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Molecular Detection of *fim H* and *papC* genes and Apigenine Effect on The Colonization Factor of Clinical Isolates of *Escherichia coli*

A Thesis
Submitted to the Council of the College of Medicine, Babylon University, in Partial Fulfillment of the Requirements for the Degree of Master in Science /Medical Microbiology

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بسم الله الرحمن الرحيم

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ

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We certify that this thesis, entitled **Molecular detection of *fim H* and *papC* genes and apigenine affect on the colonization factors of clinical isolates of *Escherichia coli*** , was prepared by **Sahar Salman Jasim Al-Dulemi** under supervision at the College of Medicine, the University of Babylon as a Partial of the Requirements for the Degree of Master in Medical Microbiology.

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Dedication

To my life……my father&my mother

To my spirit…..my husband

To my lovers…..my sisters &my brother

To my best thing happed in my life …my son Rayan

To soul my sister who she left the life but didn't leave my heart

Sahar Al-Dulemi

2023

Summary:

Escherichia coli is the bacteria that belong to the family Enterobacteriacae. Pap gene cluster and fim gene cluster are so essential adhesive factors in *E.coli* particularly pathogenic one and the assembly of these genes clusters may give rise to synthesize both types of fimbriae or pili, for instance, type1 fimbria is assembled by many genes such as Fim C,Fim D and Fim H and also for p fimbriae which is controlled by numeres genes such as papC,papR and others.

In this study, a total of 80 specimens were obtained from patients suffering from different diseses who attended to Al-Marjan teaching hospital Hilla city in Babylon province,during the period from July to October 2022.

In this study, 80 clinical samples are included for detection *Escherichia coli*. It was found that only 30 isolates were obtained belonging to *E.coli* ,where 15 isolates from urine samples ,10 isolates from vagina,and 5 isolates from wounds infections.

The effect of some antibiotics are also investigated.It was found that Fosfomycine and meropeneme are higly effective on *E.coli* isolates (98.7% and 95%) respectively ,followed by Amikacine , Kanamycine and piperacline.

Some adhesive factors are investigated in 30 isolates by using PCR techniques where *fim*H and *pap*C are included in this study.

It was found that *fim*H gene was present in 29 isolates (93.3%) when specific primer for this gene is used,where as only 24 isolates of *E.coli* gave positive results for papC gene by using specific primer at a rate 80%. These genes are more common and present among *E.coli* isolates.

One locus in *pap*C gene related to the 10 isolates of *E.coli* which give positive for this gene were subdecred for DNA sequencing.

The *pap*C amplicon of 10 isolates show high rate of similarty in DNA sequencing and the results showed that only two variants are seen in this

sequencing where one nucleatided is substituted by another 177T>C.This occurs in only one isolate, the other nine isolates gave the same sequencing with no variation.

Moreover,colonization factor antigens(CFA/1) was investigated in all clinical isolates of *E.coli*.It was observed that all isolates gave positive results for this antigen.

When epigenine is added at different concentrations(0.1%,0.2%,0.5%,and 1%).It was notized that epigenine has no effect on CFA/1 which gave an indicator that *E.coli* contains more than one adhesive factors used for attachment of different tissues.

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List of abbreviations

Abbreviation	*Meaning*
EIEC	Enteroinvasive E.coli
EPEC	Enteropathogenic E.coli
ETEC	Enterotoxogenic E.coli
EDTA	Ethylene diamine tetra acetic acid
KDa	Killodalton
µg	Micro gram
µl	Micro liter
MSA	Multiple sequence alignment
Omp	Outer membrane protein
PCR	Polymerase chain reaction
TBE buffer	Tris Borate- EDTA- buffer
TE buffer	Tris - EDTA buffer
UTI	Urinary Tract Infection
UPEC	Uropathogenic E.coli

Chapter One

Introduction and Literature Review

1.1 Introduction:

Escherichia coli is the bacteria that belong to the family Enterobacteriacae. This bacteria is gram negative bacilli present as commensal in human intestine, or opportunistic pathogen that may cause different disease in the human ,such as diarrhea, urinary tract infection, women vaginitis and others.(Hussein & Naser.,2023).

This bacteria is aerobic or facultative anaerobic which is able to ferment lactose in Macconky agar ,Moreover, *E coli* has different modes of adhesion which make it enable to adhere varios types of tissues.

There adhesive factors called pilli or fimbriae, which appear as hair_like appendages that protrude from the surface of bacteria.(Lupo,*et al.*, (2021).

The most common types of fimbriae that are studied extensively is p fimbria which is encoded by the pap gene (pytonethritis associated pilli),where it is spreading among uropathogenic *E coli* (UPEC) that causes pyelonephritis and are characterized by its being resistant to mannose.(Du,2021).

However, type 1 fimbiriae are remarkably true virulence factors of UPEC that can strength the attachement of *E. coli* to different types of cells(Mancuso, *et al*.,2023)

where, it recognize uroplakin from bladder epithelial cells and mannoside containing host proteins .This fimbriae including *fim* H are expreced by 90% of UPEC strains and encoded by *fim* H gene.(Aziz, *et al.*,2023).

The expression of fimbriae on the surface of *E.coli* strains may be affected by some antibiotics and this will reduce the bacteria to adhere the

tissues and hence reduce the ability of bacteria to cause infections(Maisto, *et al.*,2023).

Besides ,Most types of pilli or fimbriae may be produce by other types of *E.coli* strains such as Enterotoxigenic *E.coli*(ETEC) which can produce different types of adhesive factors such as colonization factor antigen (CFA) which has previously reported in most commensals and pathogenic *E.coli* (Zhang,*et al*.,2022)

Pap gene cluster and fim gene cluster are so essential adhesive factors in *E.coli* particularly pathogenic one and the assembly of these genes clusters may give rise to synthesize both types of fimbriae or pili, for instance, type1 fimbria is assembled by many genes such as Fim C,Fim D and Fim H and also for p fimbriae which is controlled by numeres genes such as papC,papR and others.(Gahlot, *et al*.,2022).

Many strains of *E.coli* can produce an array of these adhesive factors, and often inhibition of a single adhesive may cost enough to bacteria to lose its virulence.

However, the functions of pilli are not limited only to adhesion and can help in many other pathways such as evasion of bacteria from immune system and help the bacteria to survive inside host tissues(Lacerda and Ingersoll ,2020).

Many chemical and herbal compounds may play a role to influence on pilli action , one of there compounds is Apegenine which is derived from many plants and it is observed to have an effect on fim H by molecular docking(Alaa,2022).

Also , there are many mobile elements may consist of one or more adhesive factors these include the plasmids,phages,integrons and

pathogenicity islands which have genes encoding one or more virulence factors which include the colonization factor antigen and secretion systems(Desvaux,*et al*.,2020).

These mobile elements when cured from bacteria , it will reduce its ability to make attachment ,where there are many antibiotics may cause curing for these elements such as quinolone group of antibiotics which have direct effect on these elements and also on the adhesive factors present in *E.coli* strains.(Kamruzzaman, *et al*.,2021).

However,the action of adhesive factors in *E.coli* is still requiring further studies because,this bacteria has multiple colonization factor antigens such as CFA/1,CFA/2 and CFA/3 depending on what sugars should be used and what type of blood should be considered and the colonization factor antigens may be encoded by more than one gene related to gene cluster or pathogenicity islands or gene cassettes.

Aim of study:

Phenotypic and genetic study the adhesion factor associated with *E.coli* isolated from urinary tract infection.

To achieve the above aim, the following objectives were suggested:

1- isolationof *E .coli* from different clinical samples.
2- Effect of some antibiotics on bacterial isolation.
3- molecular detection of *fim* H genes and *pap* C genes.
4- Detection of colonization factor antigen (CFA/1) produced by these bacteria.
5- show the effect of Apigenine on CFA/1.
6- DNA sequencing of *pap*C gene to show its variants .

1.2 Literature Review

1.2.1 *Escherichia coli (E.coli):*

Escherichia species belong to the Enterobacteriaceae family of bacteria, and they're: *E. fergusonii, E. vulneris, E. hermanii, E. blattae, E. albertii a*nd *E. coli, Escherichia coli* belongs to a group of bacteria informally known as coliforms that are found in the gastrointestinal tract commonly found in the lower intestine of warm-blooded organisms , most *E. coli* strains do not cause disease, naturally living in the gut , but virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis , hemorrhagic colitis, septic shock, and Crohn's disease (Sora, *et al*., 2021*).*

A rod-shaped, non-spore forming, Gram-negative bacterium that uses flagella to move around. Colonies of *E. coli* are generally convex with a smooth surface. On MacConkey agar, its colonies appear flat, dry and pink coloured while they appear to have a colorful "sheen" on Eosin Methylene Blue (EMB) agar. *E. coli* O157:H7 can be differentiated from other *E. coli* serotypes by utilizing sorbitol MacConkey agar since O157:H7, unlike other serotypes, cannot ferment sorbitol. *E. coli* strains do not produce H_2S in Triple Sugar Iron agar. Most strains are capable of producing β-glucuronidase enzyme. Optimal temperature for growth is 36 - 37 °C and a pH of 4.4-9 is suitable for growth. The bacteria gives a negative result to the oxidase, urease, nitrite tests while having a positive result in the catalase test, and as for the indole test, most strains give a positive result (Hussein *& Naser*., 2023).

Uropathogenic *E. coli* (UPEC) is one of the main causes of urinary tract infections , it is part of the normal microbiota in the gut and can be introduced in many ways . UTIs are a significant public health problem

that considered one of the common sources of infections causing systemic illness in infants and children , if not treated the complication may lead to renal scarring , end stage renal failure and hypertension . UTIs are the third most common infection after respiratory and gastrointestinal infections , it causes a significant morbidity and considerable mortality that affects about 150 million people each year worldwide (González , *et al.*, 2020).

E. coli grows as normal flora in the digestive tract and is considered a pathogenic opportunistic as it can cause diarrhea, in which case it'd be called diarrheagenic *E. coli* and urinary tract infections which are caused by the type called uropathogenic *E. coli* (UPEC). It's responsible for approximately 90% of UTIs in young women.(Aziz, *et al*., 2023).

E. coli strains can be broadly grouped into three families: (1) commensal organisms that are normal residents of the gastrointestinal tract that do not cause disease, (2) strains that cause diarrheal intestinal disease and (3) strains that usually cause disease outside of the intestinal tract , Among representative strains from each of these families, only around 20% of coding sequences are shared among all strains, with 64% of sequences shared between commensal and ExPEC strains (Manohar, *et al.*, 2019).

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *coli*

The *E. coli* genome undergoes constant change due to a continual flux of genetic insertions and deletions. The genome size, which can vary by up to 1.9 Mb, encodes approximately 4000–6500 proteins. The core genome comprises only 2000 genes, and the rest of the genome determines commensalism, species specificity, pathotype, and antibiotic susceptibility profile , the core genome contains mostly nonmobile elements, while strain specific genes are more likely to be located on pathogenicity islands, which are large regions of the genome that contain virulence-related genes found in pathogenic strains but not commensal strains, and mobile elements including plasmids, bacteriophages, insertion sequence elements, transposons, integrons, and prophages , many of these mobile genetic elements are found in other pathogenic bacterial species suggesting a history of genetic transfer and exchange (Delmas,*et al*., 2022).

1.2.2 *E. coli* pathotypes:

pathotype of *E.coli* that are capable of causing disease in healthy individuals. Three general clinical syndromes can result from infection with one of these pathotypes: enteric/diarrhoeal disease, urinary tract infections (UTIs) and sepsis/meningitis(Parvez & Rahman 2018). Among the intestinal pathogens there are six well-described categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC)(Chit-Opas, *et al*.,2020).

1.2.2.1 Enteropathogenic *E. coli* (EPEC):

EPEC is a known pathotype of *E. coli* capable of causing diarrhea and also cause tissue damage to the intestine that is known as attaching and effacing lesions (AE) which results in the destruction of normal microvilli architecture(Pokharel,*et al*., 2023).

Incapable of producing shiga-toxin, heat-labile (LT) and heat-stable (ST) enterotoxins. Classified into typical and atypical EPEC, typical Enteropathogenic *E. coli* (tEPEC) that cause diarrhea to humans have a plasmid called E. coli adherence factor (EAF) plasmid with the operon bfp which encodes bundle-forming pilus (BFP), while atypical EPEC (aEPEC) strains don't have the aforementioned operon (Ahmed,*et al.*, 2021).

1-2-2-2 Enteroinvasive *E. coli* (EIEC):

The pathogenic causative agent of dysentery in humans and especially in developing countries. Dysentery occurs because of enteroinvasive *E. coli's* (EIEC) invasion and entrance into the intestinal cells causing their destruction. The bacterium attaches to the mucus layer of the large intestine by a process called endocytosis and may spread through one cell to the next. Eneteroinvasive *E. coli* serotype O96:H19 was found to be the causative agent of an outbreak in Sweden recently (Pokharel,*et al*.,2023).

1.2.2.3 Diffusely Adhering *E. coli* (DAEC):

DAEC causes unique changes on epithelial cells, resulting in diarrhea and epithelia damage which may lead to other intestinal diseases. DAEC encompasses a group of *E. coli* strains that have genes encoding for Afa/Dr adhesions that are capable of causing diarrhea in humans.

