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Molecular detection of some virulence genes in *E. coli* isolated from women with urinary tract infections

A thesis submitted to the College of Science, University of Diyala in partial fulfillment of the requirements for the Master Degree in Biology

By

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2021 A.D **1442 A.H**

بِسْمِ اللهِ الرحمنِ الرحيم

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I clarify that the thesis titled "**Molecular detection of some virulence genes in *E. coli* isolated from women with urinary tract infections**" was prepared under my supervision at the Department of Biology / College of Science /University of Diyala, as a partial requirement for the degree of Master of Science in Biology.

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We clarify that we have read this thesis titled "**Molecular detection of some virulence genes in *E. coli* isolated from women with urinary tract infections**" and we examined the student (Asia Qahtan Ahmed) in its content and in what is related with it, at (//2021) and in our opinion, it meets the standard of a thesis for the degree of Master of Science in Biology.

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Dedication

I would like to dedicate this work to…

My gracious parents, may Allah keep protecting them…

My partner in life, to my dear husband **Abdul-Basit**…

My sweet, little angels - **Reyam**, **Humam**, **Hesham**…

Those who helped and supported me, my brothers and sisters…

My brother from a different mother - dear **Ali**…

Those who accompanied me during my study - my friends…

Those who taught me even a single letter…

I give to you the humble fruition of my work.

- **Asia**.

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And finally, my thanks, gratitude and appreciation to my family, praying to The Almighty to give them good health… and to those that I forgot to mention may Allah give them the best of rewards.

- Asia.

Summary

Escherichia coli is one of the main contributors to UTIs and it's highly resistant to antibiotics and these are due to the many virulence factors which enables them to cause UTI and even severe and chronic cases in women. A total of 30 *E. coli* isolates were obtained from 200 urine samples from women suffering from UTI symptoms from Al-Sa'adiya, Jalwla'a and Khanaqin General Hospitals. This included pregnant and non-pregnant women with an age range of 15 – 40 years from eastern Diyala Province during the period from September to November. Primary diagnosing of the isolates was done by specific culture media (MacConkey agar, Eosin-Methylene Blue, blood agar). Sorbitol MacConkey agar was used to distinguish EHEC from the rest of the isolates. Biochemical tests and Vitek 2 System were used to confirm the final diagnosing. A total of 30 *E. coli* isolates were obtained, 11 being of pregnant and 19 of non-pregnant women, and 3 EHEC were also detected from the obtained isolates.

Susceptibility testing was done to test the isolates' resistance and it was noted that nalidixic acid and ceftazidime were the most resisted antibiotics tested while meropenem was the most effective antibiotic tested. Resistance patterns for the isolates was also calculated and 7 isolates were MDR and the remaining 23 were XDR. Minimum inhibitory concentration was measured for all isolates using

ceftazidime and cefepime on Muller-Hinton agar and sequence dilutions and results were 512 and 1024µg/mL, respectively. The study also includes phenotypic detection of some virulence factors. Biofilm production was tested using two methods: tube method (TM) and micro-titer plate (MTP) method. The first method showed 19 (63.3%) biofilm-producing isolates while the second method showed 25 (83.3%) biofilm producers. ESBL production was also tested using double disk synergy test and 19 isolates were producers while 11 were not. Hemolysin production was tested using sheep blood agar and it was found that 3 isolates showed β-hemolysis, 9 showed α-hemolysis and 18 showed ɣ-hemolysis. Capsule production was also investigated using nigrosin stain followed by direct microscopy and it was found that 23 (76.6%) were surrounded by a capsule. Molecular detection was carried out on 15 out of the 30 isolates for virulence genes. Multiplex PCR was used to detect the adhesion genes (*fimH*, *pap, sfa*) and *ompT* which has an important role in biofilm production. Results showed that all (100%) isolates had *fimH* while 12 (80%) had *pap*, 10 (66%) had *ompT* and *sfa* was found in only 7 (46%) isolates. Uniplex PCR was used to look for *kpsMII* which responsible for capsule formation and it was found in 8 (53%) isolates. The same method was used to look for *bla-TEM*, the gene encoding ESBL, and *eae*, a gene used to identify EHEC. It was found that 9 (60%) isolates had *bla-TEM* while 3 isolates had *eae*, accounting for 26.6% of the isolates which belong to the EHEC subtype.

Abbreviation List

Abbreviation	Key
µg/mL	microgram/milliliter
BFP	Bundle-forming pilus
CDC	Center for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
DAEC	Diffusely Adhering *Escherichia coli*
DDST	Double disk synergy test
DNA	Deoxyribase nucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EDTA	Ethylene Diamine Tetra Acitic acid
EHEC (STEC)	Enterohemorrhagic *Escherichia coli* (Shiga Toxin *Escherichia coli*)
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme-linked immunoabsorbance assay
EMB	Eosin Methylene Blue
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended-spectrum beta-lactamase
ETEC	Enterotoxingenic *Escherichia coli*
HUS	Hemorrhagic colitis
KIA	Kligler iron agar
KOH	Potassium hydroxide
LEE	Locus of Enterocyte Effacement
LPS	Lipopolysacchrides
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
MR-VP	Methyl red—Voges-Proskauer
OD	Optical density

Abbreviation	Key
OMP	Outer membrane protein
PAI	Pathogenicity island
PDR	Pan-drug resistant
QS	Quorum sensing
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
WHO	World Health Organization
XDR	Extensively drug resistant

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Introduction

Urinary tract infections (UTIs) are a worldwide issue that affect community and hospitalized individuals resulting in a decrease of the patient's life quality. The main causative agents of UTI are Gram-negative bacilli Enterobacteriaceae which include the uropathogenic *Escherichia coli* (UPEC) responsible for 80% of UTI cases worldwide (Korbel *et al.*, 2017). The bacteria has the ability to colonize and cause UTI by the virulence factors its carrying, including adhesion (encoded by genes such as *fimH, sfa* and *pap*), toxins (e.g. hemolysin encoded by *hlyA)*, capsule (encoded by *kpsMII*), invasins (encoded by *ompT*), and iron-uptake systems which are encoded on pathogenicity islands (PAIs) found on chromosomes or plasmids (Dadi *et al.*, 2020). The distribution of virulence factor-encoding genes of UPEC strains will open up a way for identifying them based on these genes' role in disease progress (Brons *et al.*, 2020). UTIs are more common during pregnancy due to a variety of changes happening to the female body and especially the urinary tract area (Ahmed and Yosry, 2021).

Antibiotic resistance is one of the main worldwide health issues responsible for thousands of deaths and that is expected to get worse over time (Adzitey, 2020). Some estimate that it will lead to 10 million deaths annually by the year 2050 (Bengsston-Palme *et al.*, 2018; Praveenkumarredy *et al.*, 2020). *E. coli* is a mutli-

drug resistant bacterium (MDR) because it has a high ability of resistance to many antibiotics by different methods, including enzymes such as β-lactamase which promotes resistance to β-lactam antibiotics and other enzymes that induce resistance to aminoglycosides, quinolones, etc. and many other methods (Kapoor *et al.*, 2017).

Due to the lack of studies covering UTI-causing E. coli and its pathotypes' spread around eastern Diyala Province, in addition to its virulence factors and antibiotic resistance, the current study aims to remedy these things by following the steps below:

1. Isolation and identification of *E. coli* from urine samples of women bearing UTI symptoms in eastern Diyala Province and determine how widespread it is.
2. Evaluate *E. coli* isolates' resistance towards antibiotic.
3. Phenotypic detection of virulence factors of *E. coli* and their correlation with antibiotic resistance.
4. Molecular detection of *E. coli* virulence genes and their relationship with the bacteria's virulence factors and antibiotic resistance.

Chapter Two: Literature Review

2.1 Enterobacteriaceae

The Enterobacteriaceae family includes many Gram-negative bacteria that live as normal flora inside the intestines of humans and animals. This heterologous family includes *Escherichia*, *Salmonella*, *Shigella*, *Enterobacter*, *Klebsiella*, *Proteous*, etc. Members of this family can cause many diseases to humans and animals, including wound infections, nosocomial infections, respiratory tract infections, urinary tract infections (UTIs), and genital infections. They're rod-shaped, Gram-negative, aerobic or facultative anaerobe. Most of the members are lactose fermenters, oxidase test negative, indole test positive, non-spore forming, flagellated, and the optimal temperature for their growth is 37 °C. They have a wide range of virulence factors like toxins, enzymes, capsules, flagella, etc. (Riedel *et al.*, 2019; Oliveira *et al.*, 2017).

2.2 *Escherichia coli*

Escherichia species belong to the Enterobacteriaceae family of bacteria, and they're: *E. fergusonii*, *E. vulneris*, *E. hermanii*, *E. blattae*, *E. albertii* and *E. coli*. The species mentioned differ from each other biochemically (Olowe *et al.*, 2017). *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* or DEC 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 (Levinson *et al.*, 2018; Riedel *et al.*, 2019).

The bacterium was first discovered by Theodor Escherich in 1885. It was named Bacterium *Coli*, or "Colon Bacterium". In 1894, it was isolated from the feces of healthy children so it was considered nonpathogenic (Sussman, 1985), and in 1945, Bray found that a certain species of *E. coli* were the main cause for diarrhea among the infants in England (Bray, 1945).

E. coli is classified in the Enterobacteriaceae family of bacteria, and as follows (Faner *et al.*, 2017):

Domain: Bacteria

Kingdom: Eubacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *coli*

2.3 Characterization of *Escherichia coli*

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 (Kodaka *et al.*, 2004; Wanger *et al.*, 2017; Riedel *et al.*, 2019).

2.4 *E. coli* pathotypes

Diarrheagenic and uropathogenic *E. coli* have been divided into 6 pathotypes depending on their characteristics, virulence factors and their mode of action (Malema *et al.*, 2018; Rivas *et al.*, 2015):

2.4.1 Shiga-toxin producing *E. coli* (STEC) or Enterohemorrhagic *E. coli* (EAEC)

A group of foodborne bacteria responsible for a wide variety of infections, from mild diarrhea to serious dysentery cases like hemorrhagic colitis (HUC). The most common serotypes of EHEC/STEC related to human infections are O24, O45, O103, O111, O121, O145 and O157. The unique characteristic of EHEC isolates is that they're capable of producing shiga-toxins — a toxin made of one active A subunit linked with one B5 subunit responsible for binding the toxin with glycolipid receptors on the surface of the target cells which induces systemic absorption (figure 2.1). Shiga-toxins prevent the production of proteins by removing the residual adenine from rRNA 28S for ribosome 60S (Melton-Celsa, 2014; Gould *et al.*, 2013; Moxley *et al.*, 2020).

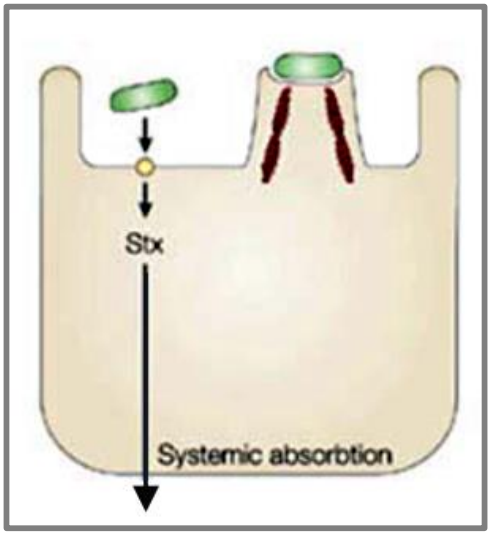

Figure 2.1 Enterohemorrhagic *E. coli* (EHEC) (Vilalta, 2018).

2.4.2 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 (figure 2.2).

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 (Mare *et al.*, 2021).

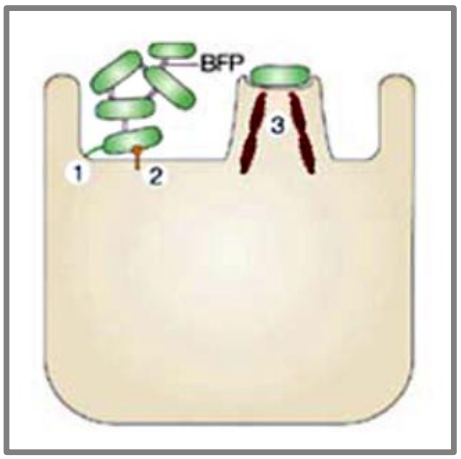

Figure 2.2 Enteropathogenic *E. coli* (EPEC) (Vilalta, 2018).

2.4.3 Enterotoxigenic *E. coli* (ETEC)

ETEC strains distinguish themselves from other *E. coli* pathotypes by their ability to produce colonization factors (CF) and enterotoxins: LT and ST. ETEC is the main cause of diarrhea in children in developing countries and anyone traveling to these areas. ETEC produce two kinds of enterotoxins: Heat-labile (LT) and heat-stable (ST). The main symptom of ETEC infection is watery diarrhea caused by the loss of ions and huge amount of water because of the

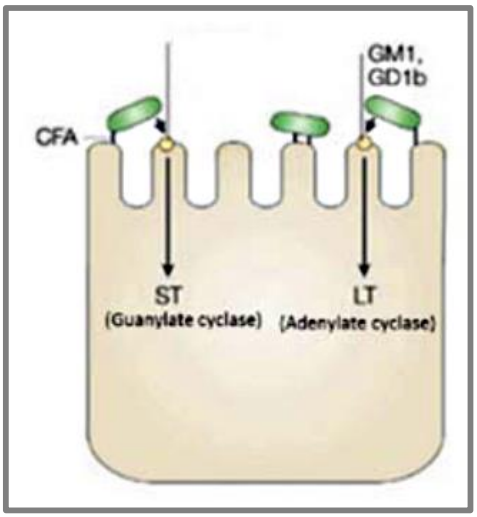

Figure 2.3 Enterotoxingenic *E. coli* (ETEC) (Vilalta, 2018).

enterotoxins which cause issues in the transmembrane channels in the intestinal epithelium (figure 2.3) (Buuck *et al.*, 2020; Fleckenstein, 2010).

2.4.4 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 (figure 2.4). Eneteroinvasive *E. coli* serotype O96:H19 was found to be the causative agent of an outbreak in Sweden recently (Michelacci *et al.*, 2020; Lagerqvist *et al.*, 2020).

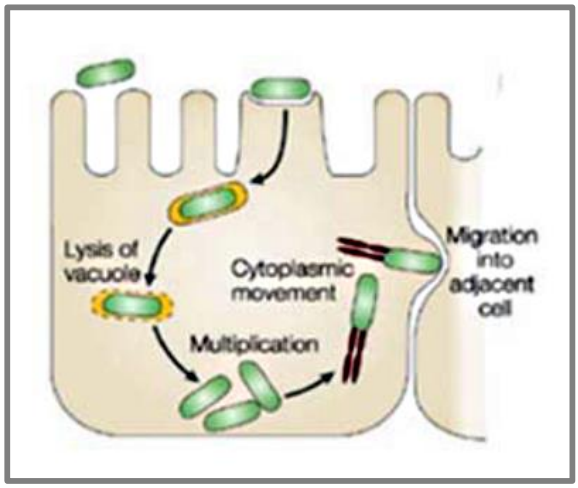

Figure 2.4 Enteroinvasive *E. coli* (EIEC) (Vilalta, 2018).

2.4.5 Enteroaggregative *E. coli* (EAEC)

Distinguished by the AA pattern it shows on epithelial cells on tissue cultures. AA pattern is described as a "stacked-brick" on top of the surface of cells in epithelial tissues culture (figure 2.5). Diarrhea caused by EAEC is watery with

mucus present, blood may or may not be present, stomach pain, low-grade fever and vomiting. In 2011, a large outbreak of a certain hybrid strain happened in Germany resulting in more than 4,300 cases of diarrhea and 50 deaths (Ellis *et al.*, 2020).

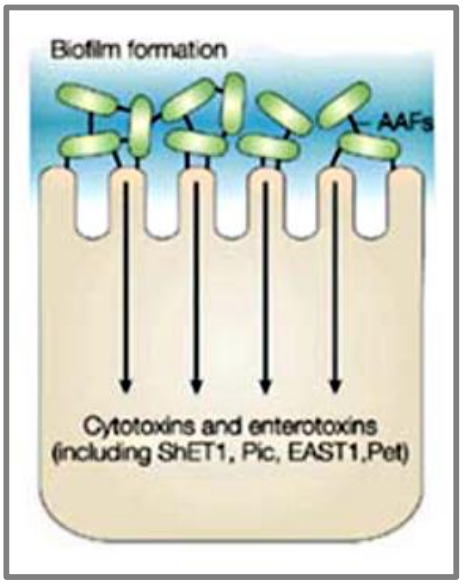

Figure 2.5 Enteroaggregative *E. coli* (EAEC) (Vilalta, 2018).

2.4.6 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 (figure 2.6). Capable of infecting children, and it's considered the second most common cause of death in children under 5 years of age (Omolajaiye *et al.*, 2020).

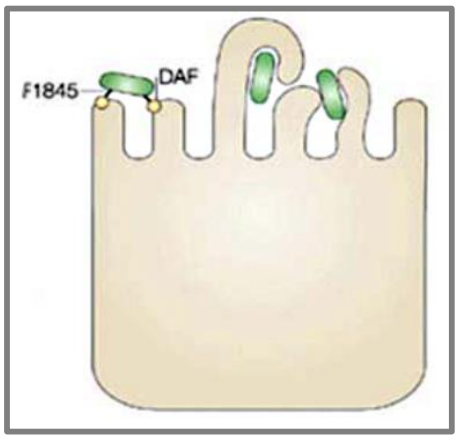

Figure 2.6 Diffusely Adhering *E. coli* (DAEC) (Vilalta, 2018).

2.5 Pathogenicity of *E. coli*

Pathological strains of *E. coli* cause various diseases in humans, including gastroenteritis, neonatal meningits, peritonitis, mastitis septicemia (Tauschek *et al.*, 2002). There are also strains that cause food poisoning, diarrheal disease and urinary

tract infections (Foxman, 2010). There are pathogenic strains such as EHEC that cause serious diseases in humans, especially EHEC. o157:H7, which has the ability to produce STEC toxins that cause hemolytic syndrome and is transmitted through foods contaminated with it (Wong *et al.*, 2000). These bacteria also cause urinary tract infections and are higher in women than in men because of the shortened urinary tract in in women, bacteria settle in the urethra and reach the bladder and kidneys and cause pyelonephritis, where the bacteria produce toxins alpha and beta-hemylicin that degrade urinary tract cells (Nicoll, 2008). UPEC bacteria have the ability to avoid the host's immune system by forming biofilms, which increases their pathogenicity and resistance to antibiotics (Ehrlich *et al*., 2005). E. coli also causes many acquired diseases for people in hospitals (Kandekar and Sekara, 2015).

2.6 Epidemiology of *E. coli*

The bacterium *E. coli* is considered the most important of the Enterobacteriaceae family of bacteria that is capable of invading the intestines and live in it as normal flora. Some strains of *E. coli* are opportunistic — they can turn pathogenic when the right conditions or opportunities are met (Gharajalar and Sofiani, 2017; Gurtler *et al.*, 2017).

It is estimated that more than 150 million people get a urinary tract infection every year, with an economic cost of about $3.5 billion per year in the United States.

According to the Center for Disease Control and Prevention (CDC), the most recent *E. coli* outbreak has happened on the 11th of March, 2021 resulting in a total of 22 cases with 1 death and further examining of the list of outbreaks of *E. coli* in the recent years, it is apparent that the number of outbreaks per year has increased for the last several years. Infection rate varies from person to person and from environment to environment depending on health conditions and geographical location and the presence of closely related UPEC groups can be responsible for a significant number of UTI cases regardless of even antibiotic resistance. The bacterium mostly infects humans in the age group of 30-39, and the rate of infection is higher in women than men (Hadi *et al.*, 2014; Forsyth *et al.*, 2018; Foxman, 2014; Matsukawa *et al.*, 2019).

2.7 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). 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). The size of these islands varies from 10-200kb and it differs from other parts of the genome in its G+C

content. 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 (Parvez and Rahman, 2018).

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 (Frankel and Ron, 2018). 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 (Nair *et al.*, 2019; Riedel *et al.*, 2019).

2.7.1 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 for

invasion (Kallas *et al.*, 2020). Adherence depends on the genes encoding the necessary adhesion factors, and these genes include *eae*, *fim*, *sfa*, *pap*, etc. 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 (Spaulding *et al.*, 2017; Neamati *et al.*, 2015).

2.7.1.1 Fimbriae

Fimbriae are activators of host immune system, extending from the cell surface and promoting bacterial colonization of the susceptible host. Fimbriae play an important role in the invasion and colonization of the host by bacteria. There many different types of fimbriae, for example type 1 fimbriae help the bacteria adhere to the intestinal epithelium, while long polar fimbriae play a crucial role in cell invasion. Different fimbriae have different assembly proteins, for example type I fimbriae are assembled by FimC and FimD, S fimbriae are assembled by SfaE and SfaF, and P fimbriae are assembled by papC and papD assembly proteins (Croxen *et al.*, 2013; Gurtler *et al.*, 2017; Volkan *et al.*, 2015).

2.7.1.1.1 Type 1 fimbriae

Type 1 pili are expressed by 90% of uropathogenic *E. coli* strains and are enconded by *fimH*. Found on the surface of the bacteria, it is 2µm in length and 10nm in width. The structure of the fimbriae is made of FimA, FimF, FimG and FimH. This type of fimbriae has a variety of functions, ranging from promotion of biofilm formation on abiotic surfaces to attachment to uroepithelial cells through a mannose-mediated attachment. This function of type 1 pili depends on the fimH adhesin found at the tip. FimH is made up of 279 amino acids, with two terminal domains: the N-terminal domain (NTD) and the C-terminal domain (CTD). NTD carries a lectin domain (FimHLD) while CTD carries a pilin domