, it affects individuals of all ages and both sex (Jhang and Kuo, 2017; Al-Tamemi, 2021).

1.2.2 *Escherichia coli*:

Escherichia coli found to be the main common causes of UTIs which is divided in to two types are pathogenic and non-pathogenic (**Figure 1.1**). The non-pathogenic *E. coli* described as commensal is present in the normal micro flora of intestine which are harmless, hinder the growth of harmful bacteria and produce vitamins, otherwise the pathogenic *E. coli* caused different disease in human and have different virulence factors (Kurdi, 2020).

1.2.2.1 Commensal *Escherichia coli*:

It is located in the gut of humans, animals and in the environment that are attributed beneficial properties as promotion of intestinal homeostasis. These strains are cause disease in humans except in immunocompromised patients or when the normal gastrointestinal barriers are breached (Lindstedt *et al*., 2018).

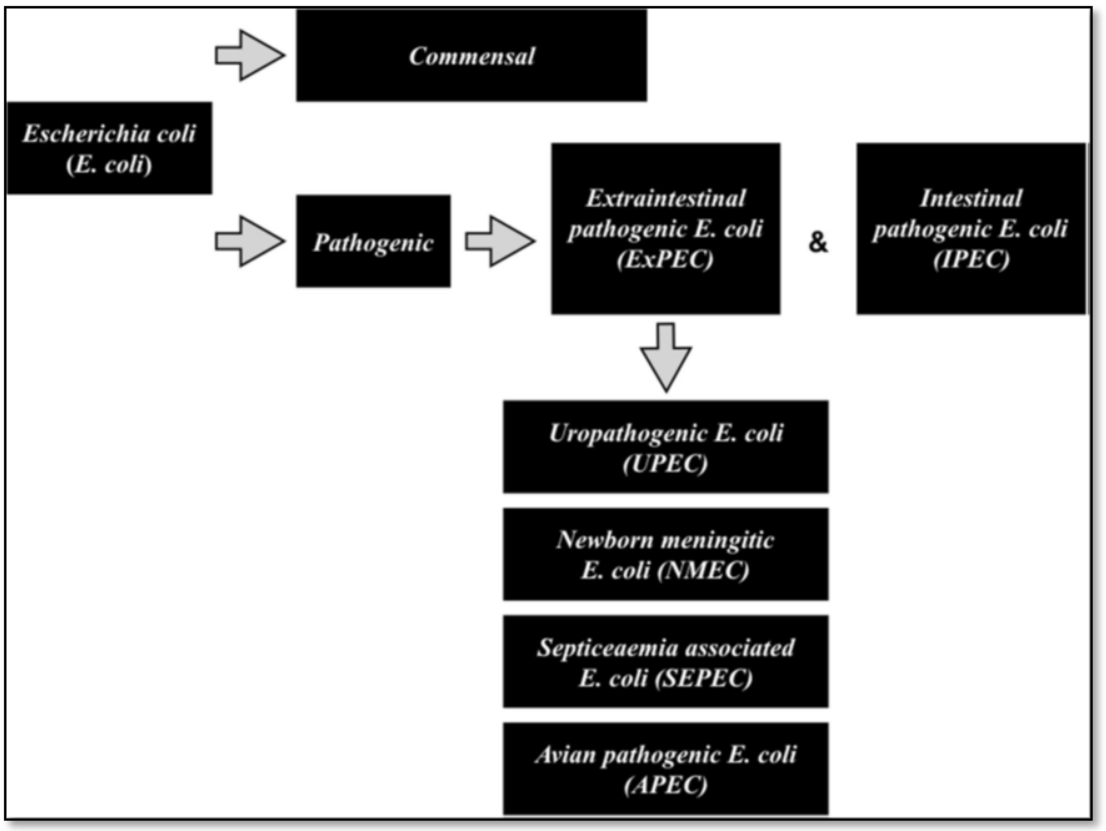

Figure (1.1): Pathogenic diversity of *Escherichia coli* strains (Sarowska *et al.*, 2019)

1.2.2.2 Pathogenic *Escherichia coli*:

Depending on the set of virulence factors acquired the pathogenic *E. coli* were classified to Intestinal Pathogenic *E. coli* (IPEC) and Extra Intestinal *E. coli* (ExPEC). This Pathogenic strains of *E. coli* were characterized by virulence factors which help them to overcome the host defenses and colonize or invade the urinary tract (uropathogenic strains) or gastrointestinal tract (DEC strains) (Ocampo *et al* ., 2021) .

1.2.2.2.1 The Intestinal Pathogenic *Escherichia coli*:

The intestinal pathogenic *E. coli* included six different *E. coli* pathotype depending on their adhesion/colonization mechanism characteristics, virulence factors and their mode of action, were Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Diffusely Adhering *E. coli* (DAEC) and Enterohemorrhagic *E. coli* (EHEC) or Shiga-toxin producing *E. coli* (STEC) (Malema *et al.*, 2018).

Enteroaggregative *E. coli* (EAEC) shows an aggregative adherence pattern to epithelial cells that cause of acute or persistent diarrhea and as a cause of linear growth faltering among children in low-income countries. The pathogenicity is due to comprise mucosal inflammation, epithelial damage and express a putative virulence factors encoded on the bacterial chromosome or the specific plasmids, also it often harbors a variable number of serine protease autotransporters of (SPATEs) that are helped it in secretogenicity (Boisen *et al*., 2020).

Enteropathogenic *Escherichia coli* (EPEC) is leading cause of infantile diarrhea particularly in developing countries and harbors a type III secretion system (T3SS) which directly injects virulence proteins into host epithelial cells, these proteins alter host structure and function likely facilitating pathogenesis (Serapio-Palacios and Finlay, 2020).

Enterotoxigenic *E. coli* (ETEC) is transmitted by fecal-contaminated food or water and produces the larger heat labile-toxin (LT) or the smaller heat stable-toxin (ST) or both enterotoxins. These enterotoxins induce the secretion of fluids and electrolytes leading to acute watery diarrhea. ETEC

pathogenesis is facilitated by a number of conserved proteins which help modulate adhesion, intestinal colonization and efficient toxin delivery (Khalil *et al*., 2021).

Entero-invasive *Escherichia coli* (EIEC) is common in low-income countries with poor hygiene in worldwide. It causes shigellosis-like symptoms in both children and adults using the same invasive mechanisms as *Shigella* spp. which result from the convergent evolution of ancestral *E. coli* that independently acquired the large invasion virulence plasmid (Van den *et al*., 2019). EIEC isolates harboring virulence genes that could induce the inflammation and extensive mucosal damages in intestinal infections especially when it encoded more than one of the virulence factors (Farajzadeh *et al*., 2020).

Diffusely adhering *E. coli* (DAEC) is a common cause of long-lasting childhood diarrhea and may induce inflammation of the intestinal mucosa contributing to the development of inflammatory bowel diseases (IBD) in susceptible children (Walczuk *et al*., 2019), it shows difference in adherence patterns, actin polymerization and motility depending on whether it is isolated from children with diarrhea or asymptomatic children (Ocampo *et al*., 2021).

Enterohemorrhagic *E.coli* (EHEC) or Shiga-toxin producing *E. coli* (STEC) is a group of foodborne bacteria responsible for a wide variety of infections from mild diarrhea to serious dysentery cases like hemorrhagic colitis (HUC). It produce Stx which a group of AB5 protein toxins that inhibit protein synthesis in cells by removing the residual adenine from 28S rRNA in large subunit ribosome 60S (Moxley *et al.*, 2020).

1.2.2.2.2 The Extra-intestinal Pathogenic *Escherichia coli:*

It forms the second group of pathogenic *E. coli* which escapes the gastrointestinal tract (GIT) and cause diseases outside the GIT with various outcomes. The ExPEC strains may result in UTIs, sepsis, meningitis and in other less frequent infections. ExPEC group is divided into Uropathogenic *E. coli* (UPEC), Neonatal Meningitis *E. coli* (NMEC) and Avian Pathogenic *E. coli* (APEC). Besides these pathogroups, a genotypic mosaic which could be stable over a variable period of time and could emerge in some strains as a result of the acquisition or loss of multiple pathogroups defining virulence factors (Tchesnokova *et al*., 2019).

Uropathogenic *E. coli* causes a large portion of hospital or community acquired UTIs, the virulence factors are often detected in it but they are non-existent or rare in strains of the normal intestinal micro flora also, they could evade the immune system of the host better and have mechanisms to attach to the urinary tract (Forsyth *et al*., 2018).

1.2.3 The pathogenesis of Uropathogenic *Escherichia coli*:

The pathogenesis of UTIs is complex and depended on the biological and behavioral factors of host with virulence factors of the infecting uropathogen. The human intestine is considered to be the primary reservoir for *E. coli*, so the intestinal *E. coli* reaches to the urinary tract system and colonizes the periurethral, vaginal areas and the urethra followed that reaching to the bladder and attaches to the surface epithelium by fimbrial and non-fimbrial adhesins **(Figure1.2)**. The adhering bacteria may be internalized into the uroepithelial facet cells and could enter the cytoplasm

followed by replication and form intracellular bacterial communities (IBCs) which considered a source of quiescent intracellular reservoirs (QIRs) (Karam *et al*., 2019).

In healthy person, the host innate response was triggered in response to the entry of *E. coli*, this response involves the production of inflammatory cytokines and chemokines. The inflammatory mediator's production results in the rapid recruitment of neutrophils in to the bladder lumen and in bacterial clearance response that leads to the exfoliation of infected bladder epithelial cells, for that the immunity cells may eliminate some of the intracellular bacterial communities (IBCs) through exfoliation of bladder surface facet cells and throwing out them with the urine but some bacteria were remain and could grow as a biofilm resistant to immune defenses and antibacterial agents (McLellan and Hunstad, 2016).

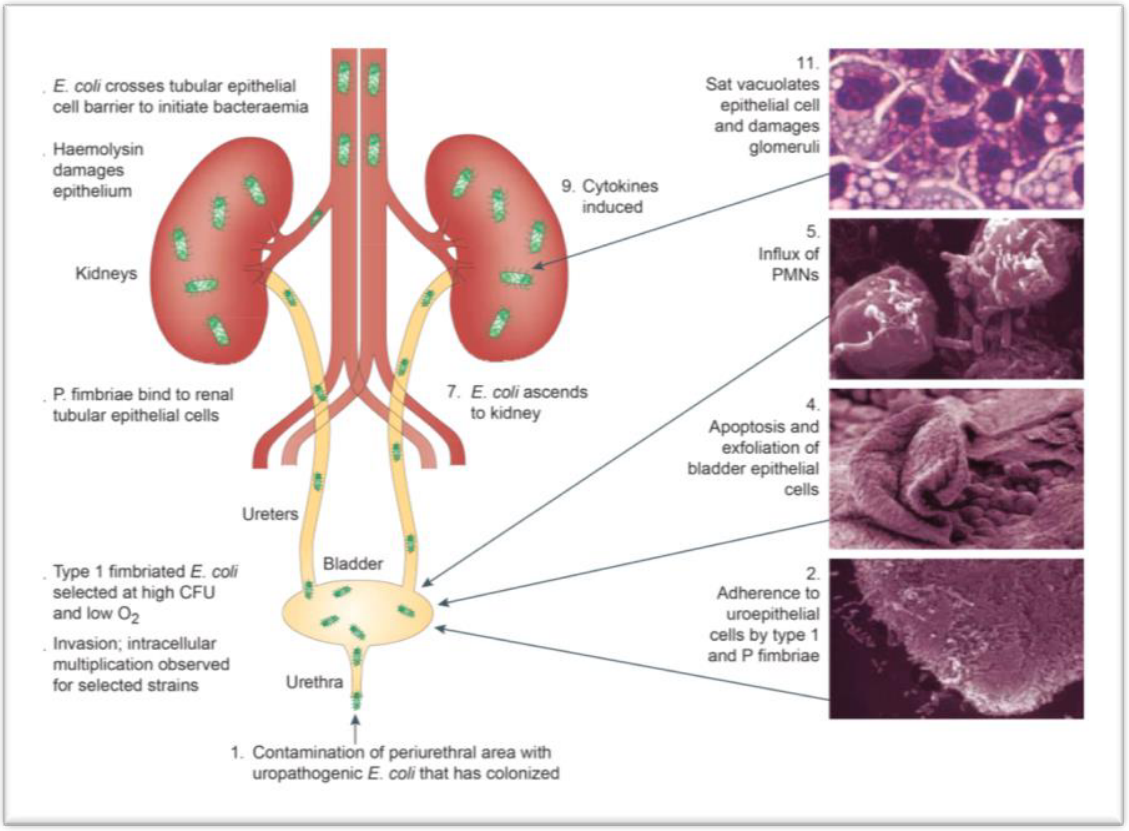

Figure (1.2): Pathogenesis of UTIs caused by UPEC. Different stages of a urinary tract infection. CFU: colony-forming units, PMNs: polymorphonuclear leukocytes (Kaper *et al*., 2004).

1.2.4 Uropathogenic *Escherichia coli* virulence factors:

Escherichia coli have many virulence genes encode fimbrial adhesins, non-fimbrial adhesion, flagella, iron acquisition systems, polysaccharide capsule, biofilm production and toxins. *E. coli* may have pathogenicity associated islands (PAIs) which carry sets of different virulence associated genes comprise large genomic regions that differ from the rest of the genome by their G + C content, their typical insertion within transfer RNA genes, and by their harboring of direct repeats, integrase determinants or other mobility loci (Desvaux *et al*., 2020). UPEC could adhere to uroepithelial

cells and establish UTIs so differ from nonpathogenic *E. coli* by the production of virulence factors which play an important role in the pathogenicity of bacteria and causing an inflammatory response that leading to occurrence the symptoms of UTIs. These virulence factors are essential to contribute significantly to *E. coli* pathogenicity (Luna-Pineda *et al*., 2019).

1.2.4.1 Uropathogenic *Escherichia coli* structural virulence factors:

There are several virulence factors that enable bacteria in causing disease such as fimbria which increase the adherence ability as it is the first step in the colonization of host tissue and progresses of disease (Bessaiah *et al*., 2021). The interaction between *E. coli* and the host urinary tract is a complex and multi-factorial event which involves several special fimbrial adhesions which aid in different steps of infection. They are typically involved in adhesion and function in a range of interactions between bacteria and other cells or bacteria and their surrounding environment. Also, serves in the colonization of surfaces, uptake of DNA or phage, adhesion to host cells, function in motility, biofilm formation and cytokine induction (Shah *et al*., 2019).

Escherichia coli have unique type of fimbriae encoded by the *pap* genes which known as pyelonephritis-associated fimbriae or P fimbriae. It confers *E. coli* with the ability to adhere to kidney withstand turbulent urine flow and colonize the urinary tract. The kidneys are less turbulent environment compared with the bladder lumen, P pili are adapted to function in this niche and facilitates attachment of *E. coli* during an ascending UTIs and is one of the initiating steps of pyelonephritis (Werneburg and Thanassi, 2018).

Curli are unique in structure that appears as aggregates on the bacterial surface and hair like projections which termed aggregate fimbriae. They are important for adherence to resistant abiotic surfaces, protected from antimicrobial peptides and have various roles during the progression of UTIs (Shea, 2017).

Lipopolysaccharide (LPS) a virulence factor in *E. coli* has been shown to induce cytokine release (e.g., IL-8) from urothelial cells through a nuclear factor-κB inflammation signaling mechanism. LPS consists of the highly conserved lipid A-core and repeating O antigen subunits that differ greatly between strains based on the sugar residues and their linkage patterns within the repeating subunits , the structure of LPS modulates life cycle and helps to form reservoirs of *E. coli*, stimulate responses of innate and adaptive immune system (Zhang *et al*., 2013; Kurdi, 2020).

Capsule is slimy layer made of polysaccharide and is considered as an important virulence factor of *E. coli* that causes attachment of bacteria to the epithelial cells or layers prior to the urinary tract invasion. K antigen-producing *E. coli* infections are always found in the upper urinary tract. Certain K types are more common among cystitis and pyelonephritis than fecal strains, the K1 polysaccharide plays an important role in intracellular bacterial communities (IBCs) development and the pathogenicity of *E. coli* in several stages (Etefia and Ben, 2020).

The Capsule formation enhanced bacterial survival which gives resistance to bactericidal host molecules (antibodies, defenses) by competition for nutrients that enhance virulence, inhibit complement-mediated killing and protect *E. coli* against phagocytosis by immune cells

such as neutrophils and monocytes, so it is anti-phagocytic and has anti-complementary activities (opsonization or lysis) (Abaas, 2018).

Flagella are filamentous organelles which composed of many protein monomeric subunits called flagellin. The flagellated *E. coli* is responsible for nearly about 70-90% of all UTIs, the benefits of having flagella mediated motility by *E. coli* during colonization of the urinary tract include the capability of dissemination to new sites of the urinary tract to obtain nutrients and in addition to escape from immune responses of the host (Bien *et al*., 2012; Kaur *et al*., 2022).

1.2.4.2 Uropathogenic *Escherichia coli* toxins:

Bacterial toxin is one of the pathogenic bacterial virulence factors and represents one of the main bacterial strategies that interact with mammalian cells (Almasaudi *et al*., 2018).

Uropathogenic *E. coli* produced toxins that may be induced an inflammatory response which is a possible pathway for UTIs symptoms, the toxins can alter the host cell signaling cascade and modulate inflammatory responses. The secreted toxins by *E. coli* including families were RTX family, serine protease autotransporters (SPATE), cyto-lethal distending toxin (CDT) and other families (Zermina *et al*., 2021).

Repeats in Toxins family (RTX) proteins are a large family of proteins secreted by many Gram-negative bacteria and are defined by a conserved Ca2+ binding domain such as Alpha hemolysin. All RTX cytolysins share several characteristic structural features were a hydrophobic pore-forming domain in the N-terminal part of the molecule, an acylated segment where the activation of the inactive protoxin to the toxin occurs by a co-expressed

toxin-activating acyltransferase, a typical calcium-binding RTX domain in the C-terminal portion of the molecule with the characteristic glycine- and aspartate-rich nonapeptide repeats finally, a C-proximal secretion signal recognized by the type I secretion system (Filipi *et al*., 2022).

In immunity, the action of RTX toxins enables bacterial pathogens to cope with the fierce host immune defenses and is often block phagocytosis, some of these toxins could reprogram the macrophages to less bactericidal cell types (Ahmad and Sebo, 2021). They are transported from the cytoplasm to the cell surface by a type one secretion system (T1SS) which have been characterized as a one-step translocation strategy whereby the secreted substrate is transported directly into the extracellular environment from the cytoplasm with no periplasmic intermediate (Smith *et al*., 2018).

Secreted auto transporters are a family of secreted proteins from gram negative bacteria that direct their own secretion across the outer membrane. In *Enterobacteriaceae*, a subgroup of these autotransporters called SPATEs (Serine Protease Autotransporters of *Enterobacteriaceae*) which constitute a superfamily of virulence factors that can cause tissue damage and cleavage of molecules of the complement system furthermore, is a key feature for the establishment of infection (Freire *et al*., 2020).

The SPATEs family plays a crucial role in survival and virulence of pathogens such as *Escherichia coli* and *Shigella* spp. which contribute to intestinal and extra-intestinal infections. These high molecular weight proteases are transported to the external milieu by using the type V or auto transporter (AT) secretion system and excreting to the extracellular space and function as proteases with diverse substrate specificities and biological

functions. SPATEs composed of three domains were an N-terminal signal peptide, a passenger domain and a C-terminal trans locator domain, the passenger domain is entirely secreted to the extracellular milieu, constitutes the mature form of the SPATE proteins and is responsible for their biological activity (Pokharel *et al*., 2019).

Amino acid sequence analysis of the passenger domains classified SPATEs in two different classes on the basis of structural and functional properties, class-1,Toxine cytopathic /cytotoxic activities (eliciting cellular changes such as cytoplasmic shrinkage, loss of membrane integrity and activation of apoptosis) while class 2 is the larger phylogenetic cluster, comprise O-glycoproteases that cleave mucin and other O-glycoproteins present on epithelial cells and the surface of hematopoietic cells (Habouria *et al*., 2019). SPATE-encoding genes are present in mobile elements such as plasmids, pro-phages and pathogenicity islands that allowing their dissemination among different *E. coli* (Freire *et al*., 2022).

1.2.4.2.1 Alpha hemolysin toxin (HlyA):

Alpha-hemolysin is a secreted lipoprotein, strong and ubiquitous cytolysin belongs to (RTX) family also, is a 1024-residue, full-length is 110-kDa in size, released by a type I secretion system (T1SS) which contains an ATP-binding cassette transporter and plays important role in pathogenesis, and act on a wide range of different host cell types (Vega *et al*., 2019).This toxin produced by numerous pathogenic as well as commensal *E. coli* isolates and has been proposed to function differentially over a gradient of concentrations, at higher toxin doses, the trans-membrane pores formed by HlyA could provoke colloid-osmotic (oncotic) lysis of eukaryotic cells

including red blood cells (RBCs), epithelial cells and leukocytes resulting in cell lysis while at low (sublytic) concentrations, hemolysin could either alter cell functions or provoke apoptotic cell death (Gu *et al*., 2021). It is produced as either a free form or an outer membrane vesicle (OMV) associated form. The free form irreversibly inserts into cell membranes of erythrocytes and epithelial cells where depending on concentration it causes ion imbalance, structural changes and cell lysis while the OMV-associated form is internalized by epithelial cells where it targets mitochondria and causes apoptosis (Strack *et al*., 2019; McCoy *et al*., 2021).

1.2.4.2.2 Secreted autotransporter toxin (Sat):

Secreted autotransporter toxin (Sat) is a 107-kDa serine protease autotransporter of Enterobacteriaceae (SPATE) presenting cytotoxic activity in renal and bladder cells (Vieira *et al*., 2020). It described as the second most prevalent SPATE gene in *E. coli* strains isolated from neonatal sepsis and detected in an *E. coli* strain causing fatal neonatal meningitis. Sat protein was first described in *E. coli* isolated from acute pyelonephritis so may have the cytopathic activity that results in the damage of the host tissue and may increase the propagation ability of the *E. coli* (Parvez and Rahman, 2018).

It is internalized by an unknown mechanism and shown to be localized specifically to the cytoskeletal fraction of bladder and kidney epithelial cells also play an important role as a cytotoxin in the pathogenicity of urinary tract infection as well as in intestinal infections. Cytotoxicity included disruption of actin, other cytoskeletal and nuclear proteins that was

dependent on the serine protease active site (Maroncle *et al*., 2006; Habouria *et al*., 2019).

Escherichia coli a leading cause of UTIs that produces Sat toxin which is cytotoxic on vero kidney cells, HK-2 human bladder and HEp-2 cell lines. Like plasmid-encoded toxin (pet) from (EAEC) and EPEC secreted protein C (espC) from (EPEC), Sat from UPEC is contact with culture cells results in cell elongation and detachment from their support so it causes kidney and bladder cells vaculation that is dependent on protease activity of Sat toxin and also on internalization (Zermina *et al*., 2021).

1.2.4.2.3 Cytolethal distending toxin (CDT):

The CDT toxin is an AB_2-type toxin produced by several pathogenic gram-negative bacteria which causes DNA damage and cell cycle arrest. The catalytic subunit CdtB is highly conserved between species and causes single and double-strand DNA breaks via DNase I-like activity, it triggers the DNA damage response via ATM kinase leading to both G2/M and G1/S cell cycle arrest. CDT increases bacterial gut colonization, promotes pro-inflammatory responses and dysregulates the immune response (McCoy *et al*., 2021).

The sequence of *cdt* genes of *E. coli* reported that the three closely linked genes named as *cdtA*, *cdtB* and *cdtC* are responsible for CDT toxin activity that could be either chromosomally or plasmid-encoded (Kurnick *et al*., 2019). All 3 genes are required for the production of this heat-stable exotoxin, the *cdtA, cdtB and cdtC* genes among different bacterial species producing this toxin show variable sequence homology and even within the

same species, several CDTs have been identified *E. coli* (Zermina *et al*., 2021).

1.2.5 Antibiotic resistance:

Antimicrobial resistance (AMR) is one of the world's most urgent public health concerns in developing and non-developing countries which may led to spread of different infections like respiratory tract infections, urinary tract infections and many other diseases that are difficult to treat due to the progress causative microorganism have made in their antimicrobial resistance abilities (Adhikari, 2020).

Gram-negative microbes develop resistance by five main ways were bacteria can carry genes coding for enzymes like beta-lactamases which hydrolyzing and inactivating beta-lactam antibiotics, changing the specific target or its function by the mutations which occur in the genes for binding sites for antibiotics, alterations of the membrane porins result in reduced permeability, bacteria can express efflux pumps to actively transport antibiotics out of the cell and alternate metabolic pathways (Hasan and Al-Harmoosh, 2020). The resistance can be acquired through intrinsic or acquired mechanisms, mutations and horizontal gene transfer. Resistant bacteria often serve as reservoir of diverse antimicrobial resistance genes (ARGs) were often spread from different species by bacteriophage transduction or spread among species using mechanisms of conjugative transfer of plasmids. The normal flora in intestine becoming a reservoir for the transmission of antibiotic resistance to other pathogenic bacteria through plasmid exchange (McInnes *et al*., 2020; Serwecinska, 2020).

Escherichia coli is one of the organisms most frequently found harbouring ESBL-genes and MDR in ESBL-producing Enterobacteriaceae is rapidly becoming a threat to the medical community. In UTIs, the increasing of antibiotic resistance and appearance of multi-drug resistant (MDR) pathogens related to high rates of inappropriate experimental treatment prescribed without testing of the antibiotic susceptibility resulting ineffective UTIs treatment (Białek *et al*., 2018).

The occurrence of antibiotic resistance is common in UTIs, this resistance is related to an increasing of mortality contribute to microbial infections. The appearance of *E. coli* strains resistant to many antibiotics due to virulence genes which enhancing the pathogenicity during infections (Chakraborty *et al*., 2017), the indiscriminate and widespread use of antibiotics in addition to the practice of prescribing antibiotics to treat UTIs without bacterial characterization led to increased resistance among uropathogens and to decreased effectiveness of oral therapies. Also, the impaired kidney function resulted from the association between regular use of antibiotics and nephrotoxicity due to acute tubular necrosis, interstitial nephritis and intra-tubular crystal deposition (Alvarez, 2020).

There are different types of antibiotics for UTIs treatment resulting from misuse and overuse of antibiotics lead to appearance and development of MDR pathogens in UTIs. The resistance to Trimethoprim-Sulfamethoxazole (TMP-SMZ), Ciprofloxacin and Amoxicillin-Clavulanate (AMC) among *E. coli* is higher in developing countries than in developed countries (Kot, 2019). So routine standard antimicrobial susceptibility testing were very important in order to reduce the rates of inappropriate empirical antibiotic

therapy of UTIs and thereby decrease the occurrence of multidrug-resistant (MDR) *E. coli* (Białek *et al*., 2018).

1.2.6 Biofilm formation by *Escherichia coli*:

Biofilm formation is another universal bacterial strategy for survival adopted by *E. coli* which found on catheters and epithelial cells, the growth in biofilms whether single species or polymicrobial protects the bacteria from engulfment by phagocytic host immune cells and are often impenetrable to circulating antibiotics, UPEC biofilm is the most crucial factor for the establishment, persistence and recurrence of UTIs which protects them against harmful conditions, antimicrobial agents and the host's immune system furthermore, could be formed on abiotic or biological surfaces including urinary catheters and uroepithelium (Naziri *et al*., 2021).

The UPEC forms intracellular biofilm communities (IBCs) and quiescent internal reservoirs (QIRs) upon invasion of the host bladder epithelial cells which are important for pathogenesis of infection and require successful entry and early trafficking into the host cell (Lewis *et al*., 2017). The virulence factors of *E. coli* aid in biofilm formation like Flagella which increase adherence, biofilm formation on bladder epithelial cells and essential for motility required for successful *E. coli* colonization of the urinary tract, Also, Type 1 fimbriae is a common virulence factor which increases the adherence ability of bacteria to host tissues led to increase the chance for antibiotic susceptibility (Spaulding e*t al*., 2017).

The first step in the biofilm formation is bacterial attachment (**Figure1.3**), the planktonic bacteria first adhere to the surface by a combination of physical forces such as attractive van der Waals and electrostatic repulsive

forces as well as chemical forces such as microbial cell cohesion. Also, environmental factors effect on bacterial adhesion like pH, temperature and Hydrophobic surfaces are more likely to attract bacteria than hydrophilic surfaces (Achinas *et al*., 2019).

Bacterial cells begin to form micro-colonies which is associated with bacterial cell aggregation and microorganism growth followed by the creation of Extracellular Polymeric Substances (EPS) after irreversible attachment by assembling previously bound cells and following cell division as a result of cell-to-cell communication, this aggregation could include the recruiting of planktonic cells from the surrounding medium (quorum sensing) (Mahdi, 2021). After that, the bacterial communicate together and this leads to triggers responses that lead to gene expression changes, up-regulating factors favoring sassily and formation of the extracellular matrix (ECM) which build three dimensional structures and complex diffusion channels formation, transporting nutrients, oxygen and other components required for bacterial growth also, removes waste products and dead cells will occurred in this stage (Choudhary *et al*., 2020).

Finally, detachment stage was occurs, biofilms become thicker after maturation and developing an anaerobic environment on the inside while external layers may begin to separate, these detachment mechanisms are essential to create new biofilms in new niches.

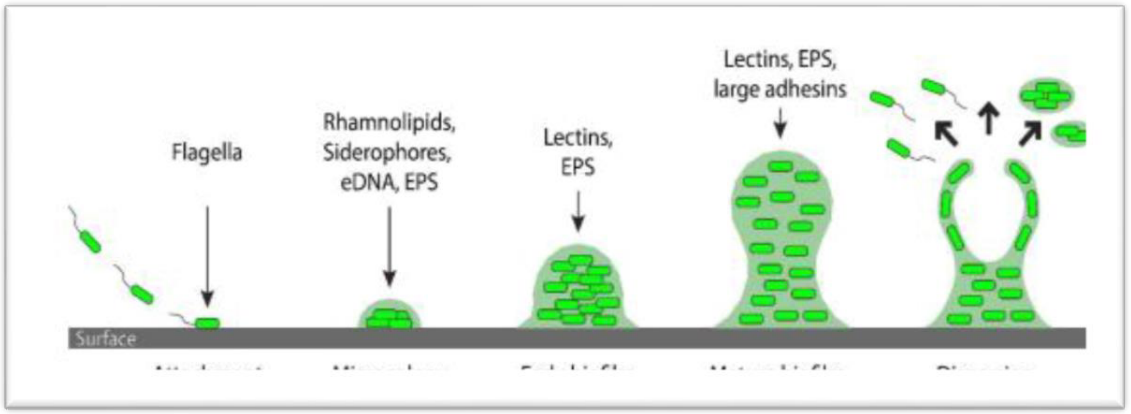

Figure (1.3): Biofilm mass formation steps (Silva *et al*., 2017).

1.2.7 Molecular assay for gene detection of *Escherichia coli*:

The concept of molecular techniques represents the study of large internal molecules represented by nucleic acids, proteins, complex carbohydrates, fats, the knowledge of molecules, materials and their use in the diagnostic process (Taylor *et al*., 2000; Muslim, 2021).

Molecular experiments that target individual microorganisms are simpler and more sensitive than culture methods but they need advanced knowledge of the pathogenic species that may be found in a sample. Polymerase Chain Reaction (PCR) is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology which has wide acceptance for rapid identification of pathogens and also for diagnosis of infectious conditions (Mishra *et al*., 2019). Because of the importance of *E. coli* in such infections, the increased incidence of infection and the possibility of the epidemic of infection as well as the riskiness of the disease and the lack of treatment have led to the focus of research in the world on improving the efficiency of molecular detection using advanced

technologies that reduce time and effort by (PCR). Currently, clinical detection methods take hours to days and limiting rapid intervention as an alternative, the use of molecular methods could improve speed and accuracy but their applicability is complicated by high genomic variability within UPEC (Brons *et al*., 2020).

1.2.7.1 Genetic variation of *Escherichia coli* toxin:

The genetic identity and frequency of *E. coli* genes were isolated and assessed for genetic variation analysis, highly genetic diversity were found among *E. coli* isolates supporting the potential for their use in epidemiological research as genes have a number of mutation recorded (Hoffmann *et al*., 2014). According to previous genetic study, the CDT-producing *E. coli* strains under examination were found to be eclectic and diversified, this observed variability may be the result of mobile genetic elements horizontally transferring virulence genes independently. This judgment is supported by results from tRNA insertion site analysis which showed that these strains do not have a common pattern of insertion while having CDT as a shared characteristic (Oloomi and Bouzari, 2008).

Genomic analysis has demonstrated a considerable genetic and phenotypic diversity between UPEC strains, which makes it difficult to pinpoint specific virulence factors associated with UPEC (Brzuszkiewicz *et al*., 2006). Supporting this, no genetic signature of putative virulence factors was found to be predictive of urovirulence in comparative genomic study of clinical UPEC isolates by (Schreiber *et al*., 2017). Common genetic changes in *E. coli* genomes ensure high diversity due to the acquisition or loss of genes through genetic modification events. Moreover, many of the virulence

factors may occur combined within the same strain and act synergistically (Braz *et al*., 2020).

Morgan *et al*., (2019) investigated that the molecular analysis of *E. coli* genome revealed that it harbors pathogenicity islands that are prevalent among *E. coli* species, the genes encoding HlyA toxin are localized within a polycistronic operon including *hlyC*, *hlyA*, *hlyB*, and *hlyD* which localized on the chromosome of pathogenic *E. coli* variants, referred to as pathogenicity islands, the operon encodes the proteins that involved in the production, activation and export of HlyA toxin.

1.2.7.2 Sequencing of *Escherichia coli* genes:

Genome sequencing is a novel tool for rapidly analyzing an organism's DNA sequence. The gene sequence has been shown to be a stable genetic marker because it is found in all bacteria and its function has not changed over time. Sanger Sequencing is designed for determining the sequence of nucleotide bases in a piece of DNA (commonly less than 1,000 bp in length). Sanger sequencing with (99.99%) base accuracy is considered the “gold standard” for validating DNA sequences including those already sequenced through next generation sequencing (NGS), this method has long been a cheap and effective method to characterize the taxonomy of bacterial strains in isolation often by sequencing the *16SrRNA* gene (Cermak *et al*., 2020). This bacterial gene is approximately 1500 bp long and contains both conserved and variable regions that evolve at different rates. The slow evolution rates of the former regions enable the design of universal primers that amplify genes across different taxa whereas fast-evolving regions reflect

differences between species and are useful for taxonomic classification (Kai *et al*., 2019).

1.2.7.3 Phylogenetic of *Escherichia coli* genes:

Phylogenetic methods have shed light on the processes of genomic evolution in the extraordinarily diverse species and revealed the origins of pathogenic *E. coli* strains. The phylogenetic classification of *E. coli* is great importance not only for understanding the populations of *E. coli* but also for clarifying the relationship between strains and diseases (Halaji *et al*., 2022).

Various diseases of the urinary tract have been linked to certain UPEC strain populations which have been extensively studied for their genetic variety. Using PCR assay, the phylogenetic groups were identified the main group in the phylogenetic study of UPEC strains (Modgil *et al*., 2020). Regarding links between strains, the phylogenetic trees produced were mostly similar to the *E. coli* analyses particularly for the future strains which were consistently clustered together but were largely separate from the other strains (Dadi *et al*., 2020).

The distribution of virulence factors may differ depending on the phylogenetic groups therefore, the analysis of virulence factors according to phylogenetic group B2 and D, which account for majority of *E. coli*. All strains in each phylogenetic group differ in the characteristics of phenotype as antibiotic resistance, growth rate-temperature relationships and ability to use certain sugars (Pergo *et al*., 2017). The production of virulence factors as well as the identification of processes by which pathogenic *E. coli* strains result in disease distinguishes them from normal microbiota that allowing them to be classified into pathotypes. Each *E. coli* pathotype has its

characteristic pathogenicity mechanisms and a specific profile of virulence factors encoded by specific gene clusters (Pakbin *et al*., 2021). However, due to microbial gene transfer, there is always exchange of virulence genes among phylogroups which may cause the accommodation of highly virulent strains in phylogroups, this could result to phylogroups containing mixed groups of strains and different clonal populations, thus, creating a more complex scenario in an attempt to establish epidemiological link set between phylogroups of *E. coli* and human infections (Dias *et al*., 2009; Etefia and Ben, 2020).

1.2.8 Extraction and partial purification of alpha hemolysin:

Protein purification is a set of processes in which one or a small number of proteins are purified from a complex compound that may be a complete cell, tissue or organism. The purification procedure of an unknown protein is usually depends on the size, physicochemical properties, binding affinity and biological activity furthermore, the protein purification process usually involves filtration and one or more chromatographic steps (Babaie, 2020).

Protein isolation and extraction from any microbial source requires determining the site of protein, either intracellular or extracellular, the extracellular toxin such as hemolysin are separated and extracted by using centrifugation to isolate the active protein from cell mass under low temperature to prevent denaturation (Daboor *et al*., 2010; Mahdi, 2021).

Generally, protein separation and purification schemes usually include one or more chromatographic steps to achieve the final separation and purification purpose, the basic step is to flow a solution containing protein

through a column containing various substances under the action of external force (Coskun, 2016**)**.

Gel chromatography is based on the as sumption that different proteins have different sizes and shapes, the protein with smaller average pore size than the gel beads must be continuously penetrated the interior of the beads (Kahle and Watzig 2018). Such small molecules not only have long movement paths but also suffer great resistance from inside the gel beads, so the elution time is different and hence elute last whereas, large molecules are excluded from all pores and come out first, for these molecules the column residence time is a direct function of their size and they can hence be separated according to this parameter (Caltabiano *et al*., 2018).

Chromatography is a useful method for acquiring very pure protein for using in very accurate experiments. Therefore, by purifying the desired molecule, it can be used in various industries such as medicine (Babaie, 2020). Also, it can be divided into High-performance liquid chromatography (HPLC), Gel Chromatography, Ion Exchange Chromatography (IEC), Hydroxyapatite Chromatography (HAC), Affinity Chromatography and other methods (Liu *et al*., 2020).

Chapter Two

Materials and Methods

2 Materials and Methods:

2.1 Laboratory materials:

2.1.1 Apparatuses and Instrument:

Laboratory Instrument and Apparatuses used in the study are listed in Table **(2.1)**

Table (2.1): Laboratory Apparatuses and Instrument used in the study

Equipment /Instruments	Company/ Origin
Aerobic incubator, Hood cabinet, Nano Drop, Oven, Water bath	Memmert/Germany
Autoclave	GallenKump/ England
Centrifuge, Light microscope	Olympus/ Japan
Gel electrophoresis system, PCR thermal cycler, UV-trans illuminator	Cleaver Scientific / U.K
High speed cooling centrifuge, Spectrophotometer	Hermle/Germany
Inverted microscope	T.C Meiji techno/Japan
Microtiter 96 wells plate	Nunclon/ Denmark
Micropipettes, PCR tubes (1.5 ml, 200µl), Sterile Eppendorff tubes (1.5ml),	Eppendorf / Germany
Tissue culture flask 25ml	SPL/ Canada
VITEK2 system	France / Biomeriux
Vortex	Gemmy/Twain

2.1.2 Chemical Materials:

Different chemical materials which used in the study are listed in Table **(2.2)**:

Table (2.2): Chemical materials used in the study

Materials	Company/ Origin
Agarose powder, Ethidium bromide, Loading dye, Nuclease free water	Promega / USA
Comassi brilliant blue G 25, Tris HCl,	BDH/ UK
Dimethyl sulfoxide (DMSO), Trypan blue	Sigma Aldrich/ USA
Tris-Borate EDTA (TBE10X)	Bio-basic/ Canada
Ethanol (70% , 90%), Hydrogen peroxide, Oxidase	BDH /England
Fetal bovine serum, Roswalled Parke Memorial Institute-1640 (RPMI-1640) powdered medium	Gibco / U.K
Gentamycin (50 mg vial)	The Arabic pharm / Jordan
MTT(3-(4,5-Dimethylthizole-2-yl)-2,5-diphnyl-2H-tetrazlium bromide) dye powdered	Germany/Roth
Phosphate buffer saline (PBS)	Sigma (USA)
Sephadex G-200	Amershamv/Pharmacia Fine Chemicals
Trypsin-Ethylene diamine tetra acetic acid (EDTA) powder	US biological/ USA

2.1.3 Culture media:

The culture media which used in the present study listed in Table (**2.3**).

Table (2.3): Cultures media used in the study

Culture media	Purpose	Company/ Origin
Blood agar base	Used for detection of hemolysin production and type of hemolysis	Oxoid / U.K
Brain heart infusion broth	Used for preservation of bacteria, biofilm formation and anti-biofilm potential of antibiotics	Himedia/ India
Chromo Agar	Used for the growth and differentiate between bacterial types in urine	Orientation/ France
Eosine methylene blue	Used for isolation and identification of negative bacteria	Oxoid/ U.K
Luria Bertani broth	Used for the *E. coli* growth	Himedia/ India
MacConkey agar	Used for isolation and identification of Enterobacteriaceae and differentiate between fermented and non-fermenting bacteria of lactose	Oxoid /U.K
Nutrient agar	the growth and isolate bacterial types	Biomark/India
RPMI-1640 Medium	Used for cell growth and viability	Gibco/ U.K
Serum-medium	Used for growth cell and inactivate Trypsin EDTA	Gibco/ U.K

2.1.4 The commercial kits:

The commercial kits used in the present study are shown in Table (**2.4**):

Table (2.4): Commercial kits used in the study

Type of Kits	Materials	Company/country
DNA extraction kit	1- Cell lysis: -GB Buffer 2-DNAbinding alcohols: -Absolute ethanol alcohol 3- Wash solution:W1Buffer, Wash buffer 4- DNA Elution: - Elution Buffer GT Buffer 30 ml In addition to proteinase K and RNase	Geneaid/ Thailand
DNA ladder	-A ladder consists of DNA bands with size 100-1500 bp	Bioneer/ Korea
Master mix 2x	-Taq DNA Polymerase - dNTPs (400 µm dATP, 400 µm dGTP, 400 µm dCTP, 400 µm dTTP) -MgCl2 3 Mm - Reaction buffer (pH 8.3)	Bioneer/ Korea

2.2 Methods:

2.2.1 Preparation of Solutions and Reagents:

The Solutions and Reagents were prepared as in Table **(2.5):**

Table (2.5): Preparation of Solutions and Reagents used in the study

Name of solution	Preparation method	Advantage	Reference
Bovine serum albumin	Dissolving (0.01g) of it in 10 ml of D.W to reach final concentration of 1mg/ml	Used to determine the amount of protein	Bradford, (1976)
Catalase Reagent	By adding 3ml of H2O2 to 100 ml of D.W.	Used to detect the ability of bacteria to produce catalase enzyme	Forbes *et al*., (2007)
Coomassie brilliant blue G-250 stain	By dissolving 0.1 g of comassie brilliant Blue G-250 in 50 mL of 95% ethyl alcohol, then 100 mL of 85% Phosphoric acid was added, mixed well and filtered using filter paper Whatman No.1 and the volume was completed to 1 litter by distilled water then saved in dark bottle	Used for total alpha hemolysin determination	Neuhoff *et al*., (1988)
MTT solution	By dissolving 0.8 g of MTT powder in 800 ml PBS. Then sterilized by filtration through a 0.2 µm filter	Used to detection the viability of cells	Meerloo *et al*., (2011)
Phosphate buffer saline with magnesium sulphate	By adding magnesium sulfate at a concentration of (0.001M) to the phosphate buffer saline	Used as a diluent in the estimation process for quantification of the effectiveness of hemolysis activity	Haque and Baldwin, (1964).
Trypsin-(EDTA) solution	By dissolving 15gm of trypsin-EDTA in 125 ml of D.W and constantly added by stirring the volume completed to 1 liter. The solution was kept by filtration using 0.22 µm Millipore filters subsequently and stored at (-80C˚).	Used for to detach and disaggregate the adherent monolayer cells from the bottom of the culture vessel	According to UAE Biological directions

2.2.2 Preparation of culture media:

The culture media listed in Table **(2.3)** were prepared according to the instructions of the company and sterilized at 121°C for 15 minutes in the autoclave (McFadden, 2000; Brown and Smith, 2017).

2.2.3 Standard curve for hemolysis using gradient concentrations of sodium chloride:

Gradient concentrations of sodium chloride solution were prepared using distilled water (Appendix I), as 3 milliliters of each concentration were mixed with 1 milliliters of washed and suspended erythrocyte cells in the physiological buffer. The tubes were then incubated at room temperature for one hour and then the tubes were precipitated at 1500 rpm for 30 minutes. The absorbance of the filtrate after separating it from the precipitate was read using the length. The wavelength 412 nm was then extracted from the concentration that caused hemolysis.

Hemolysis 50% = $\frac{A}{A1}$*100

A1: The value of the maximum absorptivity (to know the percentage of hemolysis of it)

A: The minimum absorbance value (Norddin *et al*., 2010)

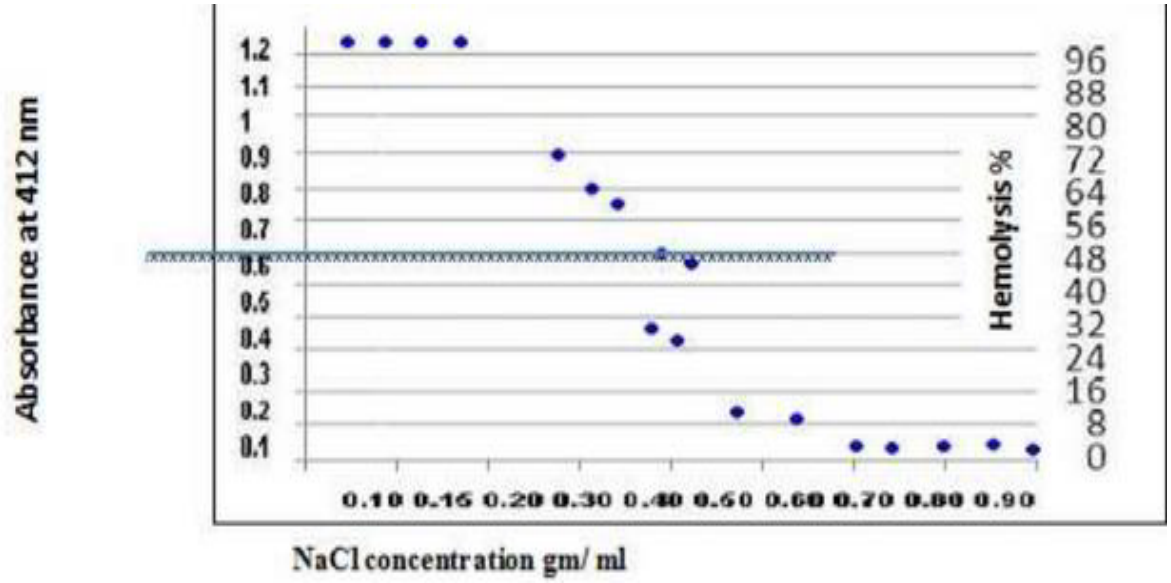

Figure (2.1): Standard curve of hemolysis using gradient concentrations of sodium chloride salt

2.2.4 Preparation of Sephadex and Gel filtration chromatography column:

Sephadex G-200 were prepared as recommended by Pharmacia Fine Chemicals Company. A quantity of Sephadex G-200 is suspended in 0.05 M phosphate buffer pH7, subjected to heating at 90C for 5 hrs. to ensure the swelling of the beads, degassed by vacuum pump under reduced pressure for 10–15 min and packed in a glass column (2×35 cm).

2.3 Study Design:

This is a cross sectional study. The study included 179 samples of urinefrom patients diagnosed with urinary tract infection, collecting under super version of specialized professional in private clinics (**Figure 2.2**).

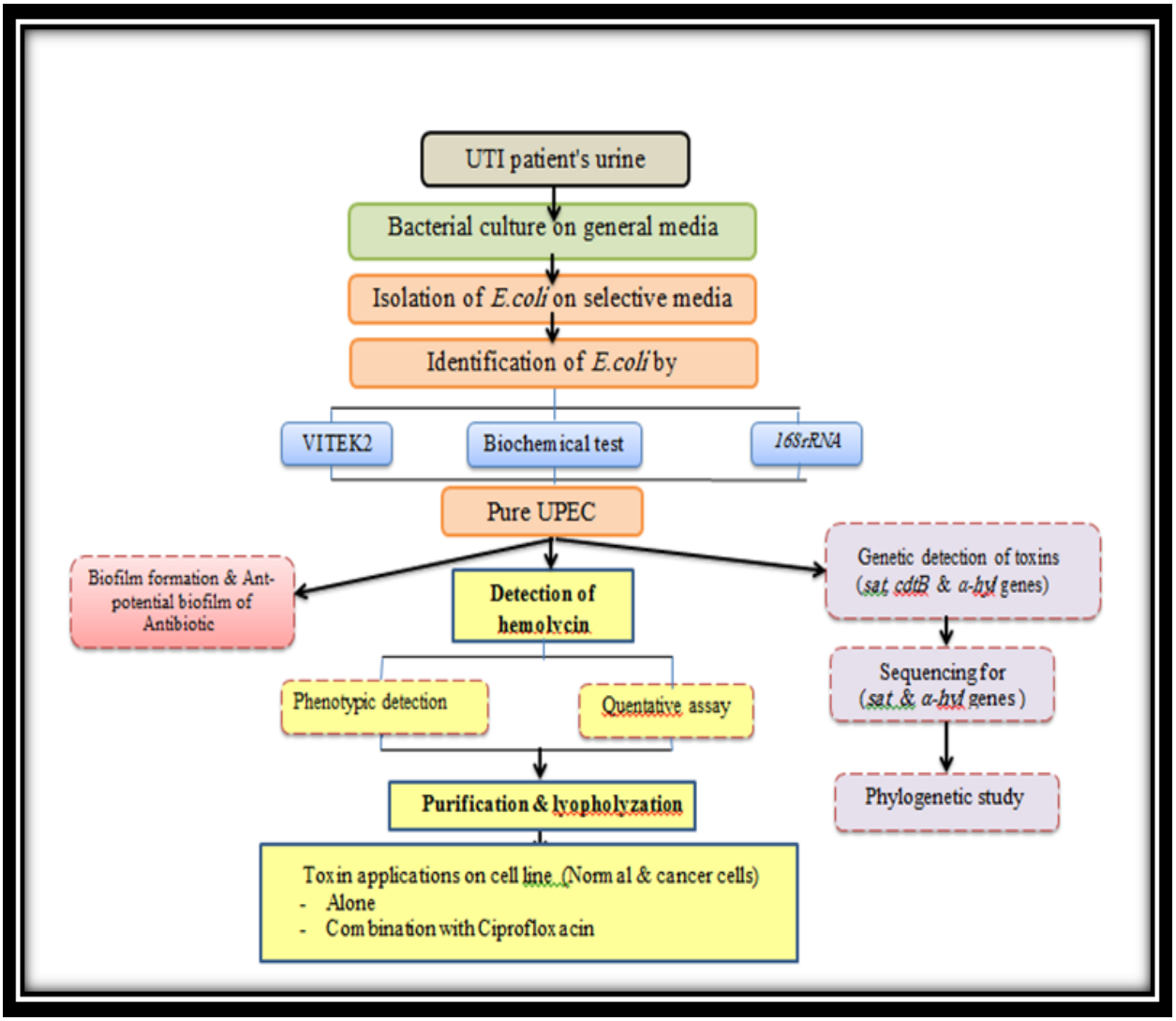

Figure (2.2): Methodology of the research project

2.3.1 Patients samples:

2.3.1.1 Urine samples collection:

The study was conducted at Al-Hilla city. A total of (179) urine samples from patients who admitted to Al Hilla General teaching hospital and Maternity and Children Hospital (Urology unit), the age arranged from (2-62) years for the purpose to isolation and identification of uropathogenic *E coli*, antibiotic susceptibility test, biofilm and anti-biofilm potential of antibiotics, detection some of virulence factors of UPEC and partial purification of alpha hemolysin toxin, finally study the effect of partial purified alpha hemolysin toxin on normal cell (Kidney) and Cancer cell (Prostate and Colon) during the period from March 2021 to December 2021.

About 10 ml of mid-stream urine samples from patients were diagnosed by a physician depending on clinical manifestation as patient with UTIs. The samples were collected in the morning in sterile containers and be surety that patient didn't take any medication for three days before collection of urine samples. The samples were diagnosed with microscopic examination transported to the laboratory in the College of Medicine. Also, general data were collected such as name, age, sex and address.

2.3.1.2 Ethical approval:

All subjects involved in this work were informed and the agreement will obtained verbally from each one before the collection of samples. This study was approved by the committee on publication ethics at College of Medicine, University of Babylon, Iraq, under the reference No. BMS/0231/016/

2.3.1.3 Isolation and identification of microorganisms:

By microscopic examination of samples excluding pus cells and other substances found in urine. The urine samples were cultured on nutrient agar, the presence of $\geq 10^5$ colony forming units (CFU/ ml) in the clean-catch midstream urine samples were considered to be significant for a diagnosis of UTIs. Then, the bacterial isolates were cultured in brain heart infusion broth to activate bacteria and incubated at 37 for 24-48 hrs. The calibrated loop technique was employed for quantitative culture and presumptive identification from each urine sample. After that, the pure colony were streaked and cultivated aerobically on Chromo agar, Eosin methylene blue agar, MacConkey agar and Blood agar at 37^{o}C for 24-48 hrs, additionally confirmation by biochemical tests like catalase and oxidase test then microscopic examination by Gram's stain. Primary identification of *E. coli* were done and isolated from other bacterial types. A subculture to get pure colonies on MacConkey agar plates were made from every single group of *E. coli* colonies (Vandepitte *et al*., 2003).

2.4 Laboratory Diagnosis:

The isolation and identification of *E coli* were performed according to (MacFaddin, 2000; Forbes *et al*., 2007) as follow:

2.4.1 Morphological examination:

The colonies were grown by sterile loop and incubated for 24hrs at 37^{o}C on different culture media. A single colony was taken from each primary positive bacterial growth and its identification depended on the morphological properties (colony size, shape, color and nature of pigments, edge, elevation and texture).

2.4.2 Microscopic examination:

Bacterial smear stained with Gram stain examines the morphology of bacterial cells to determine the form of Gram-stain reaction.

2.4.3 Biochemical tests:

The tests were performed according to (Forbes *et al*., 2007).

2.4.3.1 Catalase test:

Catalase is an enzyme that catalysis the release of oxygen from hydrogen peroxides. Gas bubbles formation indicates a positive result.

2.4.3.2 Oxidase test:

The test relies on the presence of any bacterial oxidase enzyme that would catalyze the transportation of electrons between electron donors in the bacteria and a redox dye (tetramethyl-phenylene-diamineihydrochloride).

2.4.3.3 Hemolytic reaction:

A pure culture of bacterial isolate were streaked on blood agar medium and incubated at 37C^{o} for 24-48 hrs. The appearance of a clear zone surrounding the colony is an indicator of β- hemolysis while the greenish zone is an indicator of α-hemolysis where no change of zone referred to non-hemolysis.

2.4.4 Identification and Antibiotic susceptibility test of *E. coli* isolates using VITEK 2 Compact System:

VITEK-2 is an automated microbiology system utilizing growth-based technology. It was supplied with the required identification data base for all routine identification tests that provide an improved efficiency in microbial diagnosis and antibiotic susceptibility test which reduce the time and the need to do any additional tests that will be safe for the user of system.

The kit contains the following:

- Gram negative card (ID-GNB) used for identification of gram-negative bacteria.
- Susceptibility card included:
  Ticarcillin, Piperacillin, Cefepime, Azetronam, Trimethprime-Sulfament, Piperacillin-Tazobactam, Imipenem, Meropenem, Amikacin, Tobramycin, Gentamycin, Ciprofloxacin and Minocycline**.**

2.4.5 Maintenance of bacteria:

2.4.5.1 Short term storage (McFadden, 2000):

Escherichia coli isolates were stored on nutrient agar slant at 4˚C until further tests.

2.4.5.2 Long term storage:

Fresh 24 hours, *E. coli* isolates from Nutrient agar cultures were frozen in maintenance medium that consisted of the brain heart infusion broth with glycerol by adding 15 mL of glycerol to 85 mL of the brain heart infusion broth, then distributed in sterile test tubes and autoclaving at (121°C) for (15) minutes and cooling at (45°C) and stored at -20˚C until use.

2.5 Detection of biofilm mass formation and anti-biofilm potential of antibiotics:

2.5.1 Detection of biofilm mass formation:

Microtiter Plate method (MtP) used to assess the formation of biofilms as mentioned by Babapour *et al*., (2016) with modifications in the steps:

- Bacterial suspensions of UPEC isolates were prepared from nutrient agar after 24 hrs.
- About 20 µl of bacterial suspension equal to NO. 0.5 Mcfarland standards tube used to inoculate microtiter wells containing 180 µl of BHI broth with 2 % glucose. Negative controls consisted of wells containing only 200µl of BHI broth with 2% glucose. The microtiter plate covered with parafilm during incubation at 37°C for 24 hrs.
- Unattached bacterial cells were removed after washing the wells three times with PBS pH 7.2 and dried at room temperature for 15 minutes. The adherent bacteria were then fixed in 200 µl of 99% methanol per well for 15 min. the plates were allowed to dry for 30 minutes at room temperature,
- Then 200 µl of 1% crystal violet was added for 15 min, washed three times with PBS (pH 7.2) to clear unbound dye and allowed to dry at room temperature.
- The dye bound to the adherent cells were re-solubilized with 200 µl ethanol.
- At 620 nm, the absorbance of each well were measured using an ELISA reader and the O.D value for well control has been deducted.
- From all O.D value measures, each assay were performed three times in triplicate and repeated.

- The adherence capability of the tested isolates has been categorized into four Categories: above the mean optical density of the negative control (containing broth only) were considered the cut-off optical density (ODc). Isolates were classified as follows :

(OD < ODc) …….. Non-adherent.

(ODc < OD < 2×ODc)……... Weakly-adherent.

(2×ODc < OD < 4×OD)……. Moderately-adherent.

(4×OD < OD)……. Strongly-adherent.

2.5.2 Detection of Anti-biofilm potential of antibiotics:

The antibiofilm effect of antibiotics against UPEC isolates was determined in vitro by using 96 wells polystyrene microtiter-plates method according to (Khirallah and El-deeb, 2015; Miglani and Tani-Ishii, 2021). Antibiofilm procedure include:

- Bacterial cell suspension (0.1ml) have been inoculated in 1.9 ml BHI broth and adjusted to 0.5 McFarland.
- One hundred microliter of the cultured BHI broth which supplemented with 2% glucose transferred into each well of 96- well microtiter-plate.
- One hundred microliter of antibiotic (1024 µg/ml) was added in each well of column 2. Serial dilutions were performed from column 2 to column 11, to obtain the final antibiotic concentrations, which varied from 1024µg/ml in (2^{nd} well) to 2 µg /ml in (11^{th} well).
- Ten microliter of bacterial cell suspension that prepared in step 1 was added from column 2 to column 12. Column 1 contain (BHI+1% glucose) serve as a negative control, column 12 contain (BHI+1% glucose) and

stimulated culture act as positive control. Plates were incubated at 37 C° for 24-48 hrs.

- After the incubation period, contents of the microtiter plates were emptied and the wells were washed three times with 200 microliter of PBS (pH 7.2).
- The remaining adhered bacteria were fixed with 200 microliter of methanol (99 %) per well. After 15 min, the micro titer plates were stained with 200 microliter per well of 1 % crystal violet for 5 min to clear unbound dye and allowed to dry at room temperature.
- The dye bound to the adherent cells were re-solubilized with 200 µl ethanol.
- The biofilm growth was read at 620 nm using micro plate reader.
- The percentage of biofilm inhibition were calculated according to (Kalishwaralal *et al*.,. 2010) using the equation:

Biofilm inhibition% = 1- O.D 620 of cells treated with antibiotic \ O.D 620 of cells none treated with antibiotic.

2.6 Molecular assays:

2.6.1 Genomic DNA extraction:

According to the genomic DNA purification Kit supplemented by the manufacturing company (Promega -USA), the chromosomal DNA were used as templates for all PCR experiment. The PCR reactions were performed in a thermal cycle before PCR assay. DNA extracted according to the following steps:

- One ml of an overnight culture was added to a microcentrifuge tube. Microcentrifuge tube was centrifuged at 15,000 × g for two minutes to pellet the cells. The supernatant was removed.
- About 400µl of cell lysis solution was added, then gently pipetted until the cells were re-suspended.
- Microcentrifuge tubes were incubated at 60°C for five minutes to lyse the cells; then cooled at room temperature.
- Three microliters of RNase solution was added to the cell lysate. The tube was inverted 3 times to mix.
- Microcentrifuge tube was incubated at 37°C for 30 minutes, then cooled to room temperature.
- Two hundred microliters of protein precipitation solution was added to the RNase-treated cell lysate. The tube was vortexed vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate.
- The sample was incubated on ice for five minutes.
- The tube was centrifuged at 15,000× g for three minutes.
- The supernatant containing the DNA was transferred to a clean microcentrifuge tube containing 600µl of room temperature isopropanol.
- The tube contents were gently mixed by inversion until the thread-like strands of DNA form a visible mass.
- The tube was centrifuged at 15,000× g for two minutes.
- The supernatant was carefully poured off and drained the tube on clean absorbent paper, then 600µl of room temperature 70% ethanol was added and the tube was gently inverted several times to wash the DNA pellet.

- The tube was centrifuged at 15,000× g for two minutes. Ethanol was carefully aspirated.
- The tube was drained on clean absorbent paper and allows the pellet to air-dry for10–15 minutes.
- A 50 µl of DNA rehydration solution was added to the tube and rehydrate the DNA by incubating the solution overnight at 4°C.
- The DNA was stored at –20°C to avoid degradation.
- Detection of DNA by horizontal gel electrophoresis and concentration measured by Nanodrop DNA.

2.6.2 Estimation of DNA concentration:

The extracted genomic DNA is checked by using Nano drop spectrophotometer (scan drop, analytica jena, Germany) measures DNA concentration (ng/µl) and checks the DNA purity by reading the absorbance at (260 /280 nm).

2.6.3 The mixture of PCR reaction:

Amplification of DNA was carried out in a final volume of 20µl containing the following as mentioned Table **(2.6)**.

Table (2.6): Contents of the Reaction Mixture of PCR

No.	Contents of reaction mixture	Volume
1	Master mix	10µl
2	Forward primer	1 µl
3	Reverse primer	1 µl
4	DNA template	2µl
5	Deionizer D. W	6µl
Total volume		20µl

Molecular assay in this study includes four genes, each one has specific nucleotide and product size. The primer sequences and PCR conditions that used were listed in Table **(2.7)**.

Table (2.7): The primer's sequences and optimal conditions for PCR

Gene name	Primer sequence (5´- 3´)	Size of product bp	Conditions	References
16S rRNA	F:AGAGTTTGATCCTGGCTCAG R: GGTTACCTTGTTACGACTT	1492	Step1:95ºC, 1min. Step2:94ºC, 30 sec. Step3:55ºC, 40sec. Step4:72ºC,1.5min. Step5: 72ºC, 5min.	Lin *et al*., (2008)
sat	F:CTACAGCTTGATCACCTATGGC R: CTCCCTGGTATTTCTTTGTGG	410	Step1: 94ºC, 3min. Step2:94ºC, 1min. Step 3:58ºC, 1min. Step 4:72ºC, 1min. Step5:72ºC, 5min.	Saraylu *et al*., (2012)
cdt B	F:AAATCACCAAGAATCATCCAGTTA R:AAATCTCCTGCAATCATCCAGTTTA	430	Step1: 94ºC, 5min. Step 2: 94ºC,30sec. Step 3:63ºC, 30sec. Step 4:63ºC, 3min. Step 5:72ºC,10min.	Johnson and Stell, (2000)
hly A	F:AACAAGGTAAGCACTGTTCTGGCT R:ACCATATAAGCGGTCATTCCCGTCA	1177	Step1:94ºC. 3 min. Step2: 94ºC, 1min. Step3:61ºC , 30sec. Step4:72ºC, 3min. Step5: 72ºC ,7 min.	Yamamoto *et al*., (1995).

2.6.4 Detection of chromosomal DNA and Amplified Products by Agarose Gel Electrophoresis:

DNA and duplex PCR products were resolved by horizontal agarose gel electrophoresis as follows (Sambrook and Russell, 2001):

- Mix 5 µL of loading solution to 20 µL DNA sample or PCR product, then the mixture was transferred to the pits in the agarose gel (1.5%) with ethidium bromide.
- Transfer the mold to the electrophoresis tank containing TBE (1x), the surface of the gel was immersed in the buffer solution and the DNA samples were migrated by passing them at a potential difference of 80volts for a period of 80 min for (*hlyA, cdtB* and *sat* genes) and one hrs,58 min for (*16SrRNA* gene).
- Determined the locations of the extracted DNA bundles or PCR products according to DNA ladder using an ultraviolet source.
- Photographed the gel for the purpose of confirming the presence of DNA.

2.6.5 Gene sequencing of *16SrRNA, hylA* and *sat* genes:

The resolved PCR amplicons were sequenced professionally from n terminus forward and reverse following the sequencing company instruction (Macrogen, South Korea). Particularly good chromatograph from ABI sequence file were examined extensively verifying that the annotation and variances were not caused by PCR or sequencing error. The virtual location and other details of the obtained PCR fragments were identified by comparing the observed nucleic acid sequences of local samples with the retrieved reference from the bacterial database.

2.6.6 Fasta Q sequencing data analysis:

The PCR product sequencing findings were edited aligned and assessed using BioEdit Sequence Alignment to ensure that they matched the appropriate sequences in the reference database (Blast Software NCBI). The nucleic acids found in the PCR amplicons were numbered as well as their respective locations in the reference genome.

2.6.7 Phylogenetic tree construction:

In this work, specific comprehensive tree were generated using the Maximum likelihood given by phylogeny.fr version (0001.3). The NCBI-BLASTn website was used to compare the reported variations to their homologous reference sequence neighbors. Then, using the Maximum likelihood method an inclusive tree were created that included the observed variant and visualized using. In the comprehensive tree, the sequences of each categorized phylogenetic species-group were annotated correspondingly.

2.7 Extraction and partial purification of alpha hemolysin:

2.7.1 Partial purification by gel filtration chromatography (2x35cm):

The manual procedures were used for partial extraction of alpha hemolysin, the selected isolates were grown on 100 Luria Bertani broth and incubated for 24 hrs at 37 ºC with constant shaking at 200 rpm, cultures were centrifuged at 8,000×g for 15 min at 4 ºC and filtrated by Millipore filter, then the cell pellet were dialyzed against 100 ml of 10 mM Tris HCl buffer pH 7.6 for 24 hrs at 4 ºC. The resulting supernatants were used as a source of crude and the resulting material were applied to a Sephadex G200 column (Amersham Pharmacia) for partial purification of toxin. Elution was achieved at a flow rate of 50 mL/hr and

the same buffer was used for equilibration. The Absorbance of each fraction was measured at 412 nm. The enzyme activity was also determined in each fraction and protein concentration was determined using Bradford (1976). The fractions containing the highest percentage of hemolysis were collected and lyophilized.

2.7.2 Determination of protein concentration:

Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin standard curve as follow:

- Different concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) were prepared from BSA stock solution (1mg/1ml) (Appendex II).
- A 2.5 mL of Coomassie brilliant blue G-250 dye was added to the blank and sample tubes.
- A 0.45 mL of 0.05M phosphate buffer pH 7 was added to sample tube and 0.5 of it was added to blank tube.
- After 3 minutes of incubation at room temperature the absorbance were measured at 595nm

The linearity relationship between absorbance and concentration of protein were plotted as (**Figure 2.3**) and the standard curve obtained were used to estimate the concentration of protein as the following:

Protein concentration (mg/ ml) = O.D (595 nm)/slope *1000

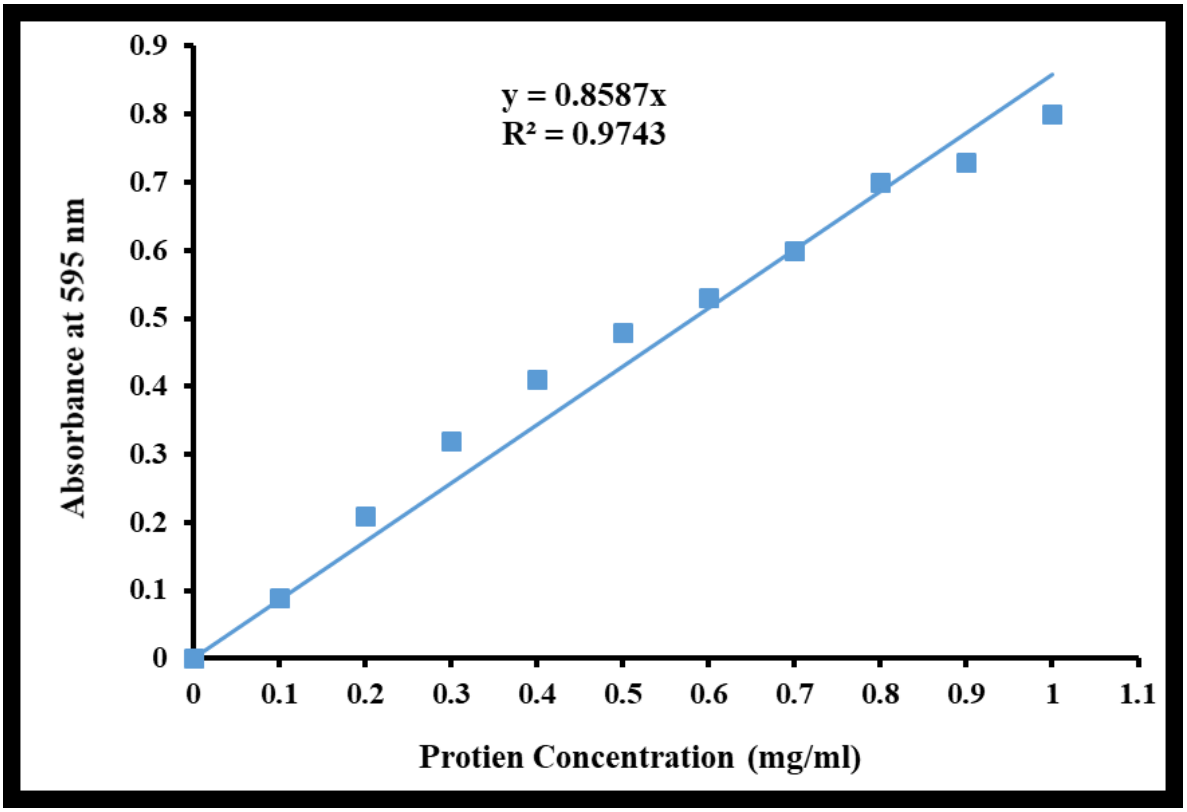

Figure (2.3): Standard curve of bovine serum albumin for the determination of protein concentration

2.7.3 Quantitative assay of alpha hemolysin:

- The photolytic activity of hemolysin was tested by a series of dilutions (Santos *et al*., 1999; Bizani and Brandelli, 2001).
- One milliliter of the crude filtrate were diluted by making a series of dilutions and using a phosphate buffer.
- Red blood cells suspended in physiological buffer were precipitated using a centrifuge center at a speed of 1500 rpm for 30 min and were washed three times with buffer solution salt phosphate with magnesium sulfate, centrifuged each time at a speed of 1500 rpm for a period of 30 min (taking into account that the device is centrifuge cooled), then the pellets were suspended (2%) in the same buffer.

- One mL of crude filtrate were mixed with one mL of obtained erythrocyte suspension. It were obtained from step 2 above in clean dry tubes and the tubes were incubated at room temperature for one hour and then at a temperature 4ºC (in the refrigerator) for one hour as well. Then two milliliters of phosphate-buffered saline with magnesium sulfate were added to each tube and mix gently.
- Sedimentation of the remnants of blood cells were carried out at a speed of 1500 rpm for 30 min and the filtrate were separate, its optical density were measured at a wavelength 412nm to measure the color of hemoglobin
- The effectiveness were determined by taking the reciprocal of the highest dilution, which gives a decomposition rate (50%) in comparison with a positive control consisting of red blood cells with a salt concentration that gives decomposition. The red blood, (50%), (100%) and negative control consisting of pellets were mixed with physiological saline solution and the relative distance to the end point was measured.

2.7.4 Alpha hemolysin and Ciprofloxacin serial dilutions:

By using D.W., two-fold serial dilution for toxin and Ciprofloxacin from the stock powder were prepared, six concentrations starting from low to high of (31.25, 62.5, 125, 250, 500 and 1000µ/ml) respectively were used in the study.

2.8 Preparation of normal (Kidney) and cancer (Colon and Prostate) cell lines for cytotoxicity assay:

Kidney, Colon and Prostate (cancer) cell lines in frozen vials were supplied from Tissue Culture Laboratory in the Rawafid Al-Eloom company /Babylon According to (Meleady and O'Connor, 2006).

2.8.1 Harvesting and sub-culturing of cell line:

Harvesting is a technique which uses the proteolytic enzyme trypsin to detach and disaggregate the adherent monolayer cells from the bottom of the culture vessel. This procedure were performed whenever the cells need to be harvested for passage and cell count (Freshney, 1994) and it includes the following steps:

- The medium were aspirated and discarded when the cell growth reaches a monolayer and before the exponential phase .
- The cells were washed with few ml of warm PBS solution.
- About 3ml of warm trypsin EDTA solution were added to cover the monolayer and with gentle rocking of the flask dish 4-5 times to flood the monolayer.
- The flask dish were incubated at 37°C until the monolayer were detached.
- Once the cells detached, the flask were removed from incubator and complete separation of the cells were done by rocking the flask from side to side.
- The cells were gently pipetted up and down to disrupt cell clumps into single cells.
- Cells were counted and re-suspended into the desired number in a growth medium with 5-10% FBS, which were used to inactivate trypsin .
- The cells were sub cultured into two flasks or cultured on culture plate.

2.8.2 Subculture of adherent cell line:

After the cells became confluent as monolayer, subculture was done according to the protocol mentioned by Brooks and Kurtti, (1971):

- Cultures were viewed by using the inverted microscope to assess the degree of confluence and confirm the absence of bacterial and fungal contaminants.
- Spent medium were removed.
- The cell monolayer were washed with PBS using a volume equivalent to half the volume of culture medium. This wash step was repeated if the cells were known to adhere strongly.
- Trypsin- EDTA were pipetted into the washed cell monolayer using 1ml per 25cm^2 of surface area. Flasks were rotated to cover the monolayer with trypsin. The excess trypsin were decanted.
- Flask returned to the incubator and left for 2-10 min.
- The cells were examined using the inverted microscope to ensure that all the cells were detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells.
- The cells were re-suspended in a small volume of fresh serum-containing medium to inactivate the trypsin. In case of cells cultured in the serum-free media, a trypsin inhibitor (e.g. soya bean trypsin inhibitor) used to inactivate the trypsin.
- The required number of cells were transferred to a new labeled flask containing pre-warmed medium.
- The cell line were incubated at 37 ºC.
- This process was repeated as demanded by the growth characteristics of the cell line.

2.8.3 Cytotoxicity assays:

2.8.3.1 MTT assay:

2.8.3.1.1 Principle:

The universal purposed of the M.T.T assay is to asses viable cells in relatively some throughput (96-well plates) without the need for celled counting. Therefore the most famous use is to determine the killing of several drugs at different dilutions.

The principled of the M.T.T assay is that for most viable cells mitochondrial action is constant and there an increase or decrease in the numbering of viable cells is linearly related to the mitochondrial action. The mitochondrial activity of the cells is reflecting by the formation of the pale red tetrazolium salt (MTT dye) into dark black formazan crystals by NADH which can be soluble for homogenous measure. Thus, any increase or decrease in viable cell numbering can be detected by measure formazan concentrations reflected in optical density (absorbance) using a plated reader at 570 nm. (Meerloo *et al*., 2011).

2.8.3.1.2 Procedure:

- According to (Meerloo et al., 2011), at the end of the during exposure period, the medium were removed from the walls and then the cells were washed with PBS. A blanking controlling was carried to assess unspecific formazaning conversion.
- A volume of 1.9 ml of MTT solution (9 mg/ ml) were added to 1 ml medium to obtain final concentration of 3 mg/mL. Then, 30 µl of the resulting solution was added in each well.
- The plated were incubated for 9 hours at 37°C until intracellular red formazan crystals were visibled under the invert microscope.

- The supernatant were removing and 10 µl DMSO were added in each wall to dissolve the resultant formazaning crystals.
- The plated were incubated at rooming temperature for 90 minutes until the cells have lyse and red crystals have dissolved.
- Absorbance were measured by a micro plate reading at 570 nm.

  The absorbance read of the blank must be taken from all samples. Absorbance read from testing samples must then be dividing by the controlling and multiplied by 100 to give percentage celling viability or proliferation. Absorbance valuable greater than the controllingling indicate cell proliferation, while little values suggest cell dead or inhibiting of proliferation. Percentage of cell viability or percentage of inhibition was calculated by the following formula:

% viability = (A.T – A.B) / (A.C – A.B) × 100%

Where, A.T = Absorbance of treat cells (drug).

A.B = Absorbance of blanks (only medium).

A.C = Absorbance of control (untreated).

% Inhibition = 100 – % viability

2.8.4 Experimental design:

According to Freshney (2010), the killing assays were applied for the determination of the event of toxin and Ciprofloxacin on cancer (Prostate, Colon) and normal (Kidney) cell lines. Different concentrations of toxin were tested for a defined timed durations.

When the growth in the flask became as monolayered before it reached the exponential phased, the cell monolayer were harvest and re-suspend with a serum-free medium in a concentration of $9*10^5$ cells/ml and seeded in a 96-

wall cell culture plate. Since the cell growth reaches 10%, the walls were exposed to serial dilutions of the toxin and Ciprofloxacin as in the following experiments.

2.8.4.1 Experiment No.1. The effect of toxin and antibiotic on Kidney cell line:

Three replicates walls in seven columns of 96-wall plate were cultured with Kidney cells in a concentration of $9*10^5$. Three replicates walls in column No.1 was considered as a control group and each one of the remaining six columns 9 walls replicates were exposed to 200 µL of each of the serial dilutions of toxin alone, Ciprofloxacin alone and toxin with Ciprofloxacin together. Then the plate was covered with a self-glass lid and incubated once for 24 hrs. After the end of the exposure, the walls washed with 200 µl of PBS. The effect of toxin on Kidney cell line growth was assessed by MTT assay.

2.8.4.2 Experiment No.2. The effect of toxin and antibiotic on Colon cell line:

As in experiment No.1 but the Colon cell were treated with the serial dilution of toxin, Ciprofloxacin and toxin with Ciprofloxacin as expressed in experiment No.1.

2.8.4.3 Experiment No.3. The effect of toxin and antibiotic on Prostate cell line:

As in experiment No.1 except that the seeded cells in the walls are Prostate cell line.

2.9 Statistical Analysis:

Normal distribution of the study parameters using Kolmogorov-Smirnov and Shapiro-Wilk normality tests to determine whether the data is within an acceptable range. The data were normal with the significant value of the Shapiro-Wilk Test as it is larger than (0.05). So that, all data were checked for the normality test and the descriptive Statistics for the study were shown in (Appendix III).

Statistical analysis was performed by using statistical package of social science (SPSS) version 25. Data were expressed as mean± Standard Deviation (SD) and statistical significances were carried out.

The data were expressed as (mean ± SD). Statistical comparison between groups were made using student's Chi-square test and a P value of ≤0.05 was considered significant; while the differences between more than two groups were analyzed using an analysis of 1variance (one – way ANOVA). Also, the relationship between the studied parameters (Niazi, 2004).

Chapter Three

Results and Discussion

3. Results and Discussion:

3.1 Characteristics of the study subject:

The study evaluated positive bacterial culture of *E. coli* from urine samples on patients who were hospitalized in the Al-Hilla Teaching Hospital (Urology unit) and Maternity and Children Hospital, all cases were prognosticated by physicians and samples were taken based on clinical evaluation for the patients. The study conducted in the period from 1st March 2021 to 1st December 2021 which included (179) samples of urine. The results revealed that dominance of *E. coli* 56\123 (45.5%) as shown in Table (**3.1**) after identification by morphological, microscopical, biochemical tests, VITEK 2 system and genetic detection by *16SrRNA*.

Table (3.1): The percentage of Uropathogens among patients

No. of urine samples	Results		No. of isolates (%)
179	Positive bacterial culture	*E. coli*	56 (45.5%)
		Other bacterial causes	67 (55.5%)
		Total	123(68.7%)
	Negative bacterial culture		56 (31.3%)

3.2 Identification and the characterization of *Escherichia coli*:

3.2.1 Conventional biochemical detection for *E. coli*:

The diagnosis of *E. coli* in laboratory were based on phenotypic characteristics including morphology of colonies were verified by Gram staining and VITEK 2 system for diagnosis of *E. coli*, (Appendix IV).

The positive bacterial culture from all samples were 123(68.7%), obtained from UTIs patients were considered true bacteriuria ($\geq 10^5$ bacteria /ml) with the remaining 56(31.3%) being negative bacterial culture may be due to other causes like fastidious bacteria, Chlamydia or viruses.

The isolates inoculated on selective media, suspected *E. coli* isolates appeared as bright pink colonies, indicating lactose fermentation when cultured on MacConkey agar and formed the green metallic sheen colonies on Eosin methylene blue (EMB) media which means that the colonies produced organic acids due to lactose and glucose fermentation in the presence of eosin and methylene gives this color (Singha and Prakash, 2008). Color and morphology characteristics on chromo agar allowed for easy differentiation of the bacteria colonies, all the isolates appeared as pink red colonies and were very easy to distinguish (Dejulius *et al*., 2004; Abdullah *et al*.,2009).

The isolates hemolysis activity were examined on blood agar, because of certain types of *E. coli* strain able to lyse erythrocytes of mammalian species which termed as hemolysis. The current isolates recorded that 16/21 (76%) of isolates gave alpha hemolysis on blood agar. Hemolysin gene is responsible for the coding of cytotoxic factor in hemolytic strains of *E. coli*

which is considered one important virulent factor in pathogenesis of UTIs (Aghemwenhio *et al*., 2017).

Several pathogens produce catalase in order to defend themselves against attacks by hydrogen peroxide, a weapon commonly used by the host's immune system in addition to oxidative stress (Iwase *et al*., 2013). Our results revealed were positive for catalase test for all isolates. However, the negative results for oxidase test present in all isolates, which was agreed to Dahwash *et al*., (2021); Abdul-Husien, (2021) and Hussein, (2022). By the end concluded that suspected 56/123 (45.5%) *E. coli* isolates were obtained of the total positive bacterial culture.

3.2.2 Identification of *Escherichia coli* by VITEK 2 system:

After identification of bacterial colonies by the conventional biochemical tests, VITEK 2 system identify using Gram negative card were done. The results produced by machine were analyzed using compact software which gave 98% probability of *E coli* characteristics with excellent confidence (Appendix V), as this device provides (47) bacterial tests as well as biochemical tests are necessary to diagnose of isolates, it is the most automated platform available with rapid results, high degree of specificities and decreases the time in comparison with manual microbial identification techniques (Abdul-Husien, 2021).

The results revealed that 56(100%) of clinical isolates were found to be *E. coli*, compared to the results of Modest *et al*., (2015) and Abdul-Husien, (2021) who reported the rate of *E. coli* within clinical isolates were (98%) and (97%) respectively.

3.2.3 Detection *Escherichia coli* by *16SrRNA*:

The DNA samples were extracted from all isolates that previously found to be *E. coli* by biochemical tests and VITEK2 system, conventional PCR carried out using the extracted DNA from samples to be amplified of *16SrRNA*(1492bp) specific for *E. coli* according to referenced sequence in the NCBI. After that, gel electrophoresis showed that all of 56(100%) samples were *E. coli* as shown in Figure **(3.1)**. This method is one of the fastest and most accurate methods for diagnosing bacteria, this gene contains the conserved regions that overlap with the variable regions that are used to determine the bacterial genus and species because*16SrRNA* is present in all bacterial species and it is slightly heterogeneous or the random change in the genetic sequence is very small over time (Srinivasan *et al*., 2015; Al-Saadi, 2019).

The results of *16SrRNA* were assessed and two of curent isolates were recorded in the gene bank with the accession number OL539543.1 and OL539542.1. So the confirmed result of *E. coli* led to toxin detection gene (*hlyA, cdtB and sat* genes). The results supported by the study of Jenkins *et al*., (2012); Lai *et al*., (2016); Maleki *et al,* (2017) and Al-Saadi, (2019) who they detected *16srRNA* in *E. coli*.

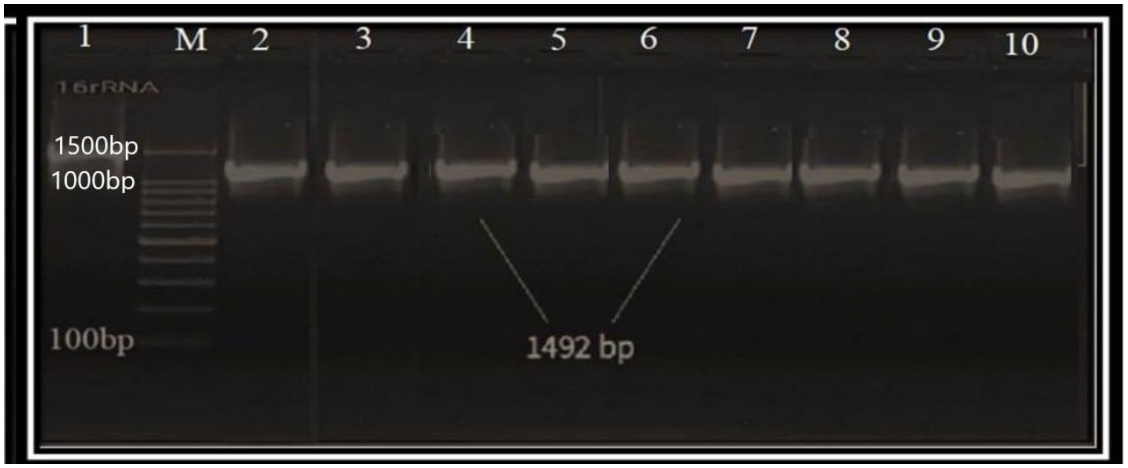

Figure (3.1): Ethidium bromide agarose gel electrophoresis for ampilification *16SrRNA* specific gene in *E. coli* was identified via PCR detection of 1492 bp (80 volt, 1hrs and 58 min). Lane M: DNA ladder. Lanes: 1-10 represent the identified *16SrRNA* gene.

3.3 The distribution of *Escherichia coli* among patients:

The results revealed high rate of UTIs by *E. coli* 56\123 (45.5%) which may be due to its natural presence as of gut intestinal tract as normal flora and to the adaptation of bacteria to live in the environment of the urinary tract, as well as the possession of strong and powerful virulence factors that increase the ability to cause infection. The most important reason for their presence as a major cause of UTIs is the transfer of bacteria from the outlet opening which is the natural environment to the urinary opening (Raeispour and Ranjbar, 2018; Al-Saadi, 2019).

The current finding are quite similar to the results of Ponnusamy and Nagappan (2013); Al-Saadi and Abdullah, (2019); Al-Saadi (2019), Ali and Rahman, (2020) and Al-Tamemi, (2021) by (54.7%), (50%), (50%), (53.85%) and (43.1%) respectively in their study for isolation of bacterial causes of UTIs, the researcher results were found to be higher than the

results presented by Saenz *et al*., (2015); Al-Hasnawy *et al*., (2018); Hamza, (2018) and Sonkol *et al*., (2020) at (38%), (23%), (36.84%) and (24.7%) respectively in urine samples with less than (60%) recorded by Al-Amri, (2019).

Different factors affect UTIs incidence and severity like sex, age, diabetes and the severity of the disease in the patient. UTIs more common in female with a high rate of recurrence that due to a different predisposing factors such as anatomical characteristics, hormonal status, pregnancy and lifestyle habits which they lead to much more common in females (Storme *et al*., 2019), which is approved with our results that a higher percent in females, 40/56(71.5%) compared to males 16/56(28.5%) with statistically significant discrepancies between the two classes (males and females) at (P=0.001) as showed in Table **(3.2)**. These results were quite similar to Tewawong *et al*., (2020) and Kurdi, (2020) who stated that the presence of *E. coli* isolates were (74%) and (73.3%) in females and (26%), (26.7%) in males, while other studies of Tabasi *et al*., (2015) and Al-Tamemi, (2021) showed a different according to sex were recoded (79.5%) and (65%) in females and (20.5%) and (34.8%) in males respectively.

About 40-50% of females during their lives may have at least one symptomatic UTIs and about 20-30% of adult females approximately can experience recurrence UTIs within 6 months after primary infection by UTIs (Kudinha, 2017; Akhtar *et al*., 2017). In males, UTIs are uncommon due to the longer length of the urethra, antibacterial properties of prostatic fluid and less colonized periurethral so the problems of infection appear in elder males where increasing prostatic hypertrophy may obstruct the urine flow and

increase the risk of developing UTIs (Shaheen *et al*., 2019; Ibrahim *et al*., 2021).

Table (3.2): Distribution of UPEC isolates in patients according to age and sex

Variable		**Patients = 56**		**P value =0.05**
		N	**Percentage**	
Age groups	2-11	7	12.5	0.38
	12-21	9	16	
	22-31	24	43	
	32-41	7	12.5	
	42-51	7	12.5	
	52-61	2	3.5	
Sex	Male	16	28.5	0.001
	Female	40	71.5	

The age incidence of UTIs in all ages has reached the maximum rate of infection (43%) in the age group (22-31) years for males and females, according to the finding of other research conducted by Sule and Kumurya, (2016); Al-Amri, (2019) and Al-Naqshbandi *et al*., (2019) were determined a high ratio of infection in (21-30) years old.

Gessese *et al*., (2017) investigated that the UTIs caused by *E. coli* affecting both males and females of all age groups during their life span but females were more susceptible than males due to wider urethra of females and its proximity to the anus and pregnancy, also the bacteria can easily move up to the urethra from the rectum and thereby cause infection.

Urethral obstruction problems are common in males in their fifties and older after the development of benign prostatic hyperplasia, incomplete urine output from the bladder and lead to a rapid increase in the incidence of UTIs (Abaas, 2018).

3.4 *Escherichia coli* antibiotic susceptibility patterns:

A various levels of susceptibility were observed in UPEC isolates towards (13) antibiotics belonged to different classes by using VITEK 2 system (Appendix VI). The results revealed that antibiotic susceptibility test were affected at high rates of sensitivity were seen with Imipenem (100%), Meropenem (100%), Amikacin (94.6%), Piperacillin-Tazobactam (71.5%), Tobramycin (71.5%) and Minocycline (51.7%) while High rates of resistance were seen with Ticarcillin(100%), Piperacillin (100%), Aztreonam (94.6%), Cefepime (94.6%) and Trimethprime-Sulfament (64.3%) as showed in Table **(3.3)**.

Table (3.3): Antibiotic susceptibility and MIC to *E. coli*

Antibiotic susceptibility	Antibiotic		No.	MIC	%
Maximum Sensitive I: Intermediate / R:Resistant / S: Sensitive	Piperacillin-Tazobactam	I	9	≤ 4	16
		R	7	32	12.5
		S	40	≥ 128	17.5
				16	12.5
				64	22.5
				8	25
	Imipenem	S	56	≤ 0.25	100
	Meropenem	S	56	≤ 4	93
				32	3.5
				≥ 128	3.5
	Amikacin	I	3	≤ 4	5.4
		S	53	20	94.6
	Gentamycin	I	4	≤ 4	7.2
		R	13	20	23.2
		S	39	≥ 128	51.2
				16	18.4
	Tobramycin	I	13	≤ 4	23.2
		R	8	8	14.3
		S	35	≥ 128	21
				16	10.4
				64	31.1
	Ciprofloxacin	I	1	≤ 4	1.7
		R	24	20	42.9
		S	31	≥ 128	52.2
				16	3.2
	Minocycline	I	9	≤ 4	16.2
		R	18	20	32.1
		S	29	≥ 128	20
				16	10
				64	1.7
				8	20
Maximum Resistance I: Intermediate / R:Resistant / S: Sensitive	Ticarcillin	R	56	≥ 128	82.1
				< 128	17.9
	Piperacillin	R	56	≥ 128	80.3
				< 128	19.7
	Cefepim	R	53	≤ 4	94.6
		S	3	20	5.4
	Azetronam	I	3	20	5.4
		R	53	≤ 4	9.8
				20	10.3
				≥ 128	1.8
				16	22.3
				64	11.2
				8	35.6
				32	2
				24	2
	Trimethprime-Sulfament	R	36	≤ 4	64.3
		I	20	20	15
				≥ 128	20.7

The results suggested that, the Carbapenems (Imipenem and Meropenem) still are a therapeutic choice in the treatment of UTIs caused by *E. coli*, these results were consistent with previous studies like a local study on patients admitted to Zakho Emergency Hospital by Polse *et al*., (2016), in Baghdad hospitals by Kadhum *et al*., (2018) and a study by Aal-Aboda and Notazy, (2018); Al-Amri, (2019); Al-Tamemi, (2021) and Dahwash *et al*., (2021). The sensitivity results to Imipenem were approved by the studies carried out in other countries for both Tabasi *et al*., (2015) and Khorshidi, *et al*., (2022) that found that (100%) of UPEC isolates were sensitive to Imipenem. However, the higher results recorded by Al-Tamemi, (2021) and Dahwash *et al*., (2021) were found both Meropenem and Imipenem exhibited antimicrobial activity as (80.8%) and (74.46%), (91.8%) respectively against *E. coli*.

Meropenem and Imipenem drug of choice as antibiotic therapy, exhibited highest sensitivity percentages among the isolates even though, were given intravenously for the control required, these drugs not commonly used for routine UTIs treatment because of economic cost.

Aminoglycosides included different agents such as Gentamicin, Tobramycin and Amikacin. The spectrum of activity, rapid bactericidal activity and favorable chemical and pharmacokinetic properties of aminoglycosides make them a clinically useful class of drugs (Serio *et al*., 2017). The resistance to Aminoglycoside may be occurring through the acquisition or up regulation of genes that encode inactivating enzymes or efflux systems so is due to the inactivation of aminoglycoside modifying enzymes (AMEs; aminoglycoside phosphotransferases, acetyl-transferases,

and nucleotidyl-transferases) by the products of genes in plasmids or transposons (Asghar and Ahmed, 2018).

The higher sensitivity rates for isolates were examined to Amikacin (94.6%) considering these results consistent with the study of Mohajeri *et al*., (2014); Tabasi *et al*., (2015); Tabidehchi *et al*., (2016); Khoramrooz *et al*., (2016); Nikzad *et al*., (2021) who they reported the sensitivity of UPEC to Amikacin which were (100%),(96.8%), (93.4%), (97%) and (96.9%) respectively.

The sensitivity results were at higher rates by *E. coli* isolates that examined to Gentamicin by (69%), in agreement to study conducted by Al-Kuriashy *et al*., (2013) who proved a good efficacy of Gentamicin against *E. coli* by (69%) where different studies by Al-Amri, (2019); Mansouri and Abbasi, (2010) and Ahmed, (2021), recorded the sensitivity to this antibiotic as (71.6%), (65.2%) and (68.1%) respectively. Moreover in another study conducted by Khadum *et al*., (2018) who mentioned that Gentamicin showed a moderate level of resistance (56%) among tested *E. coli*.

Furthermore, *E. coli* isolates were examined for Ciprofloxacin which were sensitive by (55.4%), these results were consistent with the study of Al-Amri, (2019); Mansouri and Abbasi, (2010); Ahmed, (2021), were they found the sensitivity to this antibiotic as (50%), (59%) and (43.2%) respectively but in argument with Nikzad *et al*., (2021) who found the sensitivity as (87.5%). In addition, the study applied in Babylon Government by Hindi *et al.,* (2013) described highly efficiently of Ciprofloxacin that reached (80.7%).

The resistance to Ciprofloxacin were significantly higher in developing countries such as in Ethiopia (85.5%), Pakistan (60.8%), Mongolia (58.1%) by (Munkhdelger *et al*., 2017; Shakhatreh *et al*., 2018) than in developed countries USA (5.1%), Germany (10.5%), Switzerland (17.4%), France(24.8%) by (Erb *et al*., 2018).

The sensitivity rate to β-lactam/β-lactamase inhibitor combinations including Piperacillin-Tazobactam were (71.5%) which consistent with another study result of Sonkol *et al*., (2020) who exhibited sensitivity rate by (61.9%) and lower than the result examined by Tabasi *et al*., (2015) were the sensitivity to Piperacillin-Tazobactam at (94.8%) while the lowest sensitivity recorded by Al-Hasnawy *et al*., (2019) at (38.1%)

The isolates exhibited statistically lower susceptible rates to Tobramycin and Minocycline by (62.5%) and (51.7%), these results quite similar to the study done by Aziz *et al.*, (2017) who found that *E. coli* were sensitive by (58.3%) and (45.9%) respectively and Ahmed *et al*., (2019) were found that (47%) of *E. coli* were sensitive to Tobramycin.

The higher resistance rates to antibiotic were showed by *E. coli* to different antibiotics, this resistance has been increasing dramatically and has turned out to be a serious health concern furthermore, led to emergence of resistant strains has somewhat reduced the potential of antibiotics in empiric therapies this resistance may be due to different causes included inadequate regulations and usage imprecisions, awareness deficiency in best practices which steers undue or inept use of antibiotics (Aslam *et al*., 2018).

The evaluation of antibiotic resistance profile of *E. coli* showed that isolates were resistance to Ticarcillin and Piperacillin (100%) which

approved by the study of Salehzadeha and Zamani, (2018); Allami *et al*., (2022) by (91%) and(92%, 91%) respectively, also, Abaas, (2018), Düzgün *et al*., (2019) were showed that the resistant to Piperacillin were (80%) and (61.9%) respectively.

The resistance to Cefepime were (94.6%), the results in agreement with Raeispour and Ranjbar, (2018) that they found (100%) were resistant and contracted with Ahmed *et al*., (2019) who found that (73%) of *E. coli* were resistance to Cefepime and Kurdi, (2020) who found the resistance to Piperacillin and Cefepime were (78%).

The resistant to Azetronam at (94.6%), this result agreed with Aziz *et al.*, (2017) who found that *E. coli* were resistant (95.8%) and higher than the results of Neamati *et al*., (2015) that found (51.4%) of *E. coli* were resistant to Azetronam.

The Trimethprime-Sulfament is an important and widely used first-line antimicrobial in the treatment of uncomplicated cystitis. The synergistic combination also known as co-trimoxazole (Trimethoprim and Sulfamethoxazole) was inhibiting two sequential steps in the bacterial metabolism of folic acid. The resistance to TMP-SMX was due to widely used as a first choice of treatment for UTIs, since TMP-SMX resistance is associated with the development of concomitant resistance to other antibiotics thereof, limited use of TMP-SMX may help to sustain its effectiveness over the long run (Hamza, 2018).

In current finding, *E. coli* isolates exhibited resistance to Trimethprime-Sulfament with (64.3%), these results were consistent with the study by Naji *et al*., (2017) who pointed that the resistance of *E. coli* to

Trimethoprim- sulfamethoxazole by (68%) and Al-Hasnawy *et al*., (2019) by (69.04%) but higher than Erb *et al*., (2018) were reported in Switzerland at (24.5%) among UPEC isolates resistant.

Concluding that, high resistance were observed in several countries as in Brazil with the rates of resistance (50.6%) by (Cunha *et al*., 2016). the resistance rate to TMP-SXT in India were resistance at (59.6%) by (Prasada *et al*., 2019), Iran (54%) and Ethiopia (68.5%) by (Dadi *et al*., 2018). These results indicate that in many countries TMP-SMZ should not be used in empiric UTIs due to the high frequency of *E. coli* resistant to the antimicrobial.

3.5 Biofilm formation by *Escherichia coli* and antipotential effect of antibiotics on biofilm mass formation:

3.5.1 Biofilm formation by Micro titer plate (MtP) method:

In this study, the strength of biofilm formation were investigated by the Microtiter plate method, it is a quantitative method to determine biofilm production by spectrophotometer using an ELISA reader at a wavelength of 620 nm to give a final digital value representing the quantity of biofilms produced by the bacterial suspension in the wells and considered as a standard quantitative method (Babapour *et al*., 2016).

It was found that most of the isolates were capable to form biofilm, out of 56 isolates, 50 (89.3%) were biofilm mass producers and 6(10.7%) lacked biofilm production capacity. Out of 50, 25(50%) were strong biofilm producers while 19(38%) were moderate biofilm producers, the remaining 6

isolates accounting for (12%) of the total isolates were weak biofilm producers as shown in Figure **(3.2)** shows a chart of MtP results.

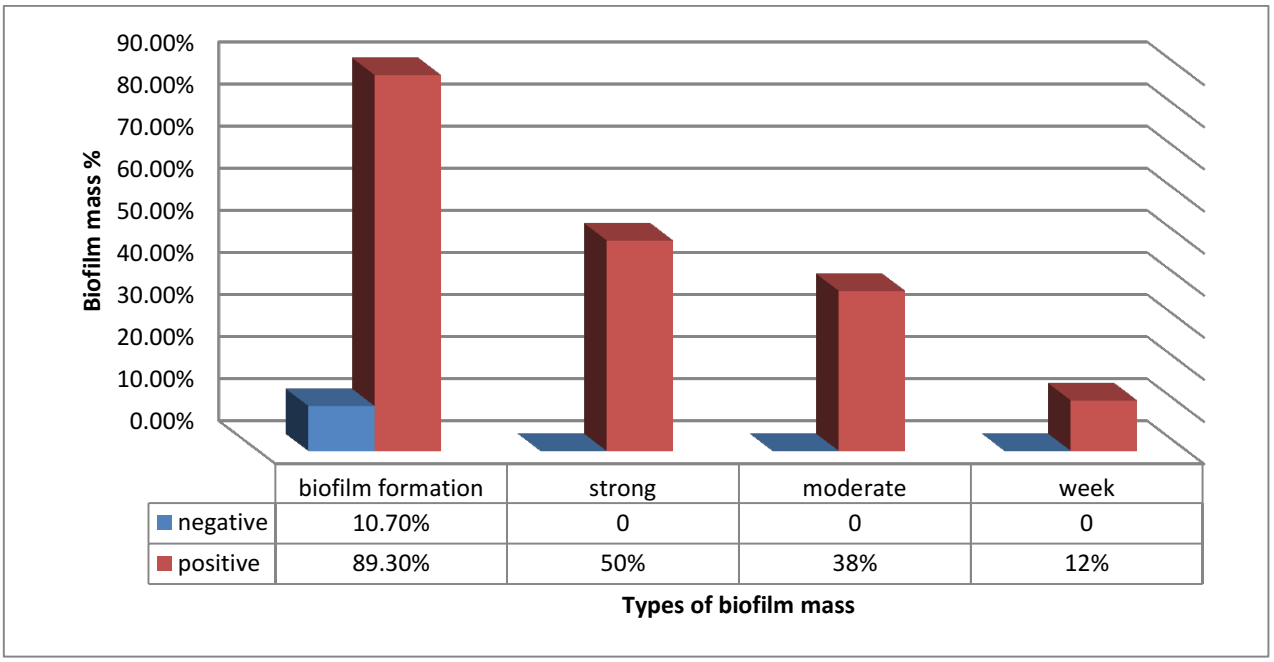

Figure (3.2): Types of biofilm mass formation by *E coli*

Biofilms are structurally and dynamically complex biological systems comprising community of microorganisms that are usually concentrated at solid–liquid interface. These are typically surrounded by an extracellular polymeric substance referred as slime consists of exopolysaccharide, proteins and DNA that facilitate adherence to the abiotic or biotic surfaces and microbial aggregation, also it is an important virulent marker attributed to the development of UTIs by *E. coli* (Miquel *et al*., 2016).

Other studies have shown a similar results when investigate biofilm production, as were found by Gawad *et al,* (2018) and Zaman *et al,*(2019) in Egypt, Al-Saadi, (2019) and Ahmed, (2021) in Iraq, Nikzad *et al*., (2021)

in Iran, who they found that (76.5%), (68%), (90%), (83.3%) and (85.8%) respectively of their isolates were actually biofilm producers and higher than Tadepalli *et al*., (2016) as they found that (35%) of their isolates were biofilm producers.

The results of the study were quit similar to Javed *et al*., (2021) who found that (40%) as strong, (38%) as moderate and (22%) as weak and comparing it with Al-Saadi, (2019) and Ahmed, (2021), were different at (4%), (20%) as strong, (12%), (63.3%) as moderate and (74%), (16.7%) as weak for biofilm production.

3.5.2 Antipotential effect of antibiotics on biofilm formation:

Microtiter plate (MtP) method used for measurement of biofilm produced on walls of wells in response to agent which effect biofilm production. Distinct antibiotics (Amikacin and Ciprofloxacin) were evaluated at different concentrations to assess the possible effect on biofilms. The isolates examined just belong to the group of strong biofilm producers.

The results in Figure **(3.3)** and **(3.4)** showed that *E. coli* were grown in the presence of antibiotics, inhibition of biofilm formation was observed. In the presence of Amikacin the optical density of the biofilm were decreased and the biofilm mass formation were inhibited began at (64 µg\ml) reaching to highest (1024 µg\ml) concentration in comparison with growth without antibiotic. Also, the exposure of bacteria to Ciprofloxacin resulted in reduction of biofilm mass formation began at (128µg\ml) reaching to highest (1024 µg\ml) concentration. The antibacterial effects of antimicrobials were observed after (48 hrs.) incubation considering that the irreversible phase of

biofilm occurs after (24–48 hrs.) in other uropathogens (Schlapp *et al.,* 2011).

Biofilms formation processes use multiple strategies to resist antibiotics, including extracellular appendages, efflux pumps, quorum sensing and an impenetrable extracellular matrix (ECM) which acts as a physical and chemical barrier to the penetration of antibiotic, anti-biofilm drugs must overcome all these barriers and achieve broad-spectrum activity against both planktonic and biofilm-forming *E. coli* cells without adverse effects (Koo *et al*., 2017; Kalsy, *et al*., 2020).

Antibiotic resistance to biofilm due to several mechanisms like a limitation of antibiotic diffusion through the matrix, horizontal transmission of resistance genes, inactivation of the antibiotic by changes in metal ion concentrations, pH values and the metabolic inactive bacterial status. The level of resistance depends on the biofilm formation stage, being more susceptible bacteria in the initial reversible step (Soto, 2014).

Amikacin and Ciprofloxacin both have reduction on biofilm formation confirming that antibiotic treatments depend on concentration level which could modulate the community structure and heterogeneity of *E. coli* biofilm. The group of aminoglycosides (Amikacin) can inhibit protein synthesis acting on the bacterial ribosome, so this may explain an inhibitory effect on the biofilm synthesis while the fluoroquinolone (Ciprofloxacin) inhibit DNA replication and able to penetrate host cell membranes and accumulate intracellularly.

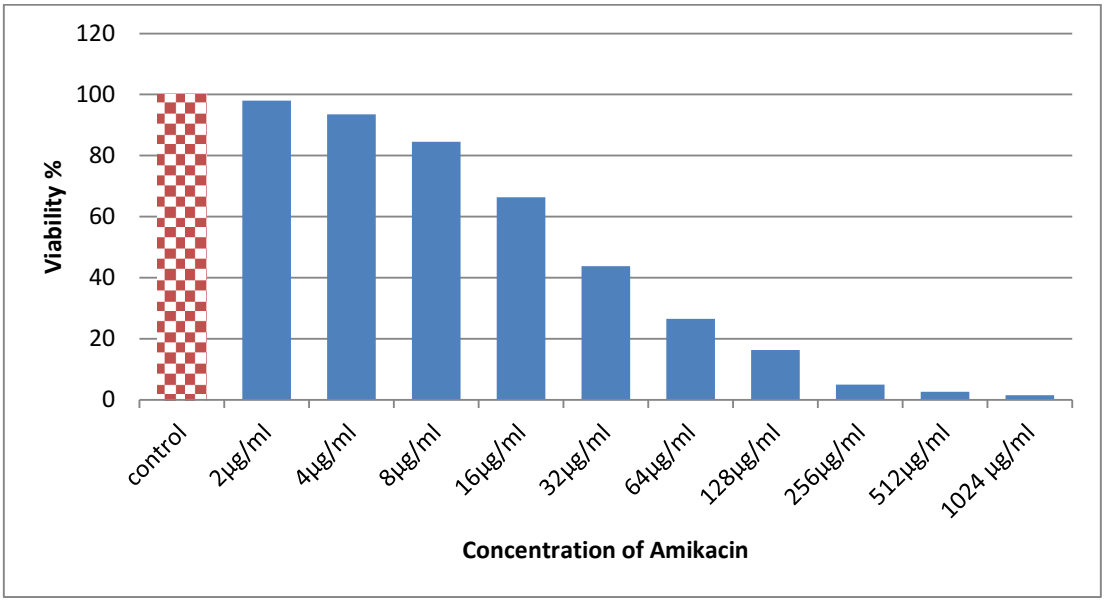

Figure (3.3): Effect of different Amikacin concentrations on biofilm mass formation

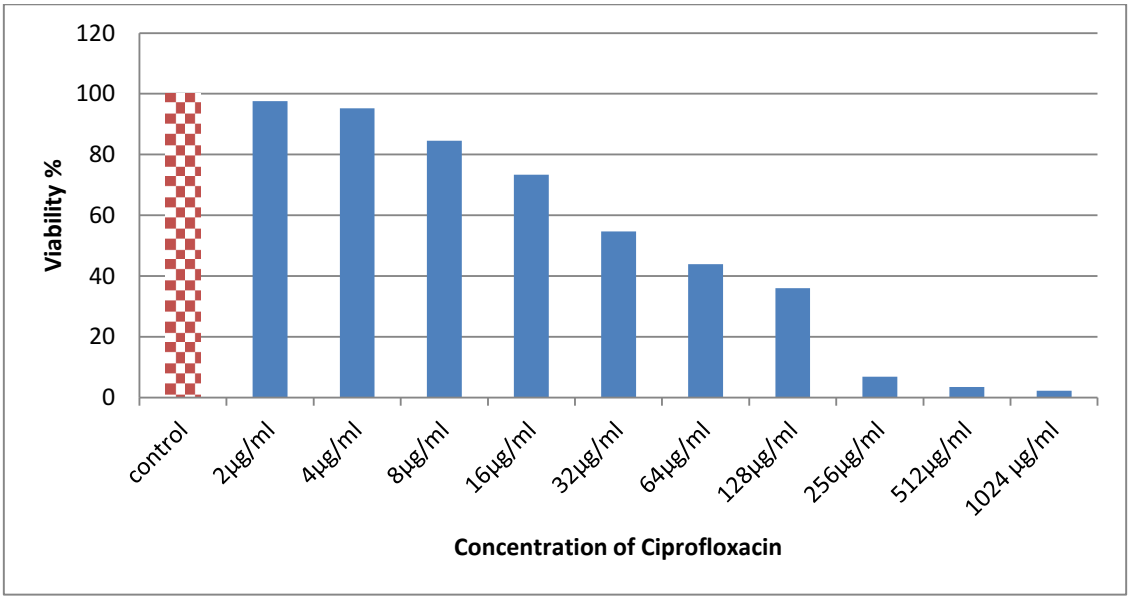

Figure (3.4): Effect of different Ciprofloxacin concentrations on biofilm mass formation.

Bret and Di Martino, (2004) and Boehm *et al.*, (2009) were shown that the concentrations of various antibiotics have been shown to both stimulate and/or impede *E. coli* biofilm formation in human infections.

Blango and Mulvey, (2010) studied the effect of different antibiotics on biofilm *in vitro* and their efficacy to eradicate biofilm, they observed that Ciprofloxacin had an inhibitory effect on biofilm persistence and promoted the reduction of pre-existing biofilm communities.

Similar results were also obtained by other researchers as Wojnicz and Tichaczek-Goska, (2013) were showed that Amikacin were the more effective antibiotic than Ciprofloxacin in eradication of biofilm, which is approved by our findings.

Moreover, González *et al*., (2017) were study the effect of some antibiotics on UPEC biofilms and found that the antibiotics, including Cephalothin, Ceftriaxone, Ceftazidime, Amikacin and Ciprofloxacin were evaluated at different concentrations after (48hrs) of biofilm formation, these antibiotics induced a significant reduction of biofilm biomass, in the case of recurrent UTIs potentially associated with the presence of biofilm, the use of third-generation cephalosporin, fluoroquinolones and aminoglycosides could be recommended and demonstrated to reduce biofilm biomass produced even by resistant strains.

3.6 Genetic detection of toxins:

3.6.1 Molecular characterization of alpha hemolysin gene (*hlyA* gene):

In the present study, PCR assay was done to detect the presence of specific *hlyA* gene as a virulence factor gene in *E. coli* isolates. The study revealed that the frequency of *hlyA* gene in UPEC isolates were detected in 21(37.5%), the positive results were detected by the presence of (1177 bp) molecular weight when compared with allelic ladder as shown in Figure **(3.5)**. Ten of current isolates were recorded in the gene bank with the accession number ON112290, ON112291, ON112292 ON112293 ON112284, ON112285, ON112286, ON112287, ON112288 and ON112289. These results similar to the results of Tiba *et al*., (2008) and Yazdanpour *et al*., (2020) who they found that the percentage of *hlyA* gene were (25.3%) and (26%) respectively in UPEC.

A local study by Abdul-Ghaffar and Abu-Risha, (2017) were recorded that prevelance of *hlyA* were 22(51.16%) in a total, 13(59.09%) in non-catheterized patients isolates, 9(42.85%) in catheterized patients isolates comparing it to other countries obtained by Aazam *et al*., (2012) and Shahbazi *et al*., (2018) who found that, the ratio of the presence of *hlyA* gene in UPEC at rate (41.7%) and (50.4%) respectively.

In contrast a high percentage was recorded among the *E. coli* isolates from UTIs patients, the prevalence of the *hlyA* were (96%) and (90.8%) by Santo *et al*., (2006); Moeinizadeh and Shaheli, (2021) with low percentage was recorded by Abaas, (2018) who found the ratio of the presence of this gene in *E. coli* was (16.6%). In phenotypic detection of hemolysin production, not all isolates carrying *hlyA* gene expressed alpha hemolytic

activity, There are 21/56 isolates have *hlyA* gene but only 16/21 (76%) of isolates expressed alpha hemolysis on blood agar, these results were agreement with Hussein, (2022).

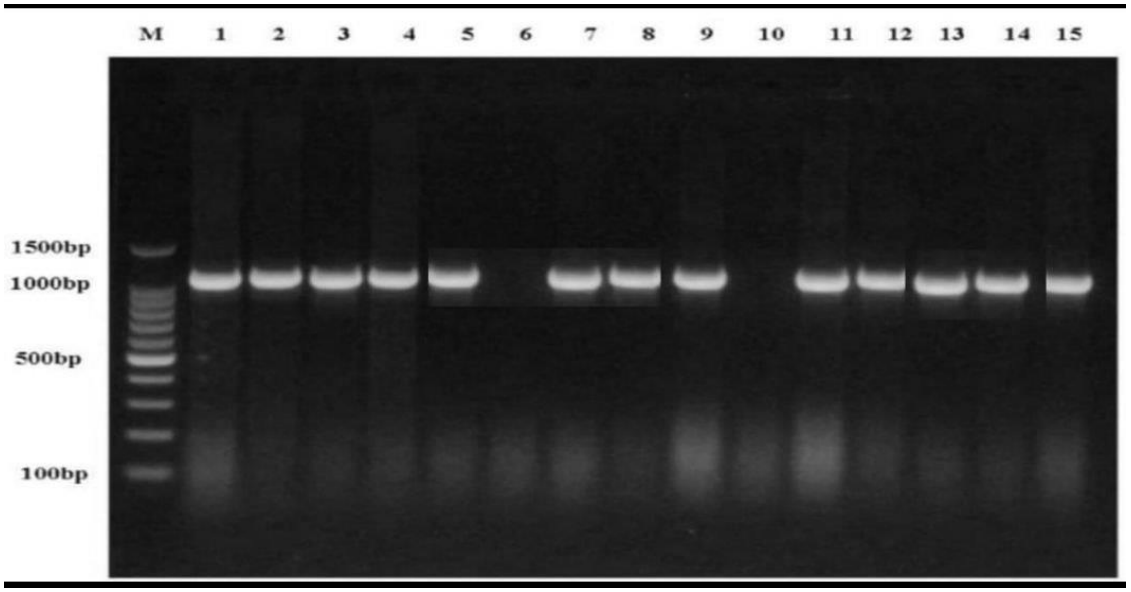

Figure (3.5): Ethidium bromide agarose gel electrophoresis for ampilification *hlyA* gene in *E. coli* by 1177 bp (80 volt, 80 min). Lane M: DNA ladder. Lanes 1,2,3,4,5,7,8,9,11,12,13,14,15 represent the identified *hlyA* gene

Hemolysin is an important virulence factor of many types of pathogenic bacteria, where helps the bacteria to survive (Raeispour and Ranjbar, 2018). HlyA disrupts cell adhesion, triggers urothelial cell death and induces inflammatory cytokines from epithelial cells or monocytes via cell signaling pathways at low concentrations (Dhakal and Mulvey, 2012). As well as, it is reported to induce kidney inflammation, injury and a higher percentage of HlyA-positive strains were isolated from pyelonephritis patients (> 70%) than from cystitis patients (31–48%), implying that HlyA is an important virulence factor in pyelonephritis (Wiles and Mulvey, 2013).

PHYML were used to create a phylogenetic tree of *Escherichia coli* strains from the alignment sequences of ten *hlyA* genes matching to 13 strains of *hlyA* genes from NCBI (Appendix VII). As it is known to be basal in the *E. coli* phylogeny, the tree has been rooted to five phylogroups. When the bootstrap value is more than 93 percent, it is reported at the nodes (500 replicates).

On the right of the picture, the main phylogenetic groups of *E. coli* species were displayed. The remaining strains were chosen as representative of *E. coli* phylogenetic diversity, as shown in the Figure **(3.6)**, with the Iraqi strain marked as a traingle, seven of the *hlyA* genes clustered together and the other separated by other international strains, revealing that the effect of the hemolysin gene varies greatly between local and international strains. Therefore, our study pointed to the number of mutation that happen with *hlyA* gene which were in agreement with the research revealing a mutation in *rfaC* that altered both *hlyA* extracellular expression and activity were examined in greater depth.

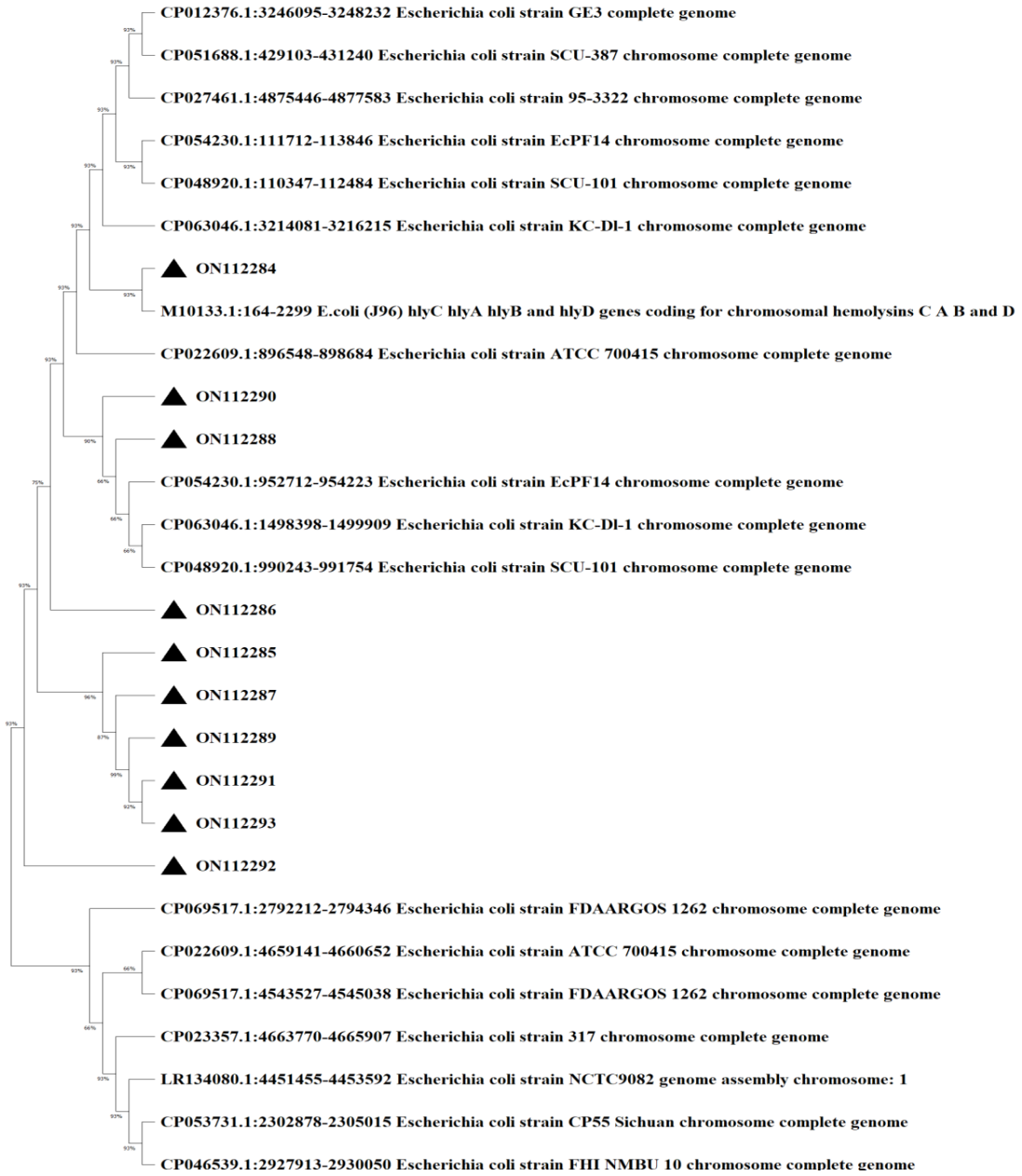

Figure (3.6): Phylogenetic Tree for *hlyA* gene of *Escherichia coli* constructed by Maximum likelihood Method with nucleotide sequences of 13 reference isolates from Gen Bank. Current isolates are indicated with black triangle

This mutation resulted in a growth-phase-dependent decrease in the stable level for extracellular *hlyA* that really was up to 16-fold lower, despite the fact that transcription plus release of *hlyA* were only reduced by a factor of two. Specific hemolytic activity in the *rfaC* mutant strain toxin was dramatically reduced in a growth-phase-dependent way (Bauer and Welch, 1997; Nhu *et al*., 2019).

Both the reduced expression and activity of *hlyA* were recovered to wild-type levels when the *rfaC* gene was provided intrans, *hlyA* from the *rfaC* mutant strain had substantially slower hemolysis kinetics, a faster rate of activity decay and higher production of apparently inactive *hlyA*-containing aggregates in culture supernatants than the wild-type strain. A model for a physical interaction between LPS and HlyA is developed in which LPS with an intact inner core participates (Bauer and Welch, 1997; Hu *et al*., 2021).

Similarly, the rate of HlyA secretion have different scenario such as HlyA is destroyed more quickly in the mutant's liquid culture media than in the wild-type strains. As a result, LPS may help to protect the released HlyA protein from destruction. Early log growth activity produced by cultures was not significantly different from wild-type activity. As growth continued, however, levels of hemolytic activity declined considerably (Stanley *et al*., 1993).

A number of mutations in various positions on the *hlyA* gene Figure **(3.7)** shows, which could explain why some strains do not display its activity while genetically present in bacteria. The explanation for the disparity in isolate percentages expressing *hlyA* could be attributable to variances in the

size and number of hospitals examined, as well as the season of sample collection and medicine taken prior to sampling.

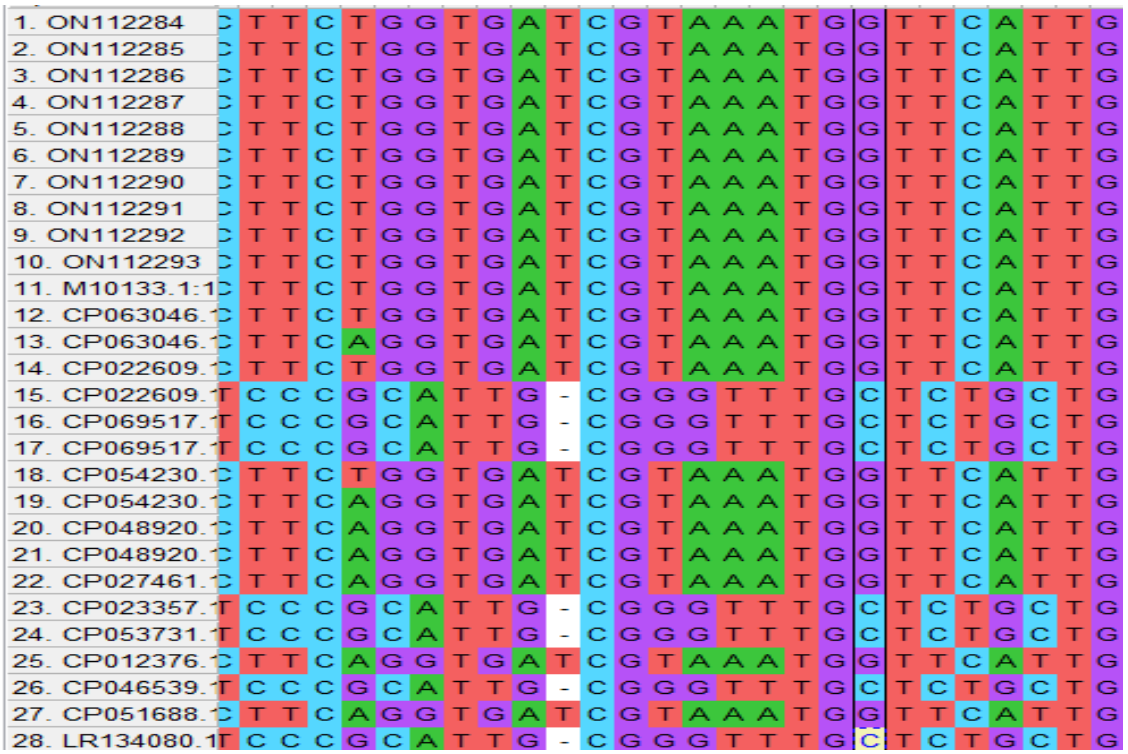

Figure (3.7): Alignment of *hlyA* gene of *E coli* with the referenced genome from NCBI (gene bank).

3.6.2 Molecular characterization of Secreted Autotransporter gene (*sat* gene):

The molecular detection of the *sat* gene by PCR technique has been done for isolates that previously detected as UPEC. The results showed that, out of 56 isolates 35(62.5%) gave positive results for this toxin gene. Positive results were detected by the presence of (410bp) molecular weight when compared with allelic ladder as shown in Figure **(3.8)** and five of current isolates were recorded in the gene bank with the accession number

ON777812, ON777813, ON777814, ON777815 and ON777816. Our finding were in between to the results obtained by Guyer *et al*., (2000) whose found (55%) pyelonephritis isolates but only (22%)fecal isolates carried the *sat* gene, Maroncle *et al*., (2006), found that *E. coli* strains associated *sat* gene with the clinical symptoms of acute pyelonephritis (68%) of strains than by fecal strains (14%). In addition to AL-Hayali *et al*., (2019), indicate the frequencies of *sat* gene were (57.5%) in UPEC isolates, Takezaki *et al*., (2019) who found that *sat* gene frequencies ranging between (25 to 70%) while the less frequency observed by Freire *et al*., (2020) who found that, *sat* gene were the most frequent gene (34.2%).

These studies indicated that detection of *sat* gene in UPEC pathotype is very important and could be an indicator for set-up a new diagnostic method. Moreover, it shows that *sat* gene has a potential value for selecting as one of candidate antigen for designing new vaccine against UTIs (Saraylu *et al*., 2012; AL-Hayali *et al*., 2019). Further to this, *sat* gene is among the most frequent SPATE-encoding genes found in UPEC, high frequency of *sat* gene in *E. coli* strains isolated from bacteremia and the cytotoxic effects of Sat on endothelial and urinary tract cells suggest that this SPATE may be involved in a different steps of bloodstream infections (BSIs) and sepsis pathogenesis (Freire *et al*., 2022).

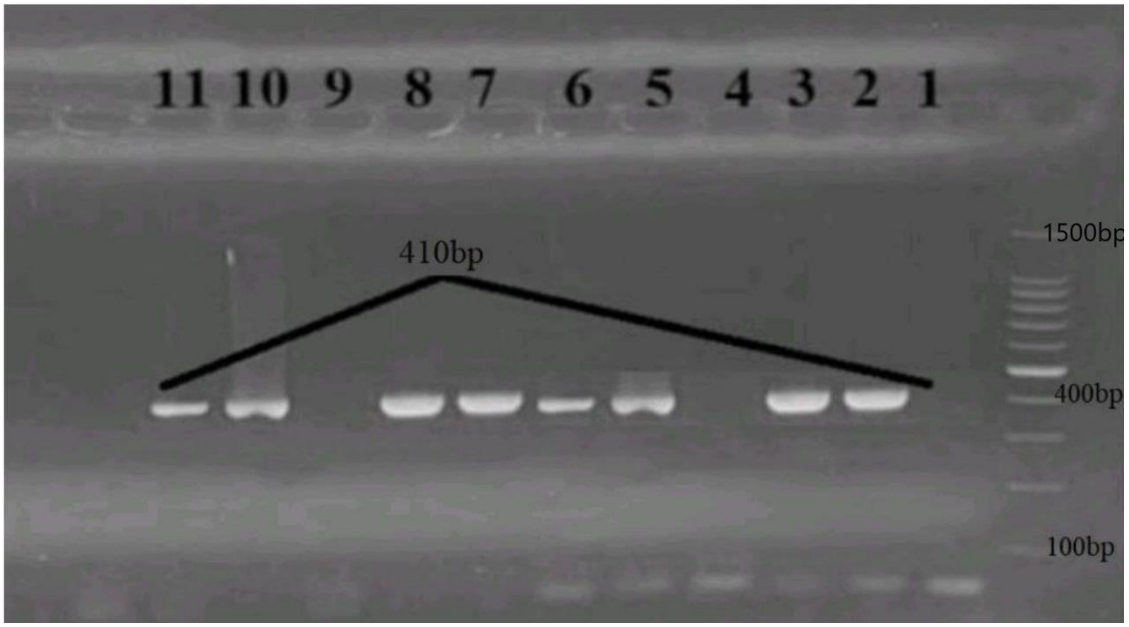

Figure (3.8): Ethidium bromide agarose gel electrophoresis for ampilification *sat* gene in *E. coli* was identified via PCR detection of 410 bp (80 volt, 80 min). Lane M: DNA ladder. Lanes: 2,3,5,6,7,8,10,11 represent the identified *sat* gene.

Previous study demonstrated the essential effect of *sat* gene which is the 107-kDa autotransported serine protease of *E. coli* as it elicits cytopathic effects on human bladder, human kidney and vero kidney epithelial cells (Tapader *et al*., 2019). The cytopathic activity displayed on various cell lines due to the association of the *sat* gene with its product with uropathogenic strains, the pathogenicity of *E. coli* were determinate by autotransporter protein SAT toxin contributing as a virulent factor. This result were supported by study revealed the ability of SAT toxin to elicit strong antibody response (inflammation or cytopathic activity) during experimental infection in the cross bag albino (CBA) mouse model of ascending UTIs (Habouria *et al*., 2019).

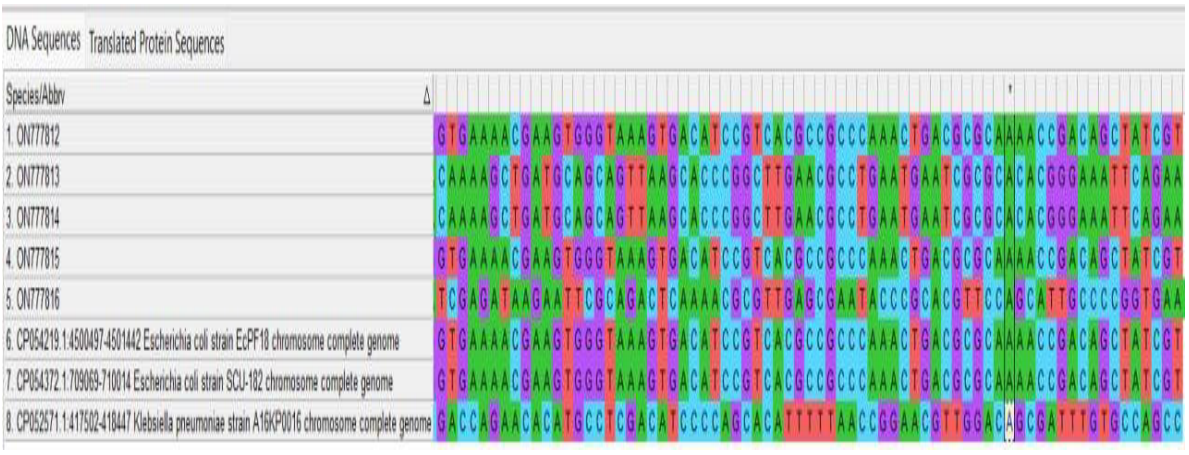

Figure (3.9): Alignment of *sat* gene of *E. coli* with the referenced genome from NCBI (gene bank).

The isolates for *sat* gene of *E.coli* were aligne using the MUSCLE algorithm (Mega 6) for both lacal and international isolates from gene bank (Appendix VIII). The current isolates indicate highly number of mutation were represented as different types of mutations as showen in the Figure **(3.9)**. Intristingly, high frequencies were presnt in the *sat* gene represented by high number of deletion so that gene activity will be affected for example genetically will be coded but phenotypically absent. However, mutation in the bacteria are becoming specialist to the enviroment they are living in, they accumulate mutation that may be deleterious or lethal in secondary enviroment, this evolution on the mutation rate have a major effect on bacterial population (Giraud *et al*., 2003). Phenotypes and genotypes of bacterial strains with clinical disease have been used to determine the relationship between Sat production and virulence characteristics of uropathogenic isolates. Virulence factors that contribute to an organism's pathogenicity are determined by the frequency of various mutation produced by organisms linked to clinical UTI symptoms (Sarowska *et al*., 2019). Sat ability to cause vacuolization inside the cytoplasm of human cell lines from

the urinary tract is a surprising discovery, given that no other known vacuolating toxin of *E. coli* strains isolated from people exists (Guyer *et al*., 2002). So that, our study pointed on the diffrences present in the gene due to mutation which could cause change in vacuolating toxin of *E. coli* strains.

Phylogenetic tree for *sat* gene of *E coli* as shown in Figure **(3.10)** that only three local strains were clustered together even thought, number of mutation present because of ON777816 (12%) were different however, this strain were similar to CP052571 in (52%). Other two strains were clustered separately to international strain. These differences for the local strain revealing the effect of mutation that observed in the alignment Figure **(3.9)**. The findings were remarkably similar to research showing that variations in tissue enterotoxic and cytotoxic activity were caused by genetic mutation. Also, reporting that Sat enterotoxic and cytotoxic activity in tissue. These are significant findings, even thought intriguing because up until this point, Sat cytotoxin was only demonstrated in bladder and kidney tissues of mouse model assays, were significant finding discoveries with in raise interesting questions because Sat cytotoxin was previously only found in animal model studies of the bladder and kidney tissues (Guyer *et al*., 2002).

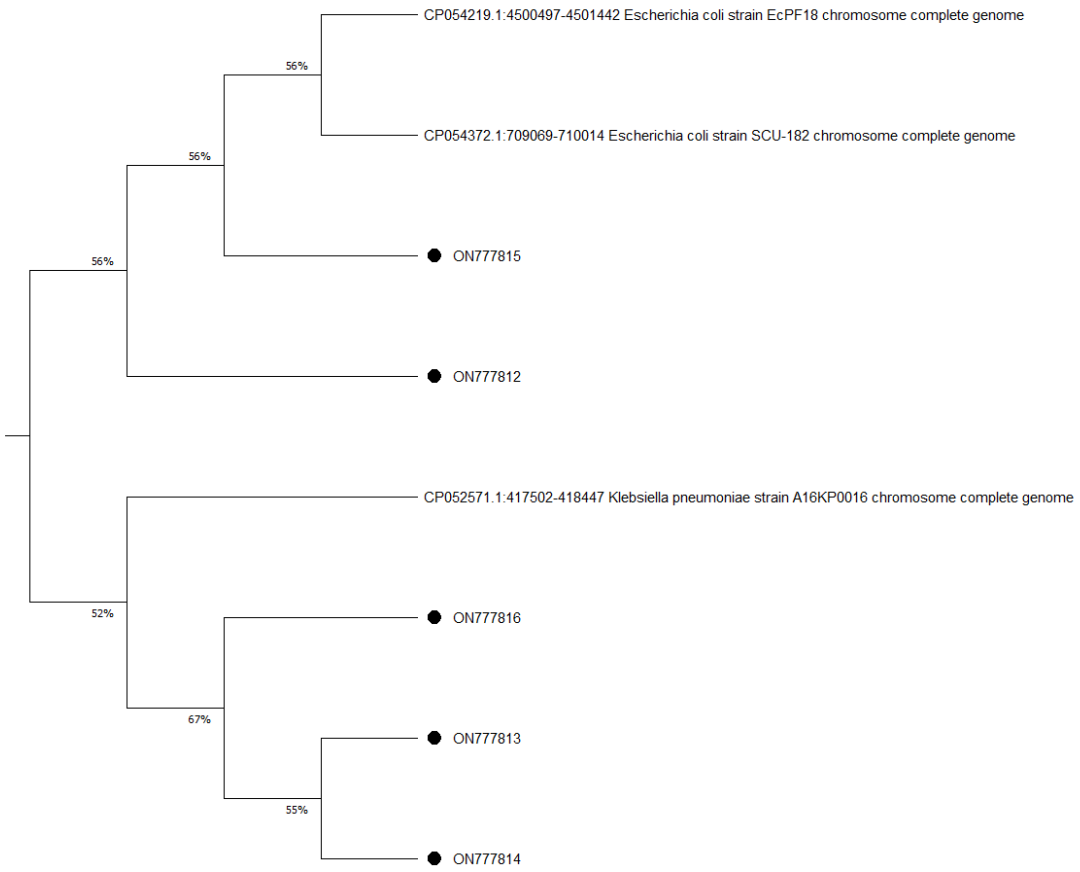

Figure (3.10): Phylogenetic Tree for *sat* gene of *E. coli* constructed by UPGMA Method with nucleotide sequences of 3 reference isolates from gene bank. Current isolates are indicated with black circle.

Genetic variation for the five isolates were examined as showed in **Table (3.4)** with the Identity an average between (98% - 93%) this variation accounted with the single nucleotide (SNP) with the minority change on the gene compared to the second isolate were found three SNPs were present on genom which is effect on the genom as showed with (95%) of identity regarding to that number of mutations. Similarly, with the third isolate three SNPs were found as well as the identity (95%). However, the same identity (95%) for fourth isolate with one (SNP) this result revealing that the locus of

mutation very important regarding to the identity. Finally, the result of fifth isolate with the genetic varation with four SNPs with (93%) of identity this result could revealing that the SNPs were present on the effective part of the gene (because of SNPs come silent sense or non silent sense). To the best of our knowledge, there is no information on the genetic variation of *sat* gene in UPEC.

Table (3.4): Genetic variation for the *sat* gene

No.	Isolate	Identity (%)	Accession Number	Genetic Variation
1	***E. coli***	98%	**ON777812**	A----T
2	***E. coli***	95%	**ON777813**	G----C G----A C----T
3	***E. coli***	95%	**ON777814**	G----C G----A G----T
4	***E. coli***	95%	**ON777815**	G----A
5	***E. coli***	93%	**ON777816**	G----T T----C A----G T----C

3.6.3 Molecular characterization of Cytolethal distending toxin:

Molecular detection of *cdtB* gene were done for isolates that previously detected as UPEC. The results showed that, none of the isolates gave positive results for this toxin gene as a *cdtB* gene were not detected by gel electrophoresis.

The *cdt* operon contains three adjacent genes, *cdtA, cdtB* and *cdtC* and expression of all the genes is necessary for maximum toxin activity while *cdtB* acts as an active subunit with DNase I activity, *cdtA* and *cdtC* facilitate binding of CDT to identified receptor molecule on susceptible cells and entry of *cdtB* into the cytoplasm. The *cdt* gene causes irreversible inhibition of cell cycle at the G2 /M and produce single nuclear giant cells (Peerayeh, 2018).

In general, extra-intestinal infections caused by strains that are transitorily present in the faecal microbiota and bear specific groups of genes encoding virulence factors. If this type of strains colonizes the perineum, they may ascend the urethra and colonize the urinary tract, thus causing disease (Abe *et al*., 2008). The results were in agreement with the investigations done in Iran Mirzarazi *et al*., (2015); Hozzari *et al*., (2020) and Zermina *et al*., (2021) were they found that none of UPEC isolates have *cdtB* gene. However, the study done by Johnson and Stell (2000); Peerayeh, (2018) were found (8% and 18%) of the uropathogenic isolates were *cdtB* positive regarding *cdtB* as possible ExPEC virulence factor. The previous study have been mention that the presence of gene were found in the bacteria were isolated from intestine (Hinenoya *et al*., 2009; Kim *et al*.; 2009).

In first time *cdt* producing *E. coli* were observed in relation to children with enteritis (Bielaszewska *et al*., 2009), while in other studies, *cdt* gene was observed in strains isolated from urosepsis *E. coli* and fecal *E. coli* and in patients with various symptoms such as diarrhea, encephalopathy (Timothy *et al*., 2008).

3.6.4 Association between genetic diversity of toxins and Uropathogenic *Escherichia coli* biofilm production:

Among 21(56) *E. coli* isolates positive for *hlyA* gene were positive for biofilm production by 20/50 (40%) and 1/6(16.6%) isolate was not former biofilm, among 35(56) *sat* gene positive *E. coli* isolates were subjected to biofilm production, 33/50(66%) isolates from former biofilm isolates while 2/6(33.3%) from not former biofilm isolates. The results were non-significant relationship were found between presence of the (*hlyA* and *sat*) genes and biofilm formation in *E. coli* isolates (P<0.162) and (P<0.114) respectively as shown in Table **(3.5)**.

Table (3.5): Relationship between biofilm formation and UPEC expression toxins

Gene	Biofilm production types			Non biofilm producer N=6	P *Value* (p<0.05)
	Strong N=25	Moderate N=19	Weak N=6		
hlyA	13(52%)	4(21%)	3(50%)	1(16.6%)	0. 114
sat	20(80%)	10(52%)	3(50%)	2(33.3%)	0.162

The prevalence of these toxins genes in former biofilm isolates were high prevalence than in non-former isolates which proves that the biofilm-forming bacteria are more pathogenic and that the formation of biofilms allows the bacteria to persist for long periods in the urogenital tract and causes an increase in the rate of bacterial virulence and the severity of the disease which leads to the difficulty of treating UTIs. Toxins assist UPEC in deeper spreading within the host tissues after damaging cell integrity,

gaining access to nutrients within the host cell or suppressing the host immune response and/or evading their antibacterial action (Arafa *et al*., 2022).

In a study by Soto *et al.,* (2007), biofilm-producing UPEC isolates which causing inflammation of prostate were often the highest hemolysin-containing isolates and study by Naves *et al*., (2008), found papC and hlyA were involved in strong biofilm production being more common at ($p<0.05$). Also, in a study conducted by Farshad *et al*., (2012); Tarchouna *et al*., (2013) and Fattahi *et al*., (2015) they found the expression of *hlyA* gene were (15.6%), (19%) and (27%) respectively.

Ballesteros *et al*., (2021) found *hlyA* and *sat* genes were (38%) and (40%) respectively in biofilm former isolates suggesting highly pathogenic UPEC which are potentially capable of causing UTIs, evading the host immune system, resisting antibiotic treatment, persisting in the urinary tract, causing recurrent UTIs and have potential to induce renal damage, gain access to the bloodstream and cause bacteremia.

However, Sat toxin not considered as colonization factor for the urinary tract so that, Sat toxin elicits just cytopathic activity which cause damages to the host tissue and lead to increasing the ability of *E. coli* to propagate. Sat cytopathic behavior suggests that it may be a significant role in *E. coli* pathogenicity in the urinary system. Centrally, suggested that one reason to speculate that specific damage to glomeruli and proximal tubules which could facilitate entry of pyelonephritogenic strains into the bloodstream (Sarowska *et al*., 2019).

3.7 Extraction and Partial purification of alpha hemolysin by Gel filtration Chromatography:

The partial purification of alpha hemolysin from crude of bacteria in Luria Bertani broth media were done by Gel filtration chromatography (Appendix IX), the active fractions were collected and applied on sephadex-200 column because the toxin have high molecular weight 110 KDa, absorbance of hemolysis at 412nm were measured, the activity of hemolysis increased in different stages of purification reaching to (1.76) at 412 nm, the activity of alpha hemolysin after dialysis increased when compared with activity before dialysis this step is important for purification as reported by (Khwen, 2021) as showed in Table (3.6).

Also, The enzyme activity, specific activity and protein concentration of alpha hemolysin toxin were measured and shows that there was an increase in the enzyme activity were (100 U/ml) and specific activity (5 U/mg) with decreasing in the other proteins concentration (20 mg/ml), the fraction which have activity hemolysis were pooled and concentrated from the fractions with numbers (24-40) these fractions were frozen at −80°C and lyophilized as shown in Figure **(3.11)**. The lyophilized pellet was re-suspended in D.W. to use in other experiments. The specific activity of toxin were very important than enzyme activity which included other different proteins in the crude mixture. Gel-filtration chromatography is a form of partition chromatography used to separate molecules of different molecular sizes. It is the simplest and mildest of all the chromatography techniques and plays a key role in the purification of enzymes, polysaccharides, nucleic acids, proteins and other biological macromolecules (Ó'Fágáin *et al*., 2017). The

purification of alpha hemolysin toxin from *E. coli* and other bacteria were reported such as from *Stachybotrys chartarum* by (Vesper *et al*., 2002), *S. aureus* by (Banimuslim, 2009) and (Al-Shammary *et al*., 2012), *Eikenella corrodens* by (Mansur *et al*., 2017) and from *E. coli* by (Razzaq *et al*., 2011) and (Wang *et al*., 2020).

Table (3.6): Partial purification of *alpha* hemolysin toxin from *E. coli*

Purification step	Absorbence at 412nm	Total volume (ml)	Enzyme activity (U/ml)	Protein Conc. (mg/ml)	Specific activity(U/mg)	Total activity (U)
Crude	0.61	100	48	60	0.8	4800
dialysis	0.79	80	64	50	1.28	5120
Gel filtration	1.76	40	100	20	5	4000

Specific activity= enzyme activity (U/ml)\ protein concentration (mg/ml)

Total activity (u) = enzyme activity (U/ml) × volume (ml)

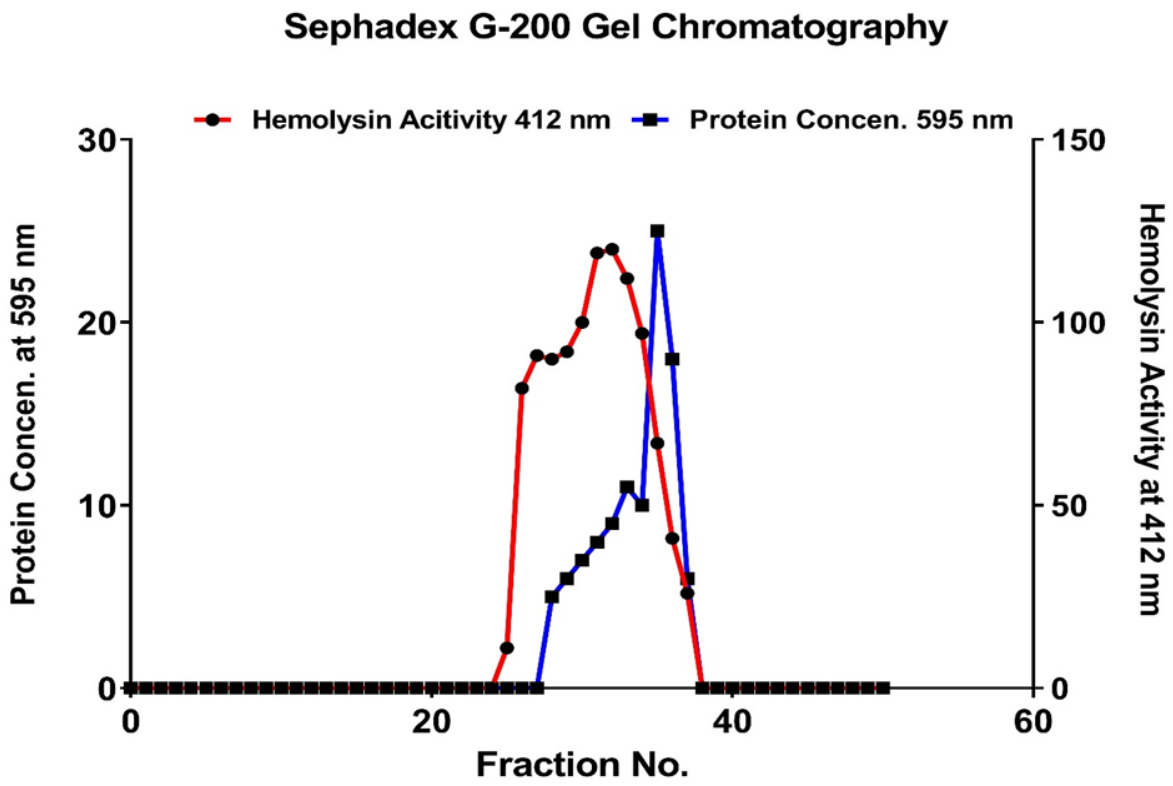

Figure (3.11): Gel filtration chromatography, alpha hemolysin purified from *E. coli* by using Sephadex G-200 column (2x35) cm

3.8 The effect of purified alpha hemolysin toxin and Ciprofloxacin on normal and cancer cell

3.8.1 The effect of alpha hemolysin and Ciprofloxacin on Kidney (Vero cell) cells prolferation:

After partial purification of alpha hemolysin by Gel-filtration chromatography from bacteria. Firstly, Vero cells were incubated with alpha hemolysin alone and another Vero cells were incubated with Ciprofloxacin alone, secondly combination between toxin and Ciprofloxacin were applied

at 37ºC for 24 hours with different diluted concentration starting from the lowest one (31.25 µg/ml) and reaching to the highest concentrations (1000 µg/ml). The results showed there were a significant decrease in kidney cells viability percent at ($p \leq 0.001$) in the higher concentrations for both toxin and Ciprofloxacin at (500 and 1000µg/ml) concentrations and with combination of (toxin and Ciprofloxacin) in (250, 500 and 1000 µg/ml) concentrations in comparison with control as showed in Figure **(3.12)**. Regarding to the finding similar action activity were found in both toxin and Ciprofloxacin whereas, in combination have a high effect starting from (250 µg/ml) concluding that one act as a suppressor and inhibitor.

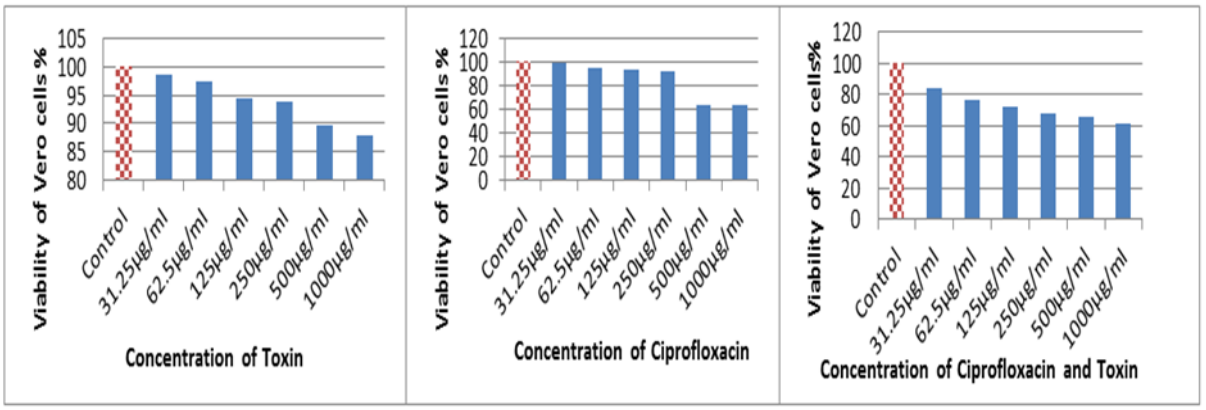

Figure (3.12): Normalized cells viability percentage of Vero cells at different concentrations of toxin, Ciprofloxacin and combination

HlyA damages the kidneys and increases macrophage accumulation so the previous investigation revealed effect of HlyA in kidney infection. Another causes necrosis, tubular casts and severe bleeding were observed in renal papillae infected with HlyA, innate immune cells such as neutrophils and macrophages were found in kidney tissues of mice infected with UPEC (Wang *et al.*, 2020). While there was no difference in infiltrating

neutrophils, significantly more macrophages were found in the HlyA group compared to the group Ciprofloxacin and no statistically significant change in bacterial titers in kidneys was reported, these findings suggest that HlyA causes kidney damage and increases macrophages during acute kidney infections, regardless of bacterial titers at the time of observation (Spencer *et al.*, 2014).

In the study of Wang *et al*., (2020) who they found that HlyA increases the mRNA and protein levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted by renal epithelial cells were up regulated by HlyA in vitro and in vivo which induced M1 macrophage accumulation in kidney. In vitro studies have shown that HlyA leaky gut phenomenon allowing the entry of luminal antigens into the sub mucosa which induce inflammation and subsequently cytokine-accelerated epithelial barrier disruption, these effects were assigned to calcium (Ca^{2+}) signaling in the epithelial cells since the HlyA pores in the host cell membrane mediate Ca^{2+} influx through the pores (Wiles *et al*., 2008; Bücker *et al*., 2014; Wiegand *et al*., 2017).

The pore-forming toxin α-hemolysin from *E. coli* did not simply induce cell death in intestinal epithelial cell but rather has a complex effect on host cell regulation, contributing to various pathologies including leak flux barrier dysfunction with focal leak formation potentiating antigen influx and the leaky gut phenomenon, propagating mucosal inflammation as well as tumorigenesis and metastasis (Schulz *et al*., 2021).

Ciprofloxacin is a second-generation of Fluoroquinolones that used to treat some infections such as severe sepsis, pneumonic plague, acute cystitis,

urinary tract infections and effective against other diseases as malaria, cancer and AIDS (Hooton *et al* ., 2012; Castro *et al* ., 2013; Apangu *et al* ., 2017) which inhibited bacterial DNA replication by disruption of the DNA topoisomerase and DNA-gyrase enzymes (Fief *et al*., 2019; Pham *et al*., 2019).

Tirumala and Roberts, (2018) showed in their study that Ciprofloxacin-induced acute kidney injury have proposed multiple mechanisms including interstitial nephritis and rhabdomyolysis or crystallisation within the renal tubules causing intra-renal obstruction.

3.8.2 The effect of alpha hemolysin and Ciprofloxacin on Colon cancer (SW) cells proliferation:

The significant results were observed in decline of cells viability percent at ($p \leq 0.001$), in all experimental groups (toxin, Ciprofloxacin and in the combination) starting from high concentrations (500 and 1000µg/ml) as showed in Figure **(3.13)**.

Colorectal cancer is the third most common cancer and has become a public health problem worldwide (Khalyfa *et al*., 2021). Carcinogenesis of colorectal cancer has been linked to *E. coli* were produce alpha hemolysin which cytotoxic to colon epithelial cells. The cultured cells are quickly killed by alpha hemolysin *in vitro* while *in vivo* environments support continuous repairmen of colonic tissues so that alpha hemolysin is carcinogenic to colonic tissues but is not as potent as to inhibit tumor development. However, *E. coli* HlyA directly initiate intestinal barrier dysfunction contributing to the diarrhea driven by a leak flux mechanism were mentiond above (Jin *et al*., 2016).

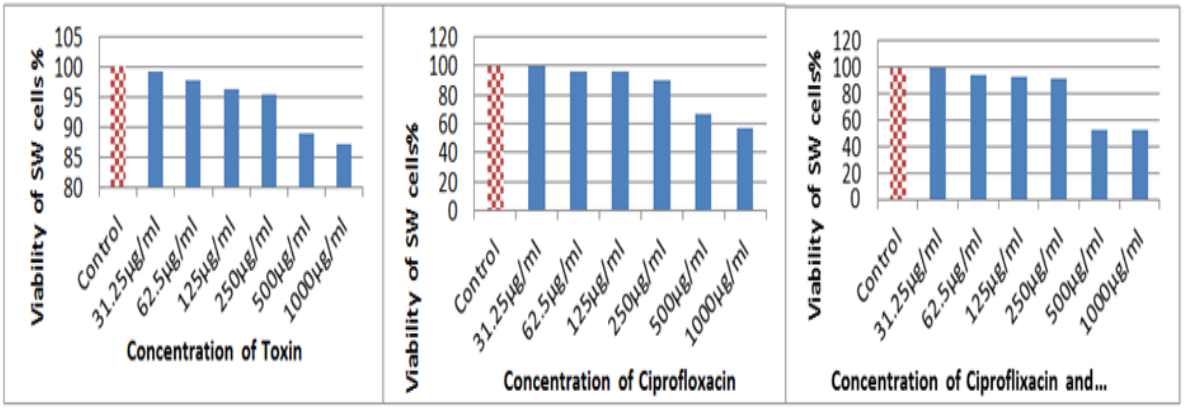

Figure (3.13): Normalized cells viability percentage of SW cells at different concentrations of toxin, Ciprofloxacin and combination

Ciprofloxacin have the ability to inhibit the proliferation of eukaryotic cell *via* mitochondrial DNA damage and reacts with the mitochondrial topoisomerase II isoform, CIP-induced cytotoxicity in cells may be also a result of free radicals generation. These properties resulted in its pro-apoptotic effects on mammalian tumor cell lines including human cells, *in vitro* anti-proliferative and apoptosis inducing activities of CIP on human prostate cancer cell lines were observed at non-clinically achievable concentrations of 50–400 µg/ml and Colorectal carcinoma cells were also sensitive to cytotoxic effects of Ciprofloxacin, when were administrated at higher doses (200–500 µg/ml) (El-Rayes *et al*., 2002; Chrzanowska *et al*., 2020).

Herold *et al*., (2002) show that Ciprofloxacin induces growth inhibition and apoptosis in colon carcinoma cell lines in a time- and dose-dependent manner, the growth arrest is mediated through inhibition of DNA-synthesis, induction of mitochondrial injury and subsequent apoptosis.

3.8.2 The effect of alpha hemolysin and Ciprofloxacin on Prostate cancer (LN) cells proliferation:

The results revealed a significant decrease at ($p \leq 0.001$) in cells viability at higher concentration of toxin, Ciprofloxacin alone and in combination at (1000µg/ml) compared to control group as expressed in Figure **(3.14)**. Interestingly, the prostate cells were found to have a lowest effect against toxin and Ciprofloxacin in the experiment this might because of prostate have an amount of zinc which may play a role in the natural resistance of the male UTIs and important to the cell nourishment (Barman *et al*., 2022) Several natural defenses against infection for the prostate gland are present, such as the production of antibacterial substances and the mechanical flushing of the prostatic urethra via voiding and ejaculation (Fair and Parrish, 1981; Xiong *et al*., 2020).

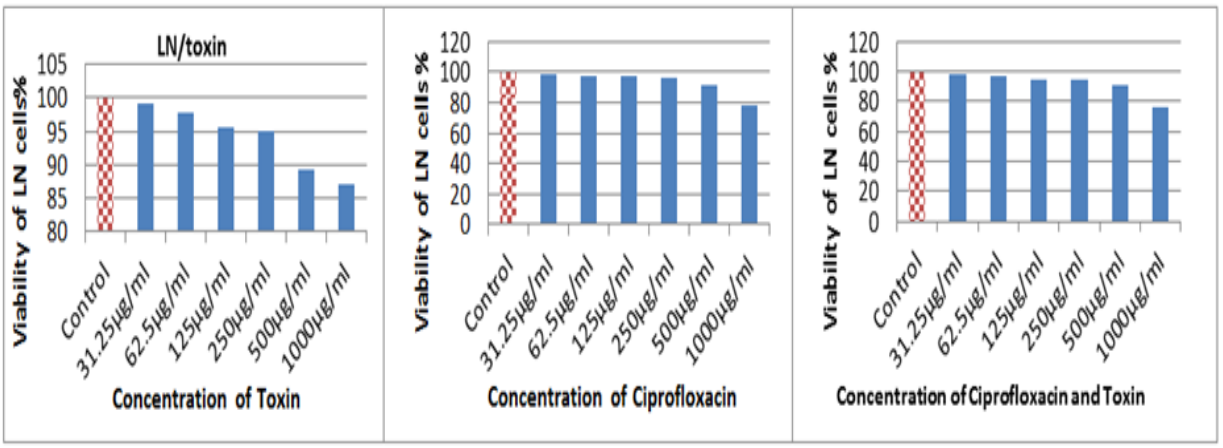

Figure (3.14): Normalized cells viability percentage of LN cells at different concentrations of toxin, Ciprofloxacin and combination

Lipsky *et al*., (2010) and Karaiskos *et al*., (2019) were investigated that only few antibiotics could penetrate the prostate and achieve sufficient effective bactericidal concentration at the field of infection. So, the pathogens at prostatic secretion and tissues must be exposed to a sufficiently high concentration of antibiotics for effective therapy that would inhibit bacterial growth or eradicate pathogens from the site of infection (Wagenlehner *et al*., 2005; Xiong *et al*., 2020).

Wang and Ji, (2019) were investigated the effect of UPEC on prostatic epithelial cell apoptosis found that, proliferation of prostate epithelial cells decreased and the progression of bacterial prostatitis were promoted by UPEC infection. Epidemiological studies from hospitalized cohorts demonstrate that *E. coli* represent up to 80% of all pathogens isolated from acute or chronic prostatitis patients (Gill and Shoskes, 2016; Lupo *et al*., 2020).

Due to virulence factors of *E. coli* such as Lps and toxins can trigger a series of inflammatory responses including cytokine production, neutrophil influx, evade host defenses, invade urinary epithelial cells and get internalized clonally to form biofilm-like intracellular bacterial communities, thereby forming a reservoir for recurrent or chronic infection and are known to invade normal human prostate epithelial cells with high efficiency and form intracellular colonies (Longhi *et al*., 2016; Ho *et al*., 2020).

Aranha *et al*., (2003) were suggesting the potential usefulness of the Ciprofloxacin as a chemotherapeutic agent for advanced prostate cancer, it showed anti-proliferative and apoptosis inducing activity on prostate cancer

cells which were mediated by cell cycle arrest at S-G2/M phase of the cell cycle.

Kloskowski *et al*., (2012) investigated the properties of Ciprofloxacin that may be allowing it to inhibit the development of prostate cancer. Firstly, Ciprofloxacin can stop the acute and chronic prostate inflammation which can lead to cancer development. Secondly, Ciprofloxacin can potentially kill prostate cancer cells in their early stage of development. Ciprofloxacin accumulates mainly in the prostate after oral intake thus Ciprofloxacin seems to be a perfect candidate as a prophylactic agent.

Kloskowski *et al*., (2021) studied the effects of Ciprofloxacin on bladder and prostate cancer cell they found that the Ciprofloxacin exhibited a toxic effect on all of the tested cell lines. In the case of non-malignant cell lines, the cytotoxic effects were weaker especially pronounced in the bladder cell line and led to an increase in late apoptotic cells and an inhibition of cell cycle mainly in the S phase.

Hangas *et al*., (2018) observed Ciprofloxacin to clearly reduce Top2 topoisomerase activity both *in vitro* and *in viv.* As Ciprofloxacin has been described to have cytostatic effects on some cancer cell lines, the impairment of mtDNA maintenance by Ciprofloxacin not only disturbed cellular proliferation and the physiological increase of mtDNA copy number during muscle maturation, it also effectively impaired the fusion of confluent myoblasts to multinuclear myotubes. So that, Ciprofloxacin have a dramatic effect on mtDNA topology, blocking replication initiation, reducing copy number and inhibiting mitochondrial transcription and has several mechanisms of mitochondrial toxicity in cells such as topoisomerase

inhibition, oxidative stress, altered calcium handling and photosensitization (Dogan *et al*., 2018). However, different efforts have been employed to overcome the limitation of rapid drug metabolism *in vivo*, which resulted in a short circulation time and a poor efficacy of known anticancer agents (Chrzanowska *et al*., 2020).

Conclusions and Recommendations

4 Conclusions and Recommendations:

4.1 Conclusions:

- Generally, current study confirmed that all age were susceptible to the UTIs also females was suffered of UTIs more than males.
- Antimicrobial effects very important in treatment against *E. coli* isolates were revealed high resistance present in treating of UTIs especially with Ticarcillin, Piperacillin, Aztreonam and Cefepime. Whereas, isolates were revealed sensitivity with Imipenem, Meropenem and Amikacin.
- Occurrence of biofilm production by *E. coli* isolates were at higher rate so that, the study examined the effect of Amikacin and Ciprofloxacin as anti-potential activity against biofilm production.
- The current study confirmed presence of *sat* and *hlyA* toxin genes among *E. coli* isolates suggestion the role of toxins in the pathogenesis however, *cdtB* were not found in all isolates.
- Phylogenic analysis of *hlyA* genes revealed a high number of mutations occurred with the *hlyA* gene, which varies substantially between local and international strains, was highlighted in the study.
- The alpha hemolysin toxin alone or with Ciprofloxacin had cytotoxic activity on both normal and cancer cell lines at higher concentration.

4.2 Recommendations:

- Correlation study for the effect of biofilm production and antibiotic susceptibility for the different types of bacteria and compared to the toxin effect.
- Study genes expression for toxin genes using real-time PCR could be more investigated for future study.
- Correlation study of alpha hemolysin toxin produced by different types of bacteria.
- The role of cytotoxic activity of hemolysin *in vivo* could be evaluated as anti- toxin.
- The effect of toxin pathway on the enzyme activity (suppressor or activator).
- Molecular study for the *cdtB* or *sat* gene and its enhancement or inhibition effect on the cell.

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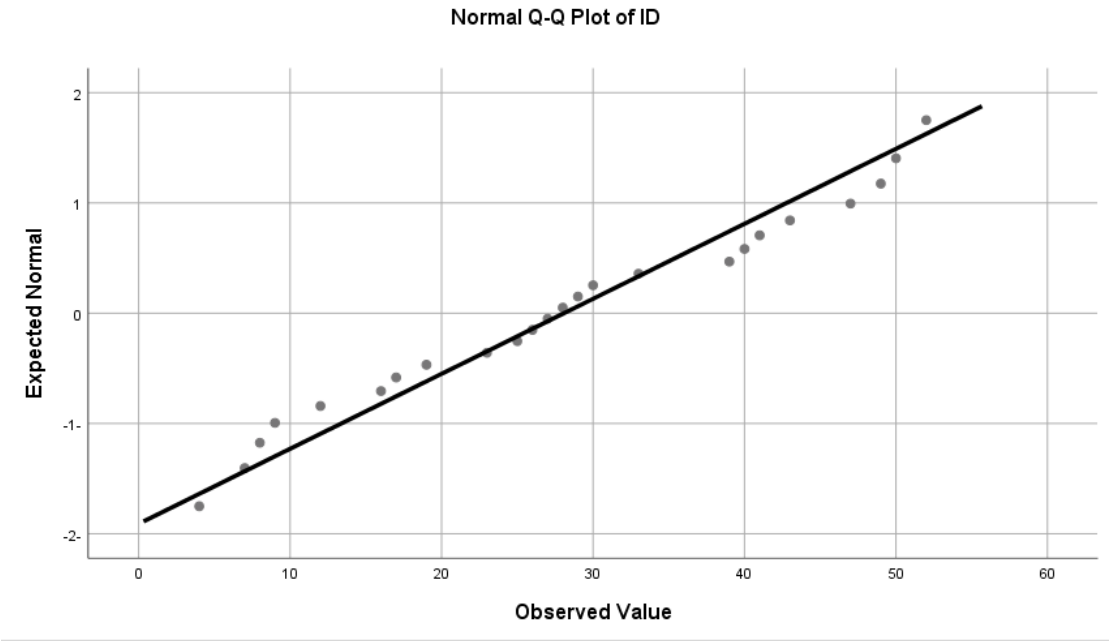
Appendix

Appendix I: Gradient concentrations of sodium chloride solution and absorbance value for each concentration and hemolysis by 50%:

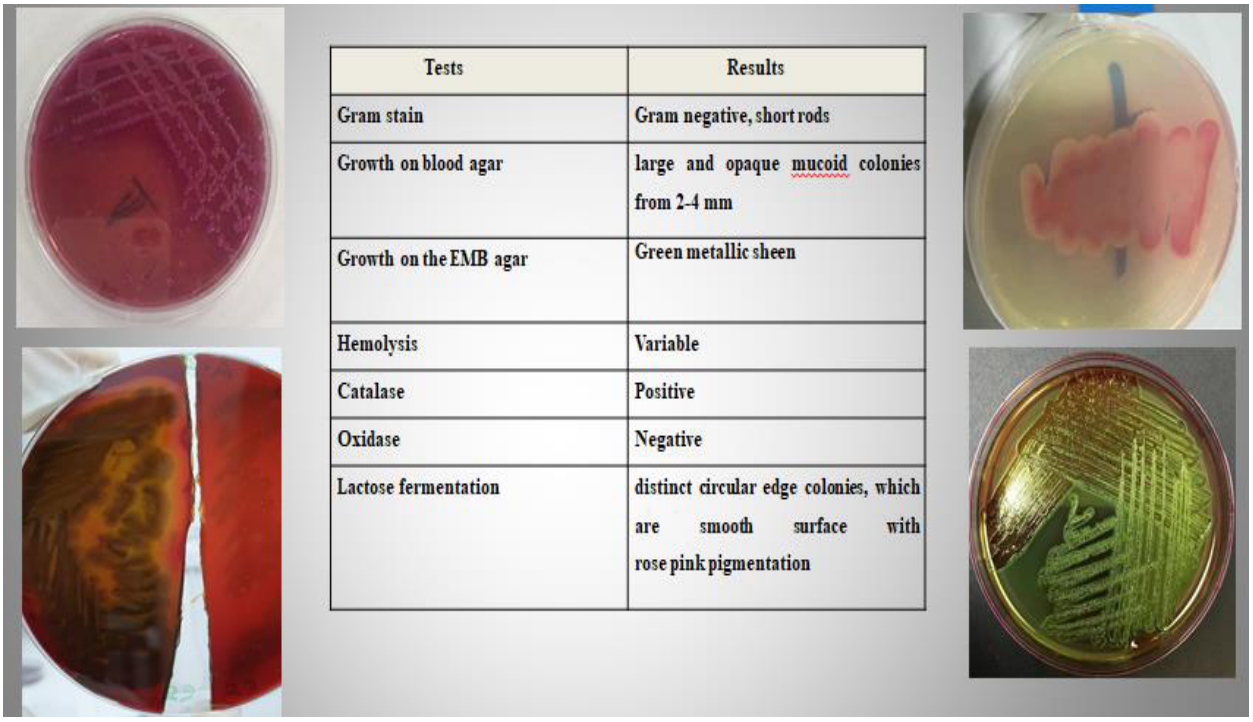
Tube No.	Sodium chloride (100concentration gm/ml)	Absorbance along a wavelength(412 nm)	hemolysis rate (50%)
1.	0.90%	0.011	0.88
2.	0.85%	0.012	0.96
3.	0.80%	0.011	0.88
4.	0.75%	0.01	0.80
5.	0.70%	0.020	1.6
6.	0.65%	0.028	2.2
7.	0.60%	0.23	18
8.	0.55%	0.33	26
9.	0.50%	0.34	27
10.	0.45%	0.62	49
11.	0.40%	0.63	50
12.	0.35%	0.79	63
13.	0.30%	0.81	64
14.	0.25%	0.90	72
15.	0.20%	1.25	100
16.	0.15%	1.25	100
17.	0.10%	1.25	100
18.	0.0	1.25	100

Appendix II: Preparation of Bovine serum albumin (BSA) concentration from stock solution of BSA (100µg/ml):

Tube No.	Volume of BSA solution (ml)	Volume of D.W. (ml)	Final volume (ml)	Final concentration (µg/ml)
1	0	1.0	1	0
2	0.1	0.9	1	10
3	0.2	0.8	1	20
4	0.3	0.7	1	30
5	0.4	0.6	1	40
6	0.5	0.5	1	50
7	0.6	0.4	1	60
8	0.7	0.3	1	70
9	0.8	0.2	1	80
10	0.9	0.1	1	90
11	1.0	0	1	100



Appendix III: Descriptive Statistics for all samples



Tests	Results
Gram stain	Gram negative, short rods
Growth on blood agar	large and opaque mucoid colonies from 2-4 mm
Growth on the EMB agar	Green metallic sheen
Hemolysis	Variable
Catalase	Positive
Oxidase	Negative
Lactose fermentation	distinct circular edge colonies, which are smooth surface with rose pink pigmentation

Appendix IV: Identification of *E. coli* isolates depended on the colonial morphology, microscopically and biochemical tests

Appendix: V

Bionumber: 0405610554526610
Organism Quantity: **Selected Organism: Escherichia coli**

Comments:	

Identification Information	**Card:** GN		**Lot Number:** 2411143203	**Expires:**	Jan 12, 2021 12:00 CST
	Completed:		**Status:** Final	**Analysis Time:**	4.83 hours
Organism Origin	VITEK 2				
Selected Organism	98% Probability **Escherichia coli**				
	Bionumber: 0405610554526610			**Confidence:**	Excellent identification
SRF Organism					
Analysis Organisms and Tests to Separate:					
Analysis Messages:					
Contraindicating Typical Biopattern(s)					

Biochemical Details

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

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

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

Page 1 of 1

Appendix: VI

Organism Quantity: **Selected Organism: Escherichia coli**

Comments:	

Identification Information			
Organism Origin	Technologist		
Selected Organism	Escherichia coli		
	Entered:	**By:**	Labadmin

Analysis Messages:
The following antibiotic(s) are not claimed:
Rifampicin,

Susceptibility Information	**Card:** AST-N222		**Lot Number:** 6221426103	**Expires:** Oct 22, 2021 13:00 CDT	
	Completed:		**Status:** Final	**Analysis Time:** 9.48 hours	
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Ticarcillin	>= 128	R	Amikacin	<= 2	S
Ticarcillin/Clavulanic Acid	>= 128	R	Gentamicin	<= 1	S
Piperacillin	>= 128	R	Tobramycin	<= 1	S
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	>= 4	R
Ceftazidime	<= 1	* R	Pefloxacin		
Cefepime	<= 1	* R	Minocycline	>= 16	R
Aztreonam	<= 1	* R	Colistin		
Imipenem	<= 0.25	S	Rifampicin		
Meropenem	<= 0.25	S	Trimethoprim/Sulfamethoxazole	>= 320	R

+= Deduced drug *= AES modified **= User modified

AES Findings:	**Last Modified:**	**Parameter Set:** Global CLSI-based+Natural Resistance
Confidence Level:	Consistent	

Appendix VII: Sequences producing significant alignments of *hlyA* gene:

Descrption	Scentific name	Max Score	Total Score	Query cover	E value	Per Ident	Acc. len	Accession
E.coli hlyA, Iraq	Escherchia coli	1013	3010	100%	0.0	100%	-	ON112292
E.coli(J96) hlyC,hlyA, hlyB and hlyD genes coding for chromosal hemolysins C, A,B and D	Escherchia coli	4107	4107	100%	o.o	100.00 %	8211	M10133.1
Escherchia coli strain KC-Dl-1 chromosome, complete genome	Escherchia coli	4053	6807	100%	o.o	99.72%	5305214	CP063046.1
Escherchia coli strain ATCC 700415 chromosome, complete genome	Escherchia coli	4049	6803	100%	o.o	99.72%	5237257	CP022609.1
Escherchia coli strain FDAARGOS_1262 chromosome, complete genome	Escherchia coli	4047	6801	100%	o.o	99.67%	5225337	CP069517.1
Escherchia coli strain EcPF14 chromosome, complete genome	Escherchia coli	4030	6790	100%	o.o	99.53%	5129852	CP054230.1
Escherchia coli strain SCU-101 chromosome, complete genome	Escherchia coli	3984	6767	100%	o.o	99.11%	5357129	CP048920.1
Escherchia coli strain 95-3322 chromosal, complete genome	Escherchia coli	3978	3978	100%	o.o	99.06%	5095223	CP027461.1
Escherchia coli strain 317 chromosome, complete genome	Escherchia coli	3978	3978	100%	o.o	99.06%	5035905	CP023357.1
Escherchia coli strain CP55_Sichuan chromosome, complete genome	Escherchia coli	3915	3915	100%	o.o	98.55%	4992761	CP053731.1

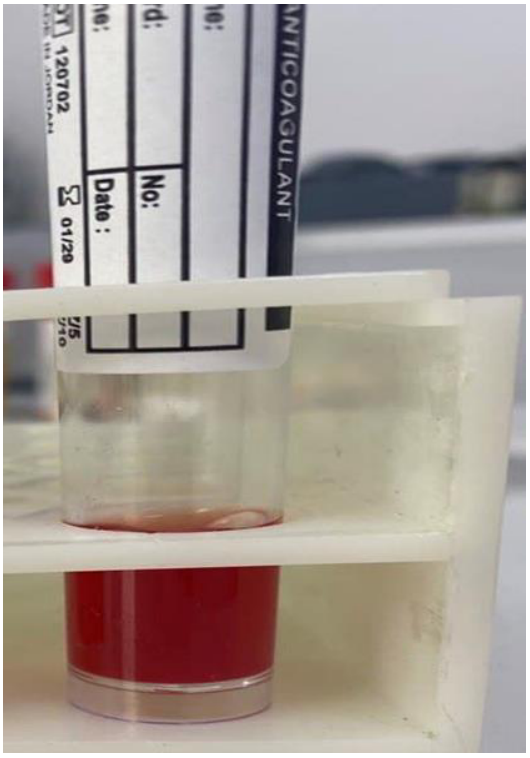
Escherchia coli strain GE3, complete genome	Escherchia coli	3915	3915	100%	o.o	98.55%	4909965	CP012376.1
Escherchia coli strain FHI_NMBU_10 chromosome, complete genome	Escherchia coli	3915	3915	100%	o.o	98.55%	5304684	CP046539.1
Escherchia coli strain SCU-387 chromosome, complete genome	Escherchia coli	3909	3909	100%	o.o	98.50%	5329017	CP051688.1
Escherchia coli strain NCTC9082, genome assembly, chromosome 1	Escherchia coli	3909	3909	100%	o.o	98.50%	5030213	LR134080.1

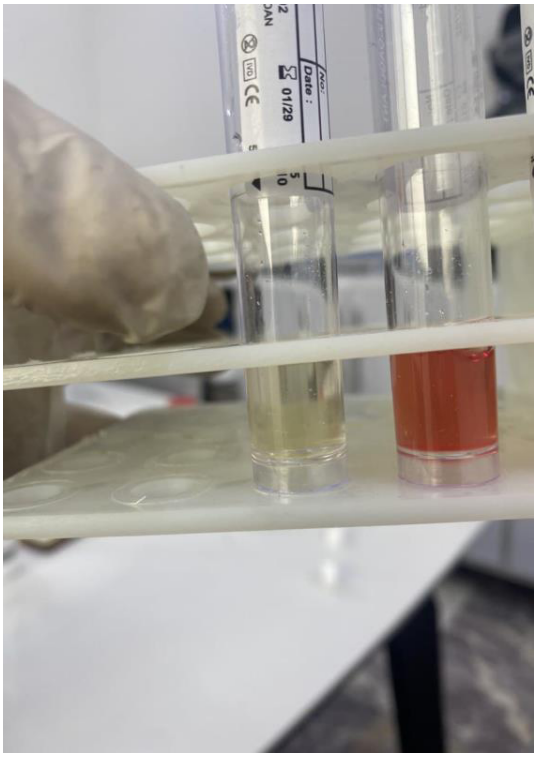
Appendix VIII: Sequences producing significant alignments of *sat* gene

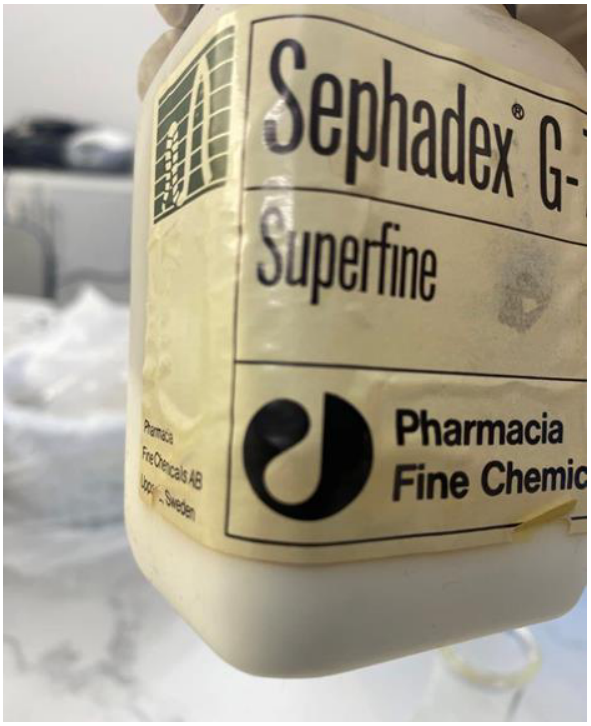
Descrption	Scentific name	Max Score	Total Score	Query cover	E value	Per Ident	Acc. len	Accession
Escherchia coli strain EoPF18 chromosome complete genome	Escherchia coli	1748	1748	100%	0.0	100%	5010549	CP054219.1
Escherchia coli strain SCU-182 chromosome complete genome	Escherchia coli	1748	1748	100%	o.o	100.00%	5015404	CP054372.1
Klebsiella pneumonae strain A16KP0016 chromosome, complete genome	Klebsiella pneumonae	1748	1748	100%	o.o	100.00%	5323556	CP052571.1
Escherchia coli strain E302 DNA, complete genome	Escherchia coli	1748	1748	100%	o.o	100.00%	5237933	AP022362.1
Escherchia coli strain CCUG 73778 chromosome, complete genome	Escherchia coli	1748	1748	100%	o.o	100.00%	5076638	CP041337.1
Escherchia coli isolate 131 genome assembly chromosome main	Escherchia coli	1748	1748	100%	o.o	100.00%	5039657	OW848785.1
Escherchia coli isolate 131 genome assembly chromosome main	Escherchia coli	1748	1748	100%	o.o	100.00%	5186323	OW849064.1
Escherchia coli strain NEC18 chromosome	Escherchia coli	1748	1748	100%	o.o	100.00%	4849845	CP091169.1
Escherchia coli strain Ec394-330266 chromosome, complete genome	Escherchia coli	1748	1748	100%	o.o	100.00%	5068759	CP056114.1
Escherchia coli strain STO_Bone4 chromosome, complete genome	Escherchia coli	1748	1748	100%	o.o	100.00%	4377204	CP054251.1
Escherchia coli strain STO_BoneB plasmid pBoneB_2 complete sequance	Escherchia coli	1748	1748	100%	o.o	100.00%	23391	CP054244.1
Escherchia coli strain STO_Bone7	Escherchia coli	1748	1748	100%	o.o	100.00%	4324717	CP054239.1

chromosome, complete genome								
Escherchia coli strain 1162invT2 chromosome, complete genome	Escherchia coli	1748	1748	100%	o.o	100.00%	5007059	CP051663.1
Escherchia coli strain 1162T7 chromosome, complete genome	Escherchia coli	1748	1748	100%	o.o	100.00%	5023946	CP051661.1
Escherchia coli strain 1162C chromosome, complete genome		1748	1748	100%	o.o	100.00%	5048620	CP051659.1
Escherchia coli strain E-T67-1 chromosome complete genome		1748	1748	100%	o.o	100.00%	5142461	CP090264.1
Escherchia coli strain E T70 chromosome complete genome		1748	1748	100%	o.o	100.00%	5159580	CP051667.1

Appendix IX: Extraction and purification of alpha hemolysin by gel filtration chromatography






Sephadex® G-
Superfine
Pharmacia
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Pharmacia
FineChemicals AB


Gel Filtration
DrNOORA

الخـــــــلاصة

شملت الدراسة (179) عينة ادرار من المرضى الوافدين الى مستشفى الحلة التعليمي العام و مستشفى الولادة والاطفال في محافظة بابل (وحدة المجاري البولية) ، وفي الفترة الممتدة من اذارالى كانون الاول(2021) وانتخبت العينات من كلا الجنسين و بأعمار مختلفة . أظهرت نتائج التشخيص الاولية ان (68.7%)123/179عينة موجبة للزرع البكتيري بعد تشخيصها باستخدام الطرق الزرعية المختبرية و الاختبارات الكيميوحيوية و التشخيص بنظام الفايتك الدقيق مع التأكيد بالتشخيص الجيني باستخدام *16SrRNA*. شكلت الاشريكية القولونية أعلى نسبة تواجد واعتبرت كمسبب رئيسي لالتهاب المجاري البولية بنسبة (45.5%)56/123 بالمقرنة مع المسببات البكتيرية الاخرى بنسبة (55.5%)67/123 معظمها من الفئة العمرية (22-31) سنة بنسبة (43%) بينما كانت الفئة العمرية (52-61) الاقل اصابة بنسبة (3.5%) و سجلت الدراسة اصابة الاناث بمعدل اعلى من الذكور(71.5% : 28.5%) على التوالي.

تضمنت الدراسة فحص الحساسية الدوائية باستخدام جهاز الفايتك للعزلات البكتيرية و مقاومتها تجاه المضادات الحيوية وبينت النتائج ان اعلى نسبة حساسية للبكتريا كانت لمضادات Imipenem و Meropenem و بنسبة (100%) و Amikacin بنسبة (94.6%) بينما Gentamycin و Tobramycin و Ciprofloxacin و Minocycline سجلت معدل حساسية أقل و بنسب (69.6% , 62.5 % , 55.4% , 51.7%) على التوالي. وتم ايجاد نسب متغايرة للمقاومة في العزلات بالنسبة لمضادات Ticarcillin و Piperacillin بنسبة (100%) و لمضادي Cefepime و Azetronam بنسبة (94.6%) وكانت أقل نسبة مقاومة لمضاد Trimethprime-Sulfament (64.3%).

تم الكشف عن قابلية انتاج الاغشية الحيوية في العزلات البكتيرية، ووجد انتاج عالي من الاغشية الحيوية بنسبة (89.3%)50 و وفقا الى قدرة العزلات لتكوين الاغشية الحيوية قسمت الى مجموعات (50%)25 قوية التكوين و (38%)12 متوسطة التكوين و (12%)6 ضعيفة التكوين. كذلك حددت فعالية مضادي Amikacin وCiprofloxacin تجاه العزلات المكونة للاغشية الحيوية باستخدام طريقة Micro titer plate. و بينت النتائج ان تثبيط انتاج الاغشية الحيوية باستخدام مضاد Amikacin كان عند التركيز(64µg\ml) في حين ان تثبيط مضاد Ciprofloxacin كان في التركيز (128 µg\ml) وصولا الى اعلى تركيز (1024 µg\ml) .

الخـــلاصة:

تم تشخيص وجود جينات سموم alpha hemolysin و secreted autotransporter و-cyto lethal distending (*hlyA, sat* and *cdtB*) باستخدام طريقة تفاعل البوليميراز المتسلسل بعد استخلاص الحمض النووي من العزلات السريرية المشخصة للأشريكية القولونية. حيث شخصت 21/56 (37.5%)من البكتريا الاشريكية المحتوية على جين الالفا الهيمولايسين بينما 35/56(62.5%) من البكتريا احتوت على جين Secreted autotransporter في حين لم يتم الحصول على عزلات تحتوي على Cyto-lethal distending. وسجلت جميع الجينات المعزولة من العزلات البكتيرية في بنك الجينات الدولي (NCBI) OL539543.1, OL539542.1, ON112284, ON112285, ON112286, ON112287, ON112288, ON112289, ON112290, ON112291, ON112292, ON112293 ON777812, ON777813, ON777814, ON777815 and ON777816 كما أظهرت النتائج عدم وجود فروق معنوية بين وجود جينات alpha hemolysin و Secreted autotransporter وقابلية البكتريا لتكوين أو عدم تكوين الاغشية الحيوية (P<0.162) and (P<0.114) على التوالي .

أظهرت الدراسة المظهرية ايجابية قدرة البكتريا على انتاج سم الهيمولايسين باستخدام اكار الدم للعزلات. وقد استخلص السم بعد تنمية البكتريا المنتجة له على وسط (Luria broth) للسم بطريقة الترشيح الهلامي باستخدام السيفادوكس G200 و بفعالية (5 وحدة لكل مل). وتم اختبار سم الهيمولايسين المستخلص على خطوط الخلايا الطبيعية و السرطانية لوحده و مع عقار Ciprofloxacin و قد بينت النتائج قدرة سم الالفا الهيمولايسين و المضاد الحيوي Ciprofloxacin على تثبيط الخلايا الطبيعية و السرطانية وبنسب أعلى في التراكيز العالية (500 &1000 µg\ml). كما وجد أن خلايا البروستات كانت أقل تأثرا بالسم والسيبروفلوكساسين في التجربة.

وزارة التعليم العالي والبحث العلمي
جـــــــــــامعة بـــــــــابل
كـــــــــــــلية الطب

التوصيف الجزيئي للسموم الخارجية في الايشيريشية القولونية البولية و تأثيرها كمضاد سرطاني

أطروحـــــــــــــة

مقدمة الى مجلس كلية الطب/ جامعة بابل وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في الأحياء المجهرية الطبية

من قِبل

تساهيل حامد كاظم الدليمي

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إشراف

أ.د. الهام عباس بنيان الساعدي

أ. د. ذكرى عدنان جواد المسلماوي

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