Capable of infecting children, and it's considered the second most common cause of death in children under 5 years of age (Mirsepasi,*et al*., 2019).

1.2.3 Pathogenicity of *E. coli*:

Bacterial pathogenicity is defined as the genetic capacity of the bacterium to cause disease, based on the virulence and resistance traits it possesses. Unfortunately, *E. coli* can easily acquire this capacity and is considered a major cause of human infectious disease worldwide.In order to better understand the double component of the pathogenicity of *E. coli*, it is important to accurately define two concepts: virulence and resistance, *E. coli* is a ubiquitous commensal of food-producing animals and humans . Most strains of this enterobacterial species are harmless commensals that live in a mutually beneficial association with their hosts and seldom causedisease . *E. coli* is, however, a particularly complex species, having diversified into pathogenic strains , based on the type of virulence factor present , and the host's clinical symptoms E. coli strains are classified into pathotypes of zoonotic intestinal pathogenic *E. coli* (IPEC) or extraintestinal pathogenic *E. coli* (ExPEC) (Braz, *et al.*,2020).

1.2.4 Virulence Factors and Genetics:

Virulence is the bacteria's ability to cause disease, and it's a measurement of pathogenicity. *E. coli* species have many virulence factors that allows the bacteria to cause infection and disease, the most important of these diseases being urinary tract infections (UTIs)(Nair,*et al*.,2019). The reason why some strains of the bacteria have the ability to overcome the immune system and cause disease is their encoding genes that are found on specific locations known as pathogenicity islands (PAIs) (Ahmed, .*et al.*, 2021). The size of these islands varies from 10-

200kb and it differs from other parts of the genome in its G-Ccontent. PAIs are present in pathogenic *E. coli* strains more than nonpathogenic strains and they code virulence factors such as adherence, toxins, siderophores, capsules, lipopolysaccharides and enzymes with each factor having its own encoding genes(Ahmed, *et al*.,2021).New strains of *E. coli* are constantly developing through processes such as mutations, gene duplication and horizontal gene transfer. Most strains of *E. coli* have a genetic backbone of approximately 4.1Mb but due to horizontal gene transfer, pathogenic *E. coli* strains have a genome that can be up to 1Mb larger than commensal *E. coli* genomes, granting them the ability to cause a wide range of diseases in different hosts (Yu, *et al*.,2021). The bacterium *E. coli* can transfer its DNA through something called bacterial conjugation, a process in which one bacterium transfer its genetic material to another through horizontal gene transfer. Transduction is another method which *E. coli* can implement to transfer its genetic material by recombining their genes with the genome of a bacteriophage that will initiate another cycle of infection in another bacteria (Arnold, *et al*.,2022).

1.2.5 Adhesion:

Adhesion is one of the most important virulence factors the bacteria *E. coli*, and the bacteria's adherence to uroepithelial cells is considered the first step forinvasion (Yang,*et al*.,2022).. Adherence depends on the genes encoding the necessary adhesion factors, and these genes include *eae, fim, sfa, pap,* etc(Behzadi,2018).Fimbriae (pili) include 3 types: F-fimbriae, S-fimbriae and P-fimbriae, and they're considered very important virulence factors as they allow the bacteria to adhere to host's cell which can then initiate colonization and allow the

formation of the biofilm, which in turn, allows the bacteria to increase its antimicrobial resistance .(Yang, *et al.*,2022).

1.2.6 Attaching & Effacing locus:

The *E. coli* attaching and effacing gene (eae gene) is located on the Locus of Enterocyte Effacement (LEE), which is a ~35-kb pathogenicity island where themain virulence genes of all strains of *E. coli* capable of inducing A/E lesions (Attaching/Effacing) are located. LEE is organized into 5 operons (LEE1 through LEE5). Located on LEE5, the eae gene is about 2800 nucleotides and encodes the adhesion protein intimin, a 94-kDa protein required for the adherence of *E. coli* to host cells at the site of A/E lesion(Whelan, *et al*., 2020).

A/E lesion are characterized by intimate adhesion of *E. coli* to the surfaces of enterocytes, on raised pedestals (pseudopodia), and destruction of nearby microvilli . The intimin protein has an N-terminus and a Cterminus. The C-terminus is highly diverse between different sources and based on differences found in the C-terminus, at least 30 intimin subtypes have been identified. The N-terminus fixes intimin in the outer membrane while the C-terminus extends from the cell's surface and binds to Tir, the cellular receptor for the bacterial transmembrane protein intimin. The intimin-Tir interaction makes way for intimate adherence and pedestal formation. Huge amounts of intimate attachment of *E. coli* to intestinal cells cause diverse signal transduction pathways — a process by which genetic material is transferred from one cell to another by the bacteria's plasmid — leading to the bringing down of many cellular process for the benefit of the bacteria (Govindarajan,*et al.*,2020).

1.2.7 *Fim* H adhesive factor:

Fim H adhesive factor is located at the tip of type 1 pilus, consists of two Ig-like domains: the lectin domain (residues 1-156) at the N terminus, which contains the carbohydrate recognition domain (CRD), and the pilin domain (residues 160-279), which connects Fimh to the pilus rod and regulates the switch between highand low-affinity states of the lectin domain While the lectin domain alone is stable.A Fim H is stable in presence of FimC (Webster, *et al*.,2022).

The first Structure of FimC-FimH complex was studied in 1999, by hund and his coworkers who reported the first co-crystallized structure of FimC-FimH with a mannose ligand , which gave important insight into the binding site(Sarshar,*et al*.,2020). Later, numerous structures of the FimH lectin domain alone or in complex with diverse mannoside ligands were published, greatly facilitating the discovery of high affinity Fimh antagonists for the treatment of UTIs (Eldridge, *et al*.,2021).

Type 1 fimbria (pilus) are expressed by a large number of *E. coli* strains, and are found in more than 95% of *E. coli* isolates from intestinal and extra-intestinal infections. Pili act as highly efficient adhesion tools for bacterial inhabiting in diverse environments, including biotic and abiotic surfaces. On the surface of UPEC, type 1 pili are uniformly distributed (Hancock, *et al.*,2022). Structurally, type 1 pili are 7 nm wide and several micrometers long rodlike fibers. The pili rods are composed of immunoglobulin like (Ig) FimA pilin subunits that are anchored into a chain, and the chain is further coiled into a helix(Kallas,*et al*.,2020).FimA helix is joined to a short 3 nm thick distal tip fibrillum that consists of two adaptor proteins, FimF and FimG. At the tip of each pilus is a single mannose-specific adhesin – FimH. The pilus rod is assembled by the

chaperone/usher pathway and in their mature form the Ig fold of each subunit is completed by an amino-terminal extension from a neighboring subunit in a process termed "donor-strand exchange"(Chatterjee, *et al*.,2021).

1.2.8 P- fimbriae:

Pili are filamentous protein polymers found on the cell surfaces of many pathogenic Gram-negative bacteria. P fimbria, a mannose-resistant adhesin of uropathogenic *Escherichia coli* (UPEC), has been shown to be associated with acute pyelonephritis. P fimbriae are chromosomally encoded by the pap gene cluster, consisting of 11 genes. (Maisto, *et al*., 2023).

An important stage in the successful colonization of the urinary tract and pathogenesis of UTI is the ability of UPEC to adhere to host uroepithelia. In 1976, Edén demonstrated that *E. coli* isolated from the urine of patients with acute symptomatic pyelonephritis adhered in greater numbers to exfoliated uroepithelial cells than *E. coli* isolated from the urine of patients with asymptomatic bacteriuria.Two years later, the ability of UPEC to attach to human uroepithelial cells was attributed to the presence of fimbriae (or pili), which appear as hair-like appendages that protrude from the surface of bacteria.These fimbriae were determined to be distinct from the common fimbriae (otherwise known as type 1 fimbriae) in that they mediated adherence to uroepithelial cells in the presence of mannose, a known inhibitor of type 1-mediated adherence.(Beebout,*et al*., (2022).

The most extensively studied adhesin, and also the first virulence-associated factor identified for UPEC is P fimbria. P fimbriae, encoded by the *pap* (pyelonephritis-associated pili) genes, are significantly prevalent

among strains of UPEC that cause pyelonephritis and are characterized by their mannose-resistant adherence to Gal(α1–4)Galβ moieties present in the globoseries of membrane glycolipids on human erythrocytes of the P blood group and on uroepithelial cells. (Sarowska , *et al.*,2019).

The ability of UPEC to colonize depends upon the expression of different fimbrial adhesins. For a successful adherence to the host cell surface, UPEC expresses many adherence factors which are crucial for attachment and thus regarded as virulence factors. Many bacterial adhesins are organized in a thin filamentous structure called fimbriae or pili although there are evidences of presence of adhesins in the cell surface of bacteria(Arafi, *et al*., (2023). Adhesins of fimbrial nature are important during Attachment process. Fimbriae, also known as pili, are long hair-like structures contained in the cell surface of bacteria that recognize specific compounds usually carbohydrates of the target host cells. Pili are the short form of fimbriae and might be used interchangeably with fimbriae. Fimbriae consist of oligomeric pilin proteins. These proteins are arranged in such a manner that they form a helical cylindrical structure and are both thinner and shorter than flagellum. These proteinaceous structures are expressed in uropathogenic strains of *E. coli* and are considered as virulence factors .(Péter,*et al.*, (2022).

Most of the receptors for these fimbriae are carbohydrates. They include type 1 fimbriae, P fimbriae, and thin aggregative fimbriae . Many bacterial pathogens can produce an array of these adhesins, and often inhibition of a single adhesin may cost enough to a bacterium to lose its virulence. Functions of pili or fimbriae are not limited only to adhesion and can help in many other crucial pathways for the microbe to survive

and evade the immune system of the host. Evolution of different types of adhesins plays a role in tissue tropism. (Kuehn, *et al*.,2020).

In *E.coli* which causeUTI, adhesins are unveiled by chaperone-usher-assisted pathway. This pathway involves two proteins, one is a periplasmic chaperone, and the other is a protein called usher. Usher act as the base of the structure, and the function of chaperone is folding and recruitment of the subunits . In absence of the chaperone, pilin proteins are degraded and misfolded and thus cannot be assembled in the form of a mature pilus. On the other hand, usher helps to mature the fimbriae and its transportation through the outer assuring integrity of the outer membrane. The constituents of usher proteins are an N-terminal domain (NTD), 24-stranded beta-barrel channel, a plug domain, and two C-terminal domains (CTD). In uropathogenic *E. coli* strains, chaperone-usher family fimbriae are more abundant.(Spaulding, *et al.*,2017).

1.2.9 *Escherichia coli* Susceptibility to Antibiotics:-

The antibiotic sensitivities of different strains of *E. coli* vary widely. Antibiotics which may be used to treat *E. coli* infection include fosfomycine ,meropenem and the amikacine. In microbial populations of *E. coli*, an important feature described is the resistance to multiple antibiotics on bacterial isolates from various origins (Denamur,*et al.*,2021). Antibiotic resistance is a growing problem. Some of this is due to the overuse of antibiotics in humans (Serwecińska, *et al*.,2020).

Antibiotic-resistant *E. coli* may also pass on the genes responsible for antibiotic resistance to other species of bacteria, such as *Staphylococcus aureus*, through a process called horizontal gene transfer. *E. coli* often carry multidrug resistant plasmids and under stress readily transfer those plasmids to other species. Indeed, *E. coli* is a frequent member of biofilms where many species of bacteria exist in close

proximity to each other. This mixing of species allows *E. coli* strains that are piliated to accept and transfer plasmids from and to other bacteria. Thus, *E. coli* and the other enterobacteria are important reservoirs of transferable antibiotic resistance. Antimicrobial resistance of *E. coli* has played an important role in clinical infectious diseases (Song, *et al*.,2021).

E. coli that produce extended-spectrum beta-lactamase (ESBL) have resistance towards most beta-lactam antibiotics, including penicillin, cephalosporins, and monobactams. *E. coli* is one of the major organisms that are capable of producing ESBL worldwide which is encoded by bla-TEM. Beta-lactamase works by cleavingthe amide bond in the beta-lactam ring which leads to the inactivation of those antibiotics. Carbapenemase is encoded on genes that are usually associated with genes that encode resistant for other non-beta-lactam antibiotics which often results in multi-drug resistance (MDR) bacteria. Carbapenemase are specific plasmidmediated beta-lactams that work against carbapenems, ertapenem, meropenem, impenem, and doripenem (Al-Sarraj,2021).

Normal intestinal flora is a reservoir for resistance genes; the prevalence of resistance in communal *E. coli* is a useful indicator of antibiotic resistance in bacteria in the community. One of the reasons of the cause of this problem is the prior indiscriminate use of antibiotics in human (Rajagopal, *et al.*,2021).

Resistance traits could be transferred by conjugation, suggesting a linkage of the corresponding resistance genes in self-transferable or mobilizable plasmids (Shan, *et al.*,2022).

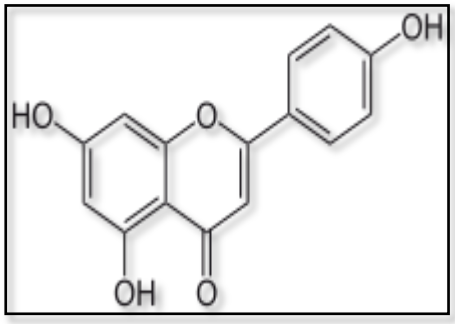
UPEC is the major causative pathogen. First line antibiotics for UTI include fosfomycine (Walker,*et al*.,2022) .

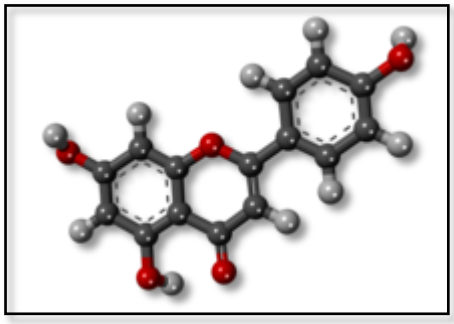
Recent studies suggest that fosfomycine UPEC strains show reduced virulence and are less capable of causing upper urinary tract infection compared with susceptible strains (Karam, *et al*.,2019).

Several studies on clinical *E. coli* have demonstrated relationship between antibiotic resistance and low virulence factors.(Yazdanpour, *et al*.,2020).

1.2.10 Apigenin

(4′,5,7-trihydroxyflavone), found in many plants, is a natural product belonging to the flavone class that is the aglycone of several naturally occurring glycosides. It is a yellow crystalline solid that has been used to dye wool.(Sajeeda , 2022).





1.2.11 Sources in nature:

Chamomile, scientifically known as *Matricaria recutita*, is a plant native to Western Europe and North Asia that is characterized by having a herbaceous bearing and flowering for medicinal use. This species has been used, since ancient times, in many European regions due to its medicinal and health-beneficial properties. It began growing in abundance in the Balkans to later be introduced in central Europe and in countries such as Italy, Bulgaria or Russia. (Némethy, *et al.*,2020).

Apigenin is found in many fruits and vegetables, but parsley,celery,celeriac,and chamomile

tea are the most common sources. Apigenin is particularly abundant in the flowers of chamomile plants, constituting 68% of total flavonoids. Dried parsley can contain about 45 apigenin/gram of the herb, and dried chamomile flower about 3-5 mg/gram. The apigenin content of fresh parsley is reportedly 215.5 mg/100 grams, which is much higher than the next highest food source, green celery hearts providing 19.1 mg/100 grams. (Aleksandra, *et al*., 2021).

1-2-12 Apigenin Benefits

Apigenin is known to offer a range of health benefits, from supporting heart and brain health to promoting healthier skin. Most of the studies involving the flavonoid use animal models, but they highlight the potential mechanisms and benefits of this plant nutrient(Komakech,*et al.*, 2019).

A-Works as an Antioxidant

Flavonoids are widely known for their antioxidant properties, and a number of studies show that apigenin, in particular, has significant antioxidant effects. It’s also an effective anti-inflammatory agent compared to other flavonoids,because of its antioxidant effects, the flavone is used to promote healthy aging and utilized to boost skin health. Topical apigenin is even used to fight acne and promote skin cell growth.(Ginwala,et al.,2019).

B. Has Antimicrobial Effects

Reports suggest that apigenin has “reverse antibiotic” activities against some bacteria, which means that it may be active against antibiotic-resistant bacteria and help reverse bacterial resistance. Researchers believe that the phytonutrient could be a candidate as a new antibiotic or as a dietary supplement to enhance the performance of antibiotics(Thomas,*et al.*,2023).

The nutrient also has proved to inhibit multiple viruses, including herpes; hepatitis C; influenza; hand, foot and mouth disease; and African swine fever.(Ugoeze, *et al.*,2022).

1-2-13 Risks and Side Effects:

Apigenin is considered safe when consumed in normal amounts through a diet rich in fruits, vegetables and herbs. If you experience stomach discomfort after consuming chamomile extract, which is sometimes taken for its high levels of apigenin, then discontinue use immediately(Zhou,et al., 2023)

Topicals containing the nutrient may cause skin irritations in some people, so if you experience any adverse reaction, stop using the product.

More research is needed to fully understand the safety of high apigenin doses. Before using extracts or supplements for a health condition, speak to your health care provider.(Hill, *et al*.,2022)

1-2-14 Dosing:

Doses in adults vary widely and range from 900 mg to 1200 mg per day, (15-25) mg per kg. Half-life may be short because it is often recommended to take chamomile three times per day (Kolanos & Stice,2021).

Chapter Two

Materials and Methods

2.Materials and Methods:

2.1Materials

2.1.1 Laboratory Apparatuses and Instruments:

Listed down in **Tables (2-1)** and **(2-2)**, the main scientific apparatus, and technical instruments with disposable materials respectively, those were employed during the course of this study.

Table (2-1): Scientific Laboratory Apparatus

Item	Company	Country
Autoclave	Herayama	Japan
Bacteriological cabinet	Labogene	Denmark
Benson burner	Dolphin	Syria
Centrifuge	Gemmy	Taiwan
Conventional PCR system	Clever Scientific	UK
Electrophoresis	Clever Scientific	Germany
Freezer	Aucma	China
Light microscope	Stermite Olympus A &D	Japan
Microcentrifuge	Becman	USA
Micropipettes 5-50 µl ,100-1000 µl, 2-20 µl	Top Dragon	Europe
Oven	GS	Taiwan
Platinum wire loop	Himedia	India
Refrigerator	Concord	Italy
Sensitive electric balance	Kern	Germany
UV-transilluminator	Clever Scientific	
Vortex	Gemmy	Taiwan
Water bath	GFL	Germany

Table (2-2): Technical Instruments and Disposable Materials

Item	Company	Country
96-well flat bottomed polystyrene Microplate	Spektar	Serbia
EDTA-tubes	Afco	Jordan
Glass slides	Sail brand	China
Microscopic Cover slide	Gitoglas	China
Millipore filters (0.45mm)	Sigem	Spain
PCR tubes 1.5 ml (Eppendrof)	Biobasic	Canada
Pettri dishes	Blastilab	Lebanon
Plastic test tubes 10ml	Dolphin	Syria
Sterile swabs	Sigem	Spain
Tips	Dolphin	Syria
Wooden sticks	Supreme	China

2.1.2 Chemical Material and Reagent:

The chemical material and reagent list in the Table (2-3)

Table (2-3): Chemical Materials and Reagents.

Item	Company	Countr Y
Catalase reagent, Indole KovacsReagent, Methyl red indicator, 5% alpha naphthol, 40%KOH, 10%KOH.	Schuchariot	Germany
Crystal violet	Sigma	USA

Eosin yellow stain solution 2% W/V.	Central brug house LTD.	India
Ethanol 70%, methanol	Fluka chemika	Switzerland
Ethanol absolute (95%)	GCC	UK
Gram stain kit and giemsa stain.	Crescent	KSA
NaCl, Na2HPO4, KH2PO4, NH4CL, MgSO4, CaCl2, FeSO4.	Merk Darmstade	Germany
Oxidase reagent (Gordon-Mcleod reagent)	Himedia	India
Phosphate buffer	Himedia	India

2.1.3 Culture Media:

Itemized down in **Table (2-4),** the main culture media utilizedin this study.

Table (2-4): Culture Media

Item	Company	Count y
1- Eosin Methylene blue (EMB) agar, MacConkey agar, Nutrient agar, the Brain heart infusion agar, Brain heart infusion broth. Peptone water broth, Muller Hinton agar, MR-VP broth 2- Urea agar base, Triple sugar iron agar, Simmon's citrate agar	Himedia Diffco	India USA

2.1.4Molecular Materials

Categorized and detailed down in **Table (2-5)**, the materials and molecular study.

Table(2-5) Molecular Materials

Materials	Company	Country
Ladder 100bp	Promega	USA
Agarose.	Promega	USA
Blue/Orange Loading Dye, 6X It is used for loading DNA samples into wells and tracking migration during gel electrophoresis.	Biobasic	Canada
Ethidium Bromide Solution, (10mg/ml).	Biobasic	Canada
Genomic DNA extraction Kit	NEX prep	UK
AccuPower PCR PreMix Kit, consist of: Taq DNA polymerase, dNTPs 400µM for each, Tris-HCl (pH 8.5-9.0)10 mM, KCl 30 mM ,$MgCl_2$ 3mM,2eppendroffs of Nuclease free water ,Stabilizer and tracking dye	Bioneer	Korea
Free nuclease water.	Bioneer	Korea
TBE Buffer (Tris-Borate-EDTA), 10X (pH 8.3)	Promega	USA

2.1.5 Antibiotics Disks:

Table (2-6): Antibiotics disks.

No.	*Groups*	*Antibiotics*	*Potency (µg per disk)*
1.	B- lactam	Pipracillin	100
2.		Carbencilline	100
3.		Meropenem	10
4.	Phosphonic	Fosfomycine	200
5.	Aminoglycosides	Amikacin	30
6.		Kanamycine	30
7.	Rifamycine	Rifampin	10
8.	Lincomycine	Clindamycin	10

2-2 Methods:

2-2-1 Preparation of Molecular Materials

2.2.1.1 Preparation of 1X TBE Buffer:

The preparation of 1X TBE buffer was performed by dilution of a concentrated 10X TBE buffer, this dilution was accomplished as 1:10(v/v); 1 volume of 10X TBE: 9 volumes of distilled water. This solution was used to prepare agarose gel and as a transmission buffer in electrophoresis process. Thus each 100ml of 10X TBE added to 900ml of sterile distal water to produce final concentration,1X TBE (Darkazanli,*et al.*,(2023).

2.2.1.2 Preparation of Agarose Gel:

This gel was prepared by adding agarose powder in 1X TBE buffer to be dissolved by boiling, and then it was left to cool to 50ºC. The dissolved amount of agarose powder is depending upon the aim for which agarose is used.

For DNA profile (visualization of the DNA after extraction), 1% agarose is used. While for visualization of PCR product (amplicon), 1.5% of agarose was employed and for single nucleotide polymorphism detection,3% agarose is used.

Ethidium Bromide stock solution with a concentration 10mg/ml was used. Only 5µl of this stock solution were supplemented to 100ml of melted agarose gel to get final concentration 0.5µg/ml.Then after the addition of ethidium bromide, mixed well and dispensed to the tray of gel electrophoresis .(Zheng, *et al*.,2023)

2.2.1.3 Rehydration of Primers:

Lyophilized primer pairs were rehydrated by DNA rehydration

solution 1X (pH 8.0) Tris- EDTA buffer (TE-buffer). Initlially, primer storage-stock tube prepared and then the working solution would prepare from primer stock tube. Consistent with the instructions of the producer (Bioneer/Korea), TE buffer was added to produce 100 picomole/microliter concentration of primer stock solution. The working solution prepared from stock as 1:10 (v/v) by dilution with TE buffer to get 10 picomole/ microliter.

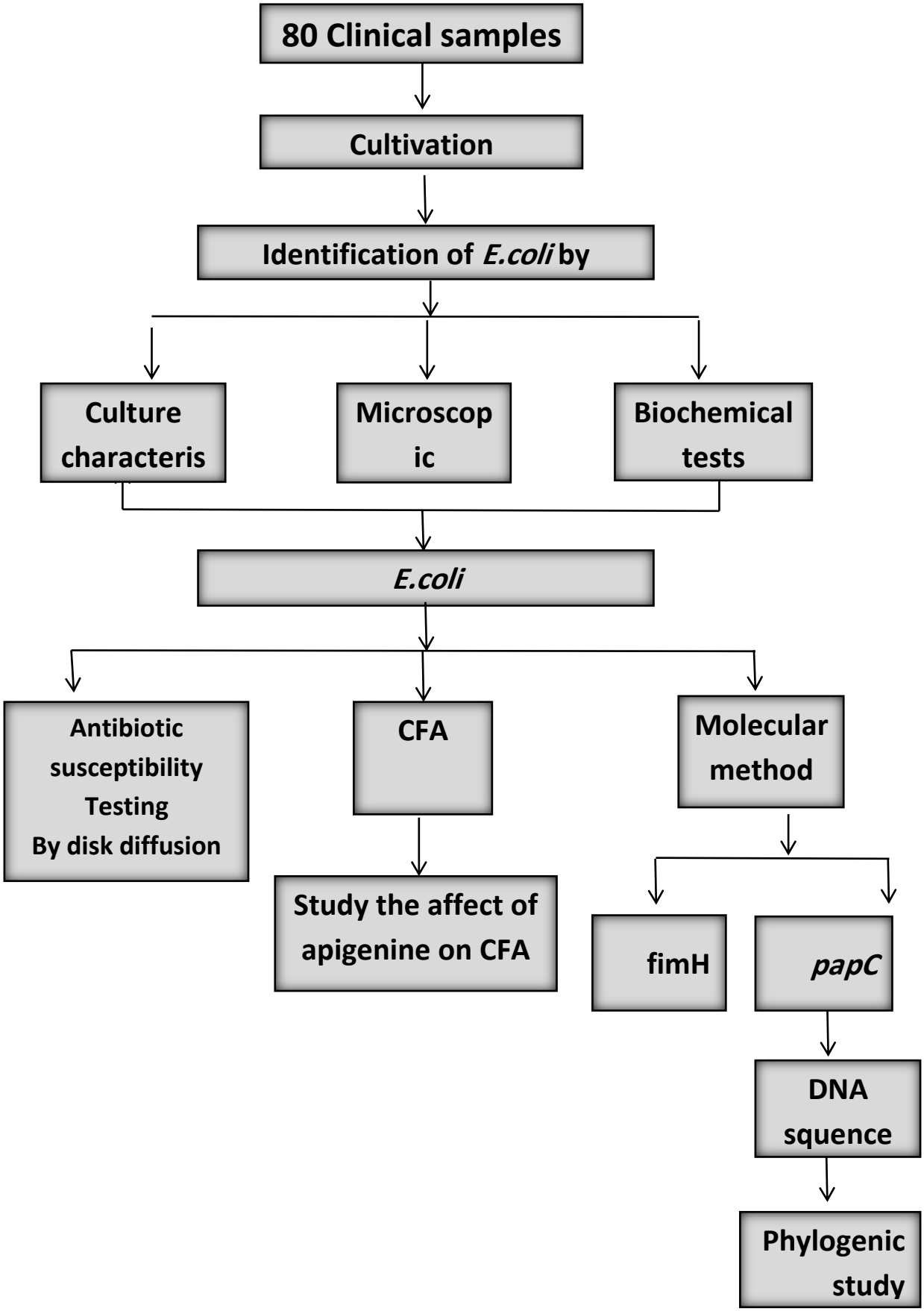
2.2.2 Study design:

This study involved (80) specimens were collected from patients with different disease,the samples collected from both sexes and different ages, who attended to AL-Marjan teaching hospital Hilla city , during the period from July to October 2022.The age of patients ranged from 20to 60 years.

2.2.3 Ethical approval:

1- The necessary ethical approval was obtained by verbal consent from patients.

2- This study was approved by the committee of publication ethics at College of Medicine, Babylon Province, Iraq.



Figure(2-1):study design

2.2.4 Collection of Specimens:

The proper specimens collected for bacteriological analysis are described below. Those specimens were collected in proper ways to avoid any possible contamination (Poore, *et al*.,2020)

1-Urine samples

The specimens were generally collected from patients suffering from UTIs. Mid-stream urine samples were collected in sterilized screw-cap containers.

2-vaginal swabs:

The specimens were generally collected from patients suffering from vaginitis. Collect the vaginal specimen using one sterile swab (CultureSwab) ,Gently press the swab into the vaginal sidewall and rotate the swab four times to thoroughly coat the swab than remove the swab and place it back in the tube. Place the tubes on dry ice or the -80°C freezer within 5 minutes of completing the transfers.

3-wound swabs:

The specimens were generally collected from patients suffering from wound infections. For a swab culture, use a sterile swab to insert the swab deeply into the wound, and gently rotate and immediately place it in the culture tube.

2.2.5 Laboratory Diagnosis:

2.2.5.1Bacterial Identification Assays:-

According to the diagnostic procedures recommended by(Ferone,*et al.*,2020) the isolation and identification of *E. coli* associated with patients under study were performed as follows:

2.2.5.2Colonial Morphology and Microscopic Examination:

A single colony was taken from each primary positive culture. Its identification depends on the morphology properties (colony size, shape,

color, translucency, edge, and elevation of texture). The colonies were

then investigated by gram stain to observe bacterial cells. Specificbiochemical tests were done to reach the final identification.

The *E coli* appeared with circular shape, entire margin, raised, small size, smooth texture, green shiny appearance, on culture media (EMB).

2.2.6 Biochemical Tests

2.2.6.1Catalase Test:

Nutrient agar medium was streaked with the selected bacterial colonies and incubated at 37ºC for 24 hrs, then the growth was transferred by the wooden stick and it was put on the surface of a clean slide, a drop of (3% H2O2) was added. Formation of gas bubbles indicated a positive result (Xu.,2022).

2.2.6.2Oxidase Test:

A strip of filter paper was soaked with a little freshly made reagent, and the colony to be tested was picked up with a sterile wooden stick and smeared over The filter paper a positive result was indicated by an intense deep purple color which appeared within 5-10sec. (Zaouri, *et al.*,2019)

2.2.6.3Indole test:

Indole test was done by adding 6-8 drops of Kovac,s reagent (p-dimethyl amino Benzaldehyde in amyl alcohol). The *E.coli* positive fo this test .The positive reaction was characterized by the formation of red color ring at the top of the broth (Ohtaki, *et al.*,2020)

2.2.6.4Methyl-red test:

The tubes of the MR-VP broth were inoculated with selective bacterial colonies and were incubated at 37oC for 24 hour. Five drops of methyl red reagent were then added to it. The appearance of red color means a positive result hydrolysis of glucose (Ohtaki, *et al.*,2020).

2.2.6.5Vogues – Proskauer test (VP):

The tubes of the MR-VP broth were inoculated with selected bacterialcolonies and were incubated at 37oC for 24 hours. The result was then read by adding 5-12 drops of alpha nephthol (reagent A) and 4 drops of 40% KOH solution (reagent B) the appearance of red color after 15 minutes -1 hrs means a positive result due to the partial hydrolysis of glucose, which produced acetoin or Acetyl - methyl - carbinol (Ohtaki, *et al.*,2020).

2.2.6.6 Citrate utilization test:

After the sterilization of Simmons Citrate slants by autoclave, the bacterial colonies were inoculated and incubated for 24 hours at 37oC. The change of color of media from green to blue indicated that the organisms were ableto utilize citrate as sole carbon source (Benson,*et al.*, 2001).

2.2.6.7 Urease test:

This test was carried out by inoculating urea medium with bacterial growth. The tubes were incubated for 24-48 hours at 37oC. The color change of medium into pink indicated a positive result (Ohtaki, *et al.*,2020).

2.2.7Antibiotic Susceptibility Testing

2.2.7.1 Disc diffusion test (DD test):

The Kirby-Bauer method is a standardized system for this test that takes all variables into consideration. It is sanctioned by the United States FDA and the Subcommittee on Antimicrobial Susceptibility Testing of the CLSI (Nguyen, *et al.*,2020).

1- It was performed by using a pure culture of previously identified bacterial organism (10^7 CFU). The inoculums to be used in grown in this test was prepared by adding growth from

5 isolated colonies on blood agar plates to 5 ml of nutrient broth, this culture was then incubated for 2 hrs. to produce a bacterial suspension of moderate turbidity that compared with turbidity of ready-made (0.5) McFarland tube standard. A sterile swap was used to obtain an inoculums from the standardized culture, this inoculums was then swabbed on Mueller–Hinton plate.

2- The antibiotic discs were placed on the surface of the medium at evenly spared intervals with flamed forceps, and then incubated at 37ºC for a full 18 hrs (Weigelt,*et al.,* 2007).

3- Antibiotics inhibition zones were measured using a transparent ruler. Zone size was compared to standard zones to determine the susceptibility of organism to each antibiotic. (Matuschek, *et al,* 2022)**.**

2.2.8 Genotyping Assays:

2.2.8.1 DNA Extraction from Gram Negative Bacteria:

The extracted of DNA from clinical isolates. One colony of each isolate cultured and inoculated into 5 ml of Brain Heart Infusion(BHI) and grown over night at 37ºC. From these isolate cultures; DNA was purified from bacterial cells using Genomic DNA kit supplemented by the manufacturing company. DNA obtained were used as templates for all PCR experiments.

The DNA concentration is measure by Nano drops machines at 260/280nm give 1.8 purify DNA. The PCR reactions were carried out in a Thermal Cycler. Before PCR assay, the DNA profile was performed by using bacterial DNA and loading buffer without thermal cycling conditions, and according to the following steps:

First- Sample preparation of Gram Negative Bacteria:

1- Overnight bacterial cells (up to 1 x 109) are transfer to a 1.5 ml micro centrifuge tube.
2- Then tubes centrifuged at 14.000 rpm for 1 min. to pellet the cells. the supernatant was Removed.
3- A total of 180µl of GT Buffer aws added, then re-suspend the cell pellet by vortex or pipette.
4- 20µl of Proteinase K is added (make sure ddH_2O was added) then, Incubate at 60ºC for at least 10 minutes. During incubation, invert the tube every 3 minutes.

Second - Cell Lysis:

5- 200µl of GB Buffer is added to the sample and mix by vortex for 10 seconds. Incubate at 70ºC for at least 10 minutes to ensure the sample lysate is clear. During incubation, invert the tube every 3 minutes. At this time, pre-heat the required Elution Buffer (200 µl per sample) to 70ºC (for step of DNA Elution).

Third- DNA Binding:

6- 200µl of absolute ethanol is added to the clear lysate and immediately mixed by shaking vigorously for 10 minutes.

7-GD Column were placed in a 2ml collection tube.

8-All of the mixture was transferred (including any precipitate) to the GD column, centrifuged at 14,000 rpm for 2 minutes.

9-The collection tube was discarded containing the flow through and the GD column was placed in a new collection tube.

Forth - Wash step:

10- 400 µl of W1 buffer is added to the GD Column. Centrifuged at 14,000 rpm for 30-60 seconds.

11-The flow-through was discarded and the GD column placed back in the 2ml collection tube.

12- 600 µl of wash buffer (Ethanol added) is added to the GD column, centrifuged at 14,000rpm for 30 seconds.

13-The flow-through was discarded and the GD column placed back in the collection tube and then centrifuged again for 3 minutes at 14,000 rpm to dry the column matrix.

Fifth - Elution step:

14-The dried GD column was transferred to a clean 1.5 ml centrifuge tube.

15-100 µl of pre-heated elution buffer or TE was added to the center of the matrix, and let stand for at least 3 minutes to ensure the elution buffer is absorbed by the matrix, centrifuged at 14,000rpm for 30 seconds to elute the purified DNA.

16-The DNA was stored at -20ºC to avoid degradation.

2.2.9 Polymerase Chain Reaction (PCR):

Polymerase Chain Reaction was performed in a final volume of 25µl as in Table (2-7). Then DNA amplification was carried out with the thermal cycler.

Table (2-7) Contents of the Reaction Mixture

No.	*Contents of reaction mixture*	*Volume*
1.	**master mix**	**12.5 µl**
2.	**Upstream primer**	**2.5µl**
3.	**Downstream primer**	**2.5µl**
4.	**DNA template**	**5µl**
5.	**Nuclease free water**	**2.5 µl**
Total volume		**25 Ul**

2.2.10 Detection of *E.coli FimH* by PCR:

Nucleic acid (DNA) that extracted from bacterial cells, was used asa template in specific PCR for the detection of virulence genes listed in **Table (2- 8)**. A single reaction mixture contained 2.5□l of upstream primer, 2.5□l of Downstream primer, 5□l of extracted DNA, 12.5□l of master mix and 2.5□l of nuclease free water. The resulting PCR products were run in 1.5% agarose gel.

2.2.10.1 Preparation of Primers:

The upstream and downstream primers are prepared according to the manufacturing company instructions (Bioneer, Korea) and stored at -20ºC.

Table (2-8): The PCR primers with their sequence and amplicon size

Primers	Sequence		Product Size
fimH genes	F	5'- ATGAAAC GAGTTAT TACCCT-3'	903bp
	R	5'- TTATTGAT AAACAAA AGTCAC-3′	

Table (2-9): PCR Thermocycler Conditions

PCR step	Temp.	Time	Repeat
Initial Denaturation	95C	3min	1
Denaturation	94C	40sec.	35 cycle
Annealing	42C	30sec	
Extension	72C	1 min	
Final extension	72C	5min	1
Hold	4C	Forever	-

2.2.11Detection of *E.coli papC* by PCR:

Table (2-10): The PCR primers with their sequence and amplicon size

Primers	Sequence		Product Size
papC genes	F	5 ' GACGGCTGTACTGCAGGGTGTGGCG- 3	328bp
	R	5 ATATCCTTTCTGCAGGGATGCAATA-3'	

Table (2-11): Cycling parameters of *Papc* gene amplification

Cycle No.	Stage	Temp. °C	Time
1	Initial denaturation	**94**	10 min.
35	Denaturation	**94**	2 min.
	Annealing	**65**	30 sec.
	Elongation	**72**	1 min.
1	Final extension	**72**	1 min.

2.2.12 Nucleic acids sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from both termini (forward and reverse directions), following the instruction manual of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Biosystem) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed nucleic acid sequences of local samples with the retrieved nucleic acid sequences, the virtual positions, and other details of the retrieved PCR fragments were identified.

2.2.13 Interpretation of sequencing data

The sequencing results of the PCR products of the targeted samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in their corresponding position within the referring

genome. The observed nucleic acids were numbered in PCR amplicons as well as in their corresponding positions within the referring genome. Each detected variant within the bacterial sequences was annotated by SnapGene Viewer ver. 4.0.4 (https://www.snapg ene.com).

2.2.14 Translation of nucleic acid variations into amino acid residues

The sequencing results of the PCR products were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit suit. The observed variations in each sequenced sample were numbered in PCR amplicons as well as in their corresponding positions within the referring genome. The amino acid sequences of the targeted proteins were retrieved online from the protein data bank (http://www.ncbi.nlm.nih.gov).

2.2.15 Deposition of sequences to GenBank

All the investigated and analyzed sequences were submitted to the NCBI Bankit portal and all the instructions described by the portal were followed as described by the server (Benson, *et al*., 2016). The submitted sequence was provided as nucleic acid sequences in the NCBI to get a unique GenBank accession number for the investigated sequences.

2.2.16 Comprehensive phylogenetic tree construction

A specific comprehensive tree was constructed in this study according to the neighbour-joining protocol described by(Sarhan *et al*. (2019). The observed variants were compared with their neighbour homologous reference sequences using the NCBI-BLASTn server (Zhang *et al*. 2000). Then, a full inclusive tree, including the observed variant, was built by the neighbour-joining method and visualized as an unrooted cladogram using the iTOL suit (Letunic and Bork, 2019). The sequences

of each incorporated species in the comprehensive tree were colored in an appropriate color.

2.2.17 SEQUENCING :

One locus was selected for genetic screening in this study. This locus is made of 328 bp, which belonged to *papC* genes, respectively. Ten samples were included in the present study. These samples were screened to partially amplify the *papC* loci sequences. Thus, the variation of the *papC* loci genes is used for *E. coli* characterization due to their possible ability to adapt to variable genetic diversities as was seen in different bacterial organisms. The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons. The NCBI BLASTn engine showed about 99% sequence similarities between the sequenced samples and the intended reference target sequences. By comparing the observed nucleic acid sequences of these investigated samples with the retrieved nucleic acid sequences of *E. coli* (GenBank acc. AP026794.1), the accurate positions and other details of the retrieved PCR fragments were identified. The total length of the targeted locus was localized in the NCBI server, and the positions of the start and end of the targeted locus were also confirmed within the most homologous bacterial target (Fig. 2-2).

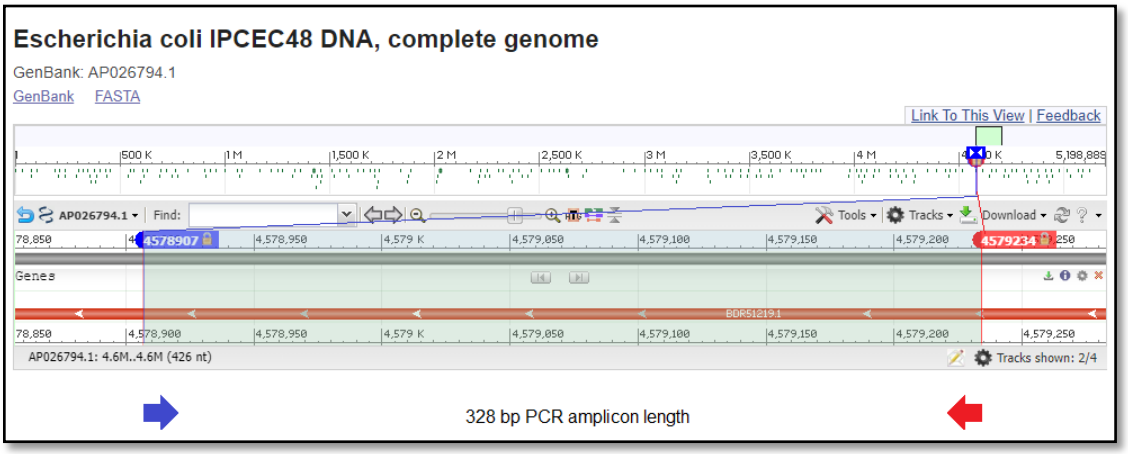


Fig.(2-2) The exact position of the retrieved 328 bp amplicon partially covered a portion of the *papC* gene within *E. coli* genomic sequences (GenBank acc. no. AP026794.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 328 bp amplicons' sequences within the genomic sequences of *E. coli*, the details of its sequences were highlighted, and the total length of the amplified amplicons was also determined (Table 2-12).

Table(2-12). The position and length of the 328 bp PCR amplicons that used to amplify a portion of the *papC* gene within *E. coli* genomic sequences (GenBank acc. no. AP026794.1).

Amplicon	**Reference locus sequences (5′ - 3′)**	**length**
papC gene	*TGTTTTCGGACTGCAGGGTGTGGCGGTTGGAT TGTCAGCCTCAAGGTCTAAATATCTGGGGCGTG ATAACGATTCTGCTTACCTGCGTATATCCGTGC CGCTGGGGACGGGGACAGCGAGCTACAGTGGC AGTATGAGTAATGACCGTTATGTGAATATGGCC GGCTACACTGACATGTTCAATGACGGTCTGGAC AGCTACAGCCTGAACGCCGGCCTTAACAGTGG CGGTGGACTGACATCGCAACGTCAGATTAATG CCTATTACAGTCATCGTAGTCCGCTGGCAAATT TGTCCGCGAATATTGCATCCCTGCAGAAAGGAT AT**	328 bp

* The forward primer is placed in a forward direction

** The reverse primer is placed in a reverse complement direction

2.2.18 Colonization Factor Antigen(CFA):-

A- Detection of (CFA/I):-

After culturing the organism on brain heart infusion broty and incubating it for 24hr. at 37°C, the agglutination of RBC with bacteria occurs in presence of D-mannose as follows:-

1- RBC suspension is prepared from the human blood(group A) and washed with phosphate buffer saline (repeated 3 times). 3% suspension from RBC(v/v) is then prepared.
2- A bacterial suspension is prepared by taking half of the bacterial growth from brain heart infusion broth and mixing it with 1ml of buffer saline to determine RBC agglutination test and vasticated colonization factor antigen type1.
3- On a clean slide, one drop of bacterial suspension is mixed with one drop of 0.1M D-mannose on one side, and with one drop of 3% suspension,and on the other slide one drop of bacterial suspension is mixed with one drop of 0.1M D-mannose on one side with one drop of 3% suspension ,and one drop of apigenine.

The agglutination of RBC with bacteria is detected after 1-2 min in room temperature which is considerd positive.(Liu, *et al*.,2020).

B-Effect of epigenine on CFA:

According to the steps done at (A).Epigenine was prepared at different concentration (0.1%,0.2%,0.5% and 1%) and observed its effect on CFA.

Only one drop of epigenine solution in different concentrations was added to the mixture of CFA solutions to show if there was agglutination or not.

Chapter Three

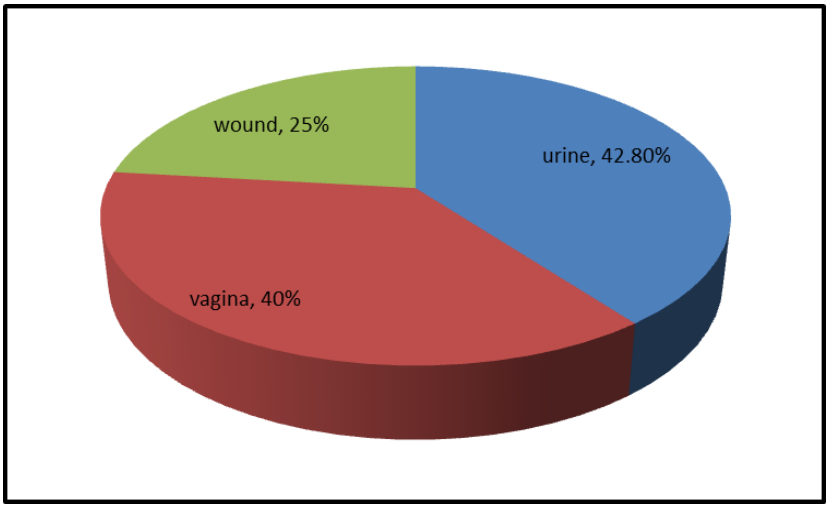
Results and Discussion

3.1 Isolation of *Escherichia coli* :

Among 80 clinical samples,50 samples were positive for bacterial growth, 30 samples were positive for *E.coli* and 20 positive for other organisms such *as Enterobacter, Klebsiela,Staphylococous, Enterococus* ,15 isolates *E.coli* were obtained from 35 urine samples ; 10 isolates from 25 samples of vagina ; 5 isolates from 20 samples of wound.

Table (3-1): Distribution of *E. coli* isolates from clinical samples

Sources of isolates	No. of samples	No. of *E. coli* isolates
Urine	35	15(42.8%)
Vagina	25	10(40%)
Wound	20	5(25%)
Total number	80	30(37.5%)



Figure(3-1): Distribution of *E. coli* isolates from clinical samples

This table indicates that *E. coli* are highly isolated from urine samples, followed by vaginal swabs samples, and then wound samples. These results are identical with Abebe,*et al*.,2019).who confirmed that *E.coli* was predominant in urinary tract infection.

According to the data obtained in this study, only 5 isolates of *E.coli* are isolated from wound infections,these isolates may be come from contamination of the wound from gut or due to hospitialization.

However,many studies have indicated that *E.coli* may be isolated from patients with UTI because this bacteria was isolated from many cases of urinary tract infections such as cystitis,pyelonephritis and urethritis(Rao, *et al.*,2022).

Also,some cases of vaginitis confirmed the presence of *E,coli* in the vagina and causes severe disease which may be associated with vaginal discharge(Wójkowska,*et al*.,2021)

On the other hand ,this bacteria may effect on wounds in male and female particulary those with post-operative wounds which may come as a result of contamination with faces or due to bad sanitation in the hospitals(Ayalew,*et al.*, (2019).

3.2 Biochemical Tests:

The main features of the isolated *E.coli* are sumerized in table (2-8) is show below.

Table (3-2): biochemical test of *E.coli*

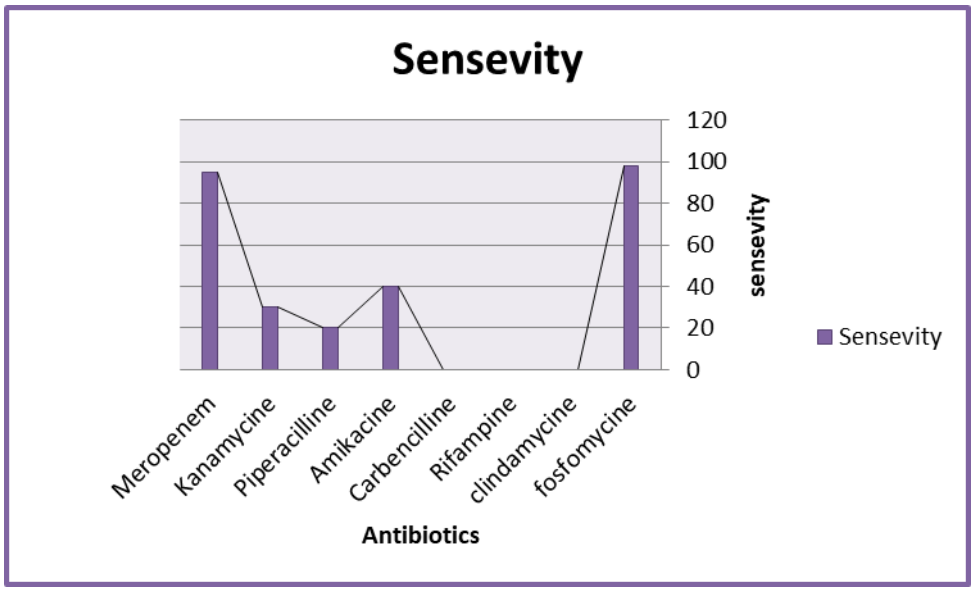
Characteristics	Test result of *E.coli*
Gram Staining	Negative
Capsule(Capsulated/Non-	Variable

Capsulated)	
Catalase	Positive (+ve)
Citrate	Negative (-ve)
DNase	Negative (-ve)
H_2S	Negative (-ve)
Hemolysis (Alfa/Beta/Gamma)	Some Strains shows Hemolysis(B)
Indole	Positive (+ve)
Lactose fermentive	Positive (+ve)
Lysine decarboxylase	Positive (+ve)
MR(Methyl red)	Positive (+ve)
Nitrate Reduction	Positive (+ve)
Motility (Motile/Non-Motile)	Motile
Oxidase	Negative (-ve)

3.3 Antibiotics susceptibility test of *Escherichia coli:*

All the identified *E. coli* isolates were subjected to *in vitro* susceptibility test by modified Kirby - Bauer disc diffusion method. Selective antibiotics were used to show their effect on *E. coli* strains isolates. It has been observed that, the highest rate of resistance is seen with clindamycine 0%, rifampin0% and carbenciline 0%, and are moderately resistant to piperaciline 20% ,kanamycine 30%, and Amikacine 40% whereas some isolates have shown highest rates of sensetivity to fosfomycine 98% ,meropenem 95%,,were susceptibility of *E.coli* strain to this antibiotics reach up to 97%. All *E. coli* isolates were

subjected to antibiotic sensitivity tests (Fig. 3-1), the susceptibility was found to be 95 and 98% for meropenem and fosfomycine .



Figure(3-2):Antibiotic sensitivity tests in *E.coli*

In this work, it was found that some of *E. coli* isolates were resistant to more than four antibiotics, which mean that an alternative choice of antibiotic is needed to eradicate E.coli associated with different infection(Zalewska,*et al.*, 2020).

Moussa, *et al*.,(2021) Reported that 40% was resistence to clindamycine. The results of this study showed the presence of high rate of sensitivity toward the antibiotics meropenem and fosfomycine at a rate 100%.

The precence of high rate of sensitivity to those antibiotics will encougrage to use them in treatment of *E.coli* associated with urinary tract infection ,where Nabrive,*et al*.,(2020) indicate the importance of fosfomycine in treatment of UTI caused by *E.coli.*

However, Amikacine is also used for treatment *E.coli* infection but the rate of sensitivity is 40%, this means that there are some mutants of *E.coli* which have ability to resist the action of this antibiotics is mentioned by Hossain,*et al*.(,2020).

Besides,the bacteria showed high resistance rate (100%) against rifampicine ,carbenciline and clindamycine and this results are identical to those results obtained by Kurpina,*et al*.,(2022) who found that the bacteria may reveal high resistance to rifampin and carbenciline.

However,clindamycine is not widely used to treat infections caused by gram negative bacteria and it is restricted to treat infections caused by gram positive infections particularly in case of vaginitis(Bungau,*et al.*,2021).

3.4 molecular detection of *Fim*H gene in *Escherichia coli* Isolates*:*

E coli isolates were identified using biochemical tests and were screened by PCR. Reported by(Al-Fatlawi, *et al.*,2020)The *fim* H gene was amplified using specific primers and showed a band about 903bp. The *fim*H gene was found in 28 isolates of the *E.coli* strains at a rate 93.3%. The type 1, or mannose-sensitive, fimbriae are produced by >80% of *E. coli* reported by (Bessaiah,*et al*.,2022) .It is now well established that the expression of type 1 fimbriae by E. coli is a virulence factor for pathogenesis of the urinary tract. Meysam,et.al,(2020), have been reported that more than 95% of all E. coli isolates express type 1 fimbriae. This pilus is defined as mannose-sensitive, because it is able to

interact with the mannosylated receptors expressed by epithelial cells, particularly urothelial cells .This specific function relies on the expression of the adhesion Fimh located at the tip of the type 1 pilus (Flament, *et.*

al.,2019) , when observing the presence of the adhesin Fimh in the total isolates of Escherichia coli in urine cultures of isolates**.**

In this study, fimH gene is detected in most *E.coli* isolates (29 isolates) by using PCR technique,Where specific primer is used for this study.The results found as shown figure (3-2) that only 29 isolates gave positive results for the presence of fim H gene where as this gene is not observed in other isolates.

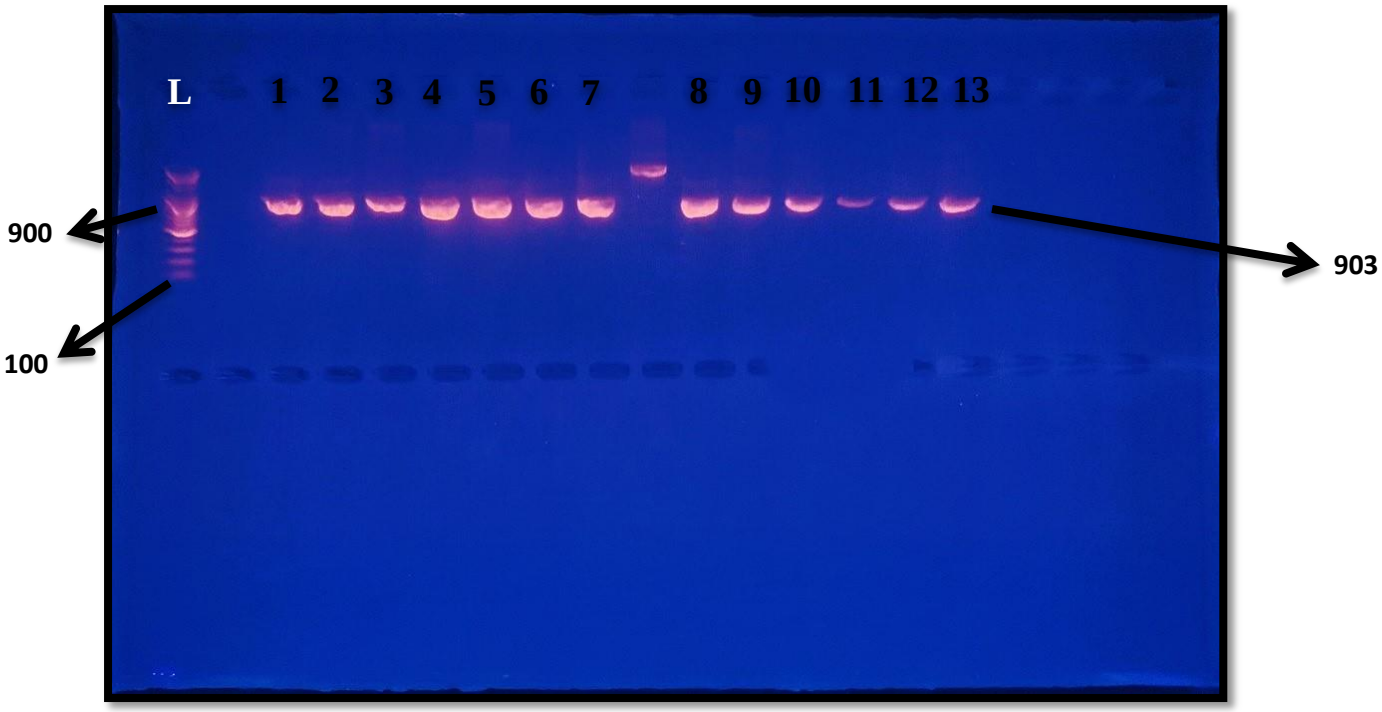


Figure (3.3): **1% Agarose gel electrophoresis at 70 volt for 50 min for *Fimh* PCR products visualized under U.V light at 301 nm after staining with ethidium bromide.the size of product is 903 bp.**

3.5 molecular detection of *pap*C gene in Escherichia coli Strains Isolates*:*

In this study, The *E coli* isolates are screened by PCR to detect papC gene. The papc gene was amplified using specific primers and showed a band about 328bp. The papc gene quality was found in 24

isolates of the *E coli* isolates as shown in figure( ) which means that papc gene, considered the most important virulence factors in bacteria *E coli.(*Yazdanpour, *et al*., (2020)

Aldawood,*et al*., (2020).reported that E coli isolates were identified using biochemical tests and were screened by PCR. The papc gene was amplified using specific primers and showed a band about 328bp.

The papc gene was found in 24 isolates Of 30 isolates positive for *E coli.*

El-Baz, *et al.*, (2022) reported that more than 90% of all *E. coli* isolates express type 2 fimbriae papc .

Basri, *et al.,(*2022).show the type 2 P fimbriae, are the most commonly implicated bacterial cell surface virulence factors,these virulence factors in E.coli bacteria causes chronicity, persistence, and recurrence of infections that cause high morbidity .

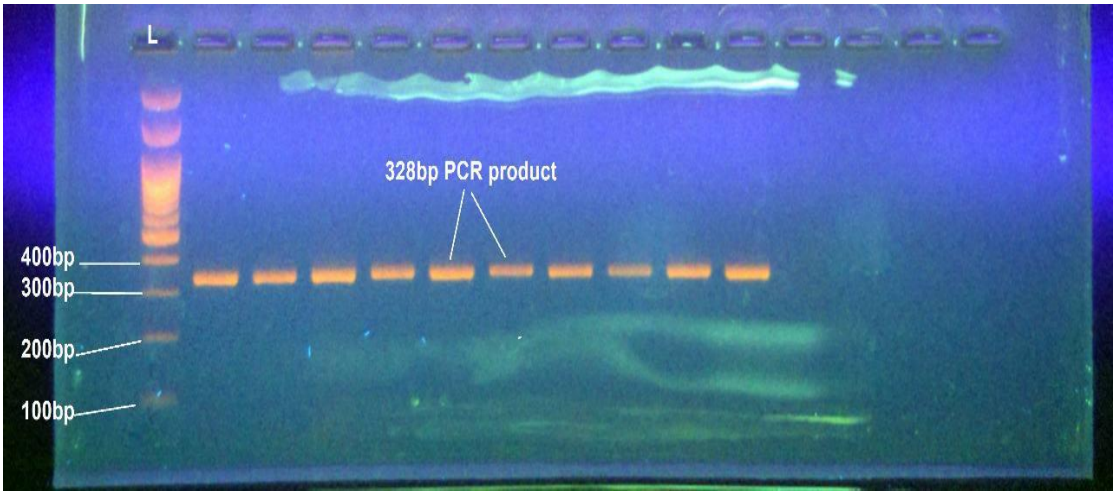


Figure (3.4): **1% Agarose gel electrophoresis at 100 volt for 5 min then 70 volt for 30 min for *papc* PCR products visualized under U.V light at 301 nm after staining with ethidium bromide. the size of product is 328 bp.**

3.6 DNA sequenc of *Papc* Gene :

In current study, DNA sequencing tested on 10 clinical *isolates*. The Sequencing reactions were performed on the *papc* PCR products.

The alignment results of the 328 bp samples revealed the presence of two nucleotide variants represented by one nucleotide substitution compared with their referring reference nucleic acid sequences (Fig. 3-5). These two nucleic acid substitutions were only identified in the S3 sample, while the other nine samples did not exhibit any alteration compared with the reference sequences with which they were compared.

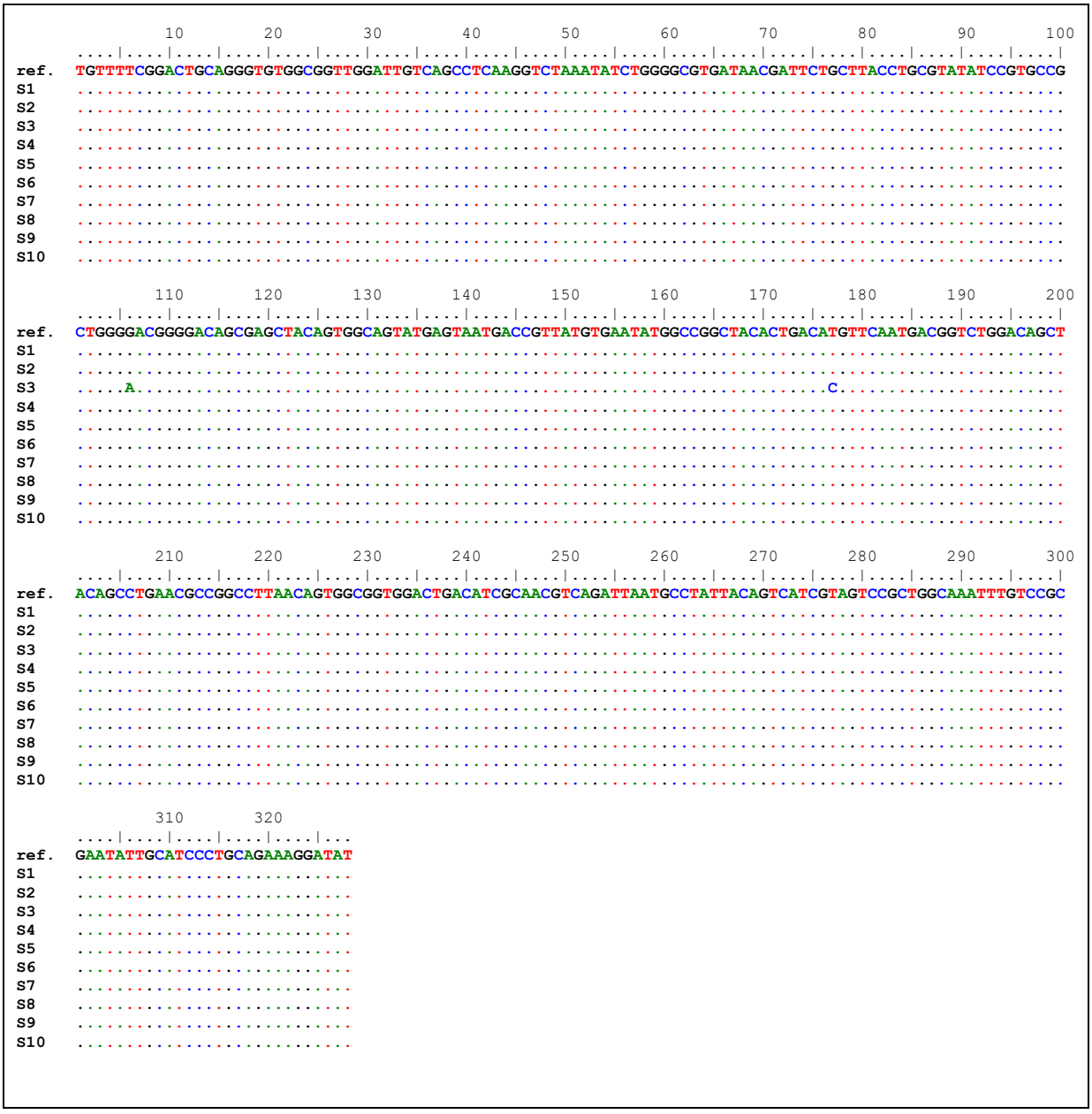


Fig.(3-5) Nucleic acid sequences alignment of ten samples with their corresponding reference sequences of the 328 bp amplicons of the *papC* gene with *E. coli* sequences. The symbol "ref" refers to the NCBI referring sequence, letter "S", followed by a number refers to the sample number.

The results of this study indicated the presence of two nucleic acid variants, namely 106G>A and 177T>C. To confirm this sort of variation, the sequencing chromatograms of the investigated samples, as well as their detailed annotations, were verified and documented, and the chromatograms of their sequences were shown according to their positions in the PCR amplicons (Fig. 3-6).

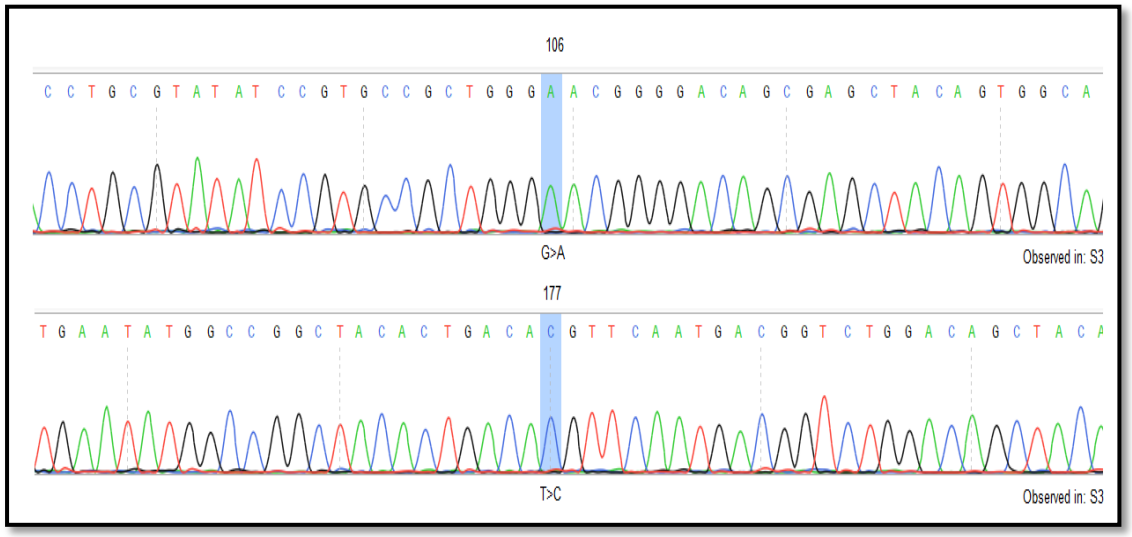


Fig.(3.6) The chromatogram of the *E. coli* DNA sequences of the *papC* sequences. The symbol ">" refers to the "substitution" variation.

All the investigated sequences were deposited in the NCBI web server, and unique accession numbers were obtained for the analyzed S1 – S10 sequences. The deposited sequences received the GenBank accession number represent the S1, S2, S3, S4, S5, S6, S7, S8, S9, and S10 samples, respectively.

The identified nucleic acid sequences were further analyzed to identify their accurate positioning of the detected 106G>A and 177T>C variants in their corresponding positions in the encoded outer membrane usher protein PapC. The amplified nucleic acid sequences were translated to their corresponding amino acid sequences using the Expasy translate suite. The amino acid alignment of these amino acid sequences with their references showed the exact positions of the utilized amplicons within the outer membrane usher protein PapC. Translation results showed that the amplified fragment had occupied 109 amino acid residues of the entire protein which is made of 836 amino acid residues (Fig. 3-7). The translation of the *papC* amplicons showed that the detected 106G>A variant was positioned in the amino acid Ser that is situated in 576 of the entire length of the protein. This variant caused a silent (synonymous) effect in the encoded protein, namely p.567Ther=. Whereas the translation of the identified 177T>C variant caused a missense

(nonsynonymous) effect on the entire amino acid sequences of the same protein, namely p.599Met>Thr.

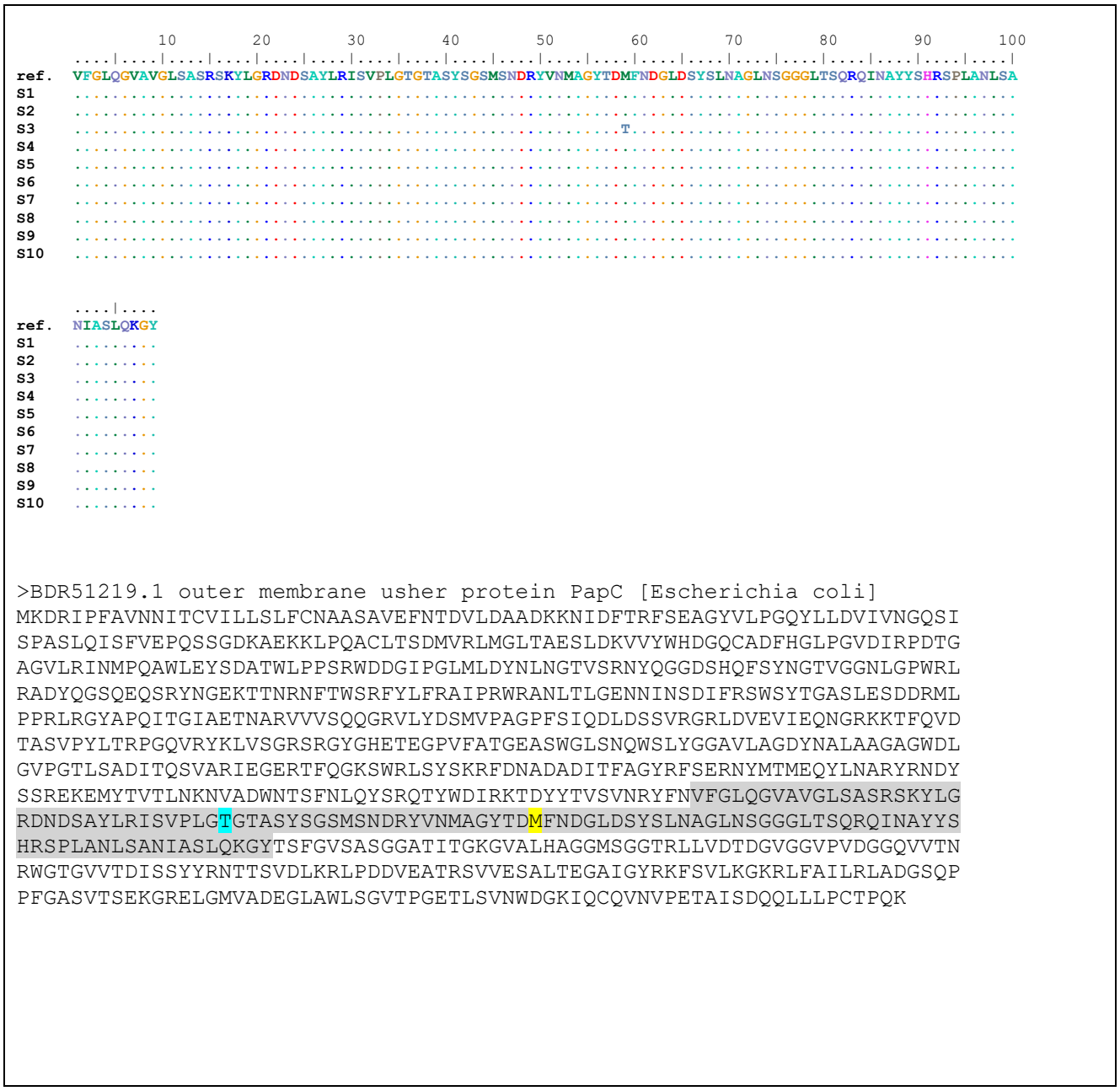


Fig.(3-7). Amino acid residues alignment of the investigated sequences of genomic sequences of *papC* gene of *E. coli* sequences. The amplified amino acids found in the encoded proteins are highlighted according to their corresponding positions within the amplified fragment and the entire protein. The grey highlights refer to the amplified region of the encoded protein in the chart, while the cyan and yellow colors refer to the detected synonymous and nonsynonymous variants, respectively.

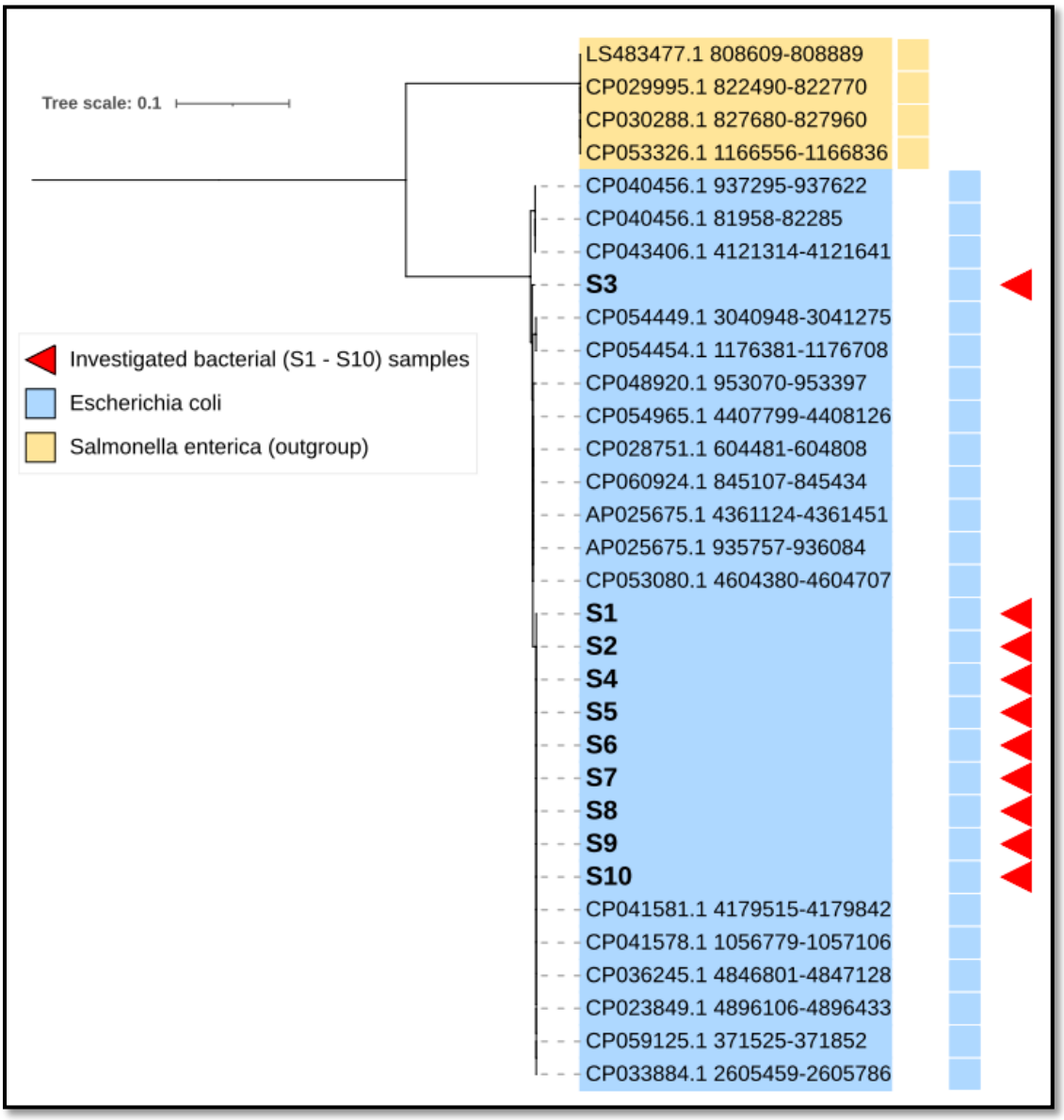


Fig..(3-8)A comprehensive rectangular cladogram phylogenetic tree of genetic variants of the *papC* sequences of one *E. coli* sample. The red-colored triangle refers to the analyzed bacterial variants. All the mentioned numbers referred to GenBank accession number of each referring species. The number "0.1" at the left portions of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letter "S#" refers to the code of the investigated samples.

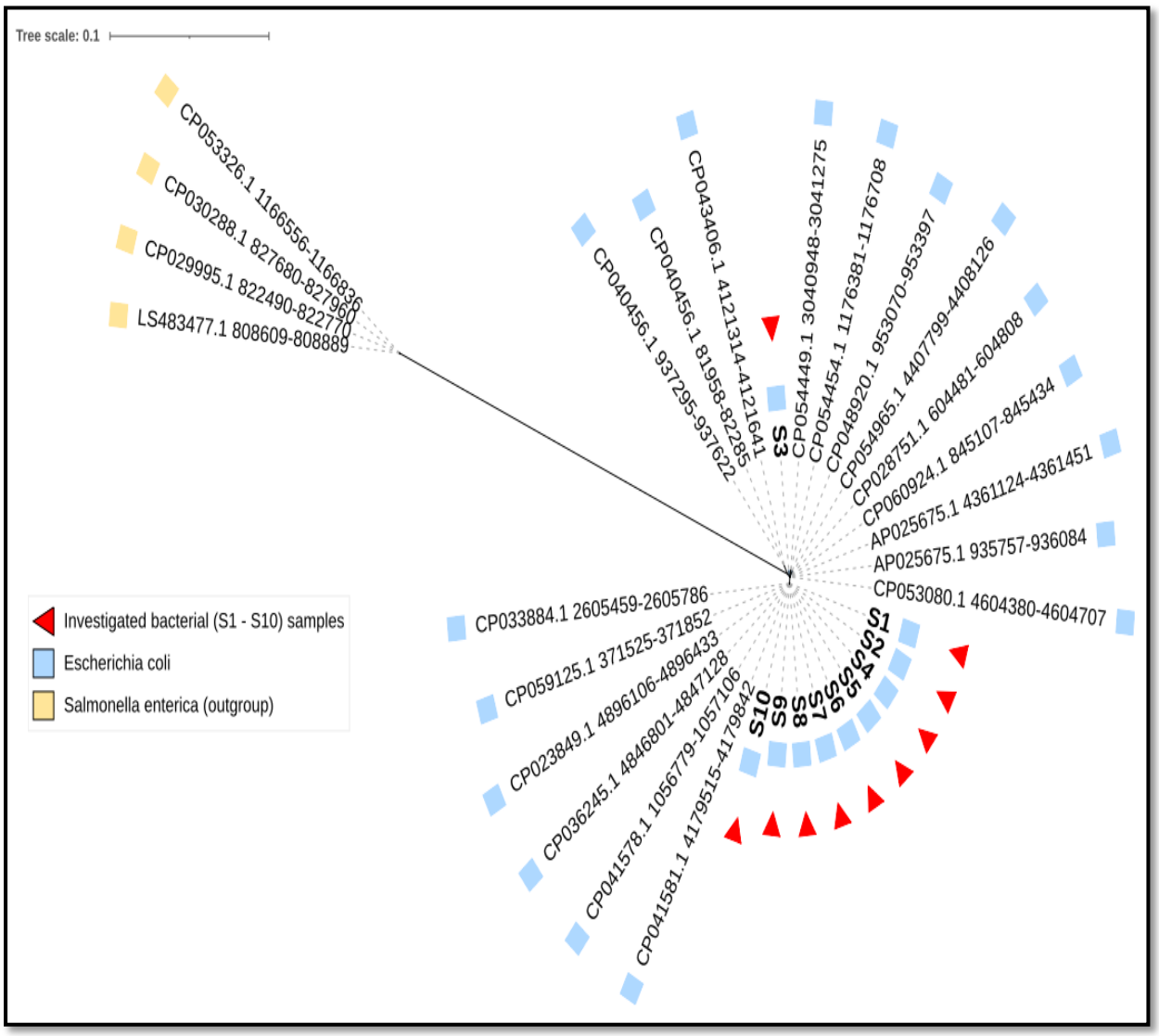


Fig. (3-9) A comprehensive unrooted cladogram phylogenetic tree of genetic variants of the *papC* sequences of one *E. coli* sample. The red-colored triangle refers to the analyzed bacterial variants. All the mentioned numbers referred to GenBank accession number of each referring species. The number "0.1" at the left portions of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letter "S#" refers to the code of the investigated samples.This belongs to E.coli strains but because of mutation it will away from other strains

A comprehensive phylogenetic tree was generated in the present study according to nucleic acid variations observed in the amplified 328 bp of the *papC* amplicons. This phylogenetic tree contained the investigated samples alongside other relative nucleic acid sequences of *E. coli* sequences.

The investigated sample was incorporated alongside other relative sequences to constitute two main phylogenetic positions of incorporated sequences within the generated cladogram. One other related organism was found to exhibit high homology to the incorporated sequences of *E. coli*. These related sequences were represented by the *Salmonella enterica* sequences, which were placed as outgroup sequences in the vicinity of the *E. coli* sequences. Within this tree, five bacterial samples belonging to *S. enterica* were added as an outgroup to assess the extent of the *E. coli* variations. However, our investigated samples were clustered away from the incorporated clades of *Salmonella enterica*. This aggregation was found away from the clade of the outgroup of the *Salmonella enterica* clade since there was a considerable phylogenetic distance between both clades. This notion indicated the presence of lower homology of *papC* sequences between both *E. coli* and *Salmonella enterica* sequences. Owing to this obvious discrimination, it is rational to depict the ability of the *papC* sequences to differentiate between both species. Accordingly, no interactions of the *papC* gene-based amplicons in terms of the detect the currently investigated bacterial sequences of the S1 – S10 samples were seen with any other bacterial species. Thus, no confusing overlap with other sequences of any outgroup species was observed. In this thorough tree, there were thirty-two aligned nucleic acid sequences in all.

As indicated above, our investigated samples were clustered into two main phylogenetic positions of close phylogenetic distances within the *E. coli* clade. However, two types of cladograms were generated to explain two different representations of the incorporated *E. coli* sequences, a rectangular cladogram (Fig. 3-8), and an unrooted cladogram (Fig. 3-9). In both cases of the constructed cladograms, the investigated samples were clustered into two main clades. The most interesting fact observed in our investigated bacterial isolates is correlated with the phylogenetic potentials of the utilized *papC* gene sequences-based amplicons to detect the presence of no high homology between *E. coli* and other related bacterial sequences, such as the *S. enterica* outgroup sequences.

Within one of the two major clades of *E. coli*, our investigated sequences exerted two patterns of phylogenetic distributions within the generated tree. The S1, S2, S4, S5, S6, S7, S8, S9, and S10 samples were suited in one phylogenetic place in close vicinity to various strains of the *E. coli* sequences that have been deposited from the USA (GenBank acc. no. CP041581.1, CP041578.1), Australia (CP036245.1), Sweden (GenBank CP023849.1), Ghana (GenBank CP059125.1), and Norway (GenBank CP033884.1). On the other side of this clade, a slight tilt was observed between the S3 sample and these related strains. The reason behind this slightly different positioning was attributed to the detected variations (106G>A and 177T>C) in the S3 sample. It was inferred from the constructed tree that the detected nucleic acid substitutions showed a tiny evolutionary effect of the variations observed in the bacterial sample in comparison with the other investigated wild-type bacterial samples. Nevertheless, the S3 sample was positioned in a distinct phylogenetic position compared with the other investigated nine samples. Within this

position, the S3 sample was located beside variable strains deposited from Norway (GenBank CP043406.1), China (GenBank CP040456.1 and CP040456.1), USA (GenBank CP054449.1 and CP054454.1). Furthermore, the presence of significant evolutionary distances between *E. coli* and *S. enterica* clades indicated the high resolution of the currently utilized PCR products of the *papC* sequences in the identification of *E. coli* and its discrimination from the other related organisms. The current observation of this phylogenetic tree has confirmed sequencing reactions because it explained the real neighbour-joining-based positioning in such observed nucleic acid variations. Interestingly, the multiple geographical distributions of our investigated bacterial samples could not be ignored.

It is interesting to note that using the *papC* gene sequences in this study has provided additional evidence for the existence of correct identification of the true identities of the bacterial species now under investigation. These data, therefore, confirm our discovery of the divergence of these infectious pathogenic bacterial genomes from distinct geographical sources and are consistent with one another. This in turn provides more evidence of the capacity of the *papC* locus sequence-specific primers currently in use to describe the examined *E. coli* and their phylogenetic positions.

3.7 Detection of colonization factor antigen(CFA) by *E.coli* isolates:

Colonization factor antigen (CFA) was investigated in all E.coli isolates.The method of detection showed that all clinical isolates of this bacteria gave positive results of agglutination in the presence of mannose sugar.This positive results mean that this bacteria may have different colonization factor that give rise to the ability of bacteria to adhere different types of tissues at various rates where the bacteria seems to

show resistance to mannose sugar which is one of the most important sugar in the structure of numerous tissues in the human.

Epigenine is also used in this study to show its affect on CFA at various concentrations .The results showed that Epigenine has no effect of CFA where there is no effect on agglutination results of CFA detection.

This means that Epigenine may have no effect of CFA because colonization factor antigen may not include in its structure the Fim H protein ,where the later has strongly influenced by Epigenine or showed by molecular docking done by previous study conducted in Babylon province by (alaa,*et al*.,2022).

table(3-3):Effect of Apigenine on CFA

Bacteria Name	CFA in the absence of Epigenine	CFA in the present of Epigenine			
		0.1%	0.2%	0.5%	1%
E.coli(30)	Positive agglutination (30 isolates)	No effect	No effect	No effect	No effect

The presence of agglutination after exposure to Epigenine may be not related to fim H but may be to other colonization factors such p fimbiriae,s fimbiriae and Pap proteins.(Lamees.*et al*.,*(*2005).

Conclusions and Recommendations

Conclusion:

1- *E.coli* could be a cause of different clinical diseases.
2- Fosfomycine and Meropenem are high effective on E. coli isolates.
3- *Fim* H& *pap* C are important virulence genes , detected among most isolates.
4- gene sequencing for pap C showed only one isolate has silent mutation.
5- Epigenine has no effect on CFA which is positive in all isolates.

Recommendations:

1- molecular docking should be study for *pap* C to show the effect of epigenine on this adhesive factor.
2- study of DNA adduct to show the effect of different compounds on adhesive factors in *E.coli.*
3- Investigation on other genes associated with bacterial colonization.
4- Fosfomycine and merpenem should be considered in treatment of *E.coli* infections.

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الخلاصة:

الأشريكية القولونية هي بكتريا تنتمي الى عائلة البكتريا المعوية.تعد مجموعة جينات ال *pap* و*fim*جين من العوامل اللاصقة الاساسية في الاشريكية القولونية وخاصة المسببة للأمراض ,حيث يؤدي تجمع هذه الجينات الى تصنيع كلا النوعين من الشعيرات على سبيل المثال يتم تجميع انواع من الخمل بواسطة العديد من الجينات مثل *fim*H,*fim*C,*fim*D وايضا بالنسبه ل p fimbriaeالذي تتحكم فيه الجينات *pap*R,*pap*C وغيرهم.

تم في هذه الدراسة الحصول على 80 عينة من المرضى الذين يعانون من امراض مختلفة والذين راجعوا مستشفى المرجان التعليمي في مدينة الحلة في محافظة بابل خلال فترة من تموز الى اكتوبر 2022.

جمعت 80 عينة سريرية في هذه الدراسة للتحري عن الاشريكية القولونية,وقد وجد أنه تم الحصول على 30 عزلة فقط تعود لبكتريا الأشريكية القولونية ,منها 15 عزلة من عينات البول,10 عزلات من المهبل و 5 عزلات من التهابات الجروح.

درس تأثير بعض المضادات الحيوية حيث وجد أن Fosfomycineو وMeropenem فعالان بشكل كبير على عزلات الأشريكية القولونية (98.7%,95%) غلى التوالي,يليهما Amikacineو Kanamycineو Piperacline.

درست بعض عوامل الألتصاق في 15 عزلة بأستخدام تقنية PCR حيث تم تضمين *fim*Hو*pap*C في هذه الدراسة.

وجد أن جين *fim*H كان موجودا في 29 عزلة (93.3%) عند أستخدام بادئ خاص لهذا الجين ,في حين أن 24 عزلة فقط من بكتريا الأشريكية القولونية أعطت نتائج أيجابية لجين *pap*C بأستخدام بادئ خاص بنسبة 80%.هذه الجينات هي الاكثر شيوعا ووجودا بين عزلات الأشريكية القولونية.

حددت موضع واحد في جين *pap*Cالمتعلق بالعزلات العشرة لبكتيريا الأشريكية القولونية التي تعطي ايجابية لهذا الجين لتسلسل الحمض النووي.

أظهرت أمبليكون,10 عزلات معدل تشابه عالي في تسلسل الحمض النووي وأظهرت النتائج أنه تم رؤية متغيرين فقط في هذا التسلسل حيث تم استبدال نواة واحدة177T>C. حدث هذا في عزلة واحدة فقط,بينما أعطت نتائج التسعة الأخرى نفس التسلسل بدون تغيير.

مع ذلك,تم فحص مستضد عامل الاستيطان (CFA/1) في جميع العزلات السريرية لبكتيريا الأشريكية القولونية.وقد لوحظ أن جميع العزلات أعطت أيجابية لهذا المستضد.

عند أضافة الابجينين بتراكيز مختلفة (0.1%,0.2%,0.5%,1%) لوحظ أن الأبجينين ليس له أي تأثير على CFA/1 مما يعطي مؤشرا على أن الأشريكية القولونية تحتوي على أكثر من عامل لاصق يستخدم لربط الانسجة المختلفة.

وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية الطب
فرع الأحياء المجهرية

التشخيص الجزيئي لجينات *fimH* و pap C و تأثير الابجينين على عوامل الاستيطان لعزلات سريرية من الاشريشية القولونية

رسالة مقدمه إلى مجلس كلية الطب / جامعة بابل
وهي جزء من متطلبات نيل درجة ماجستير في العلوم /الأحياء المجهرية الطبية

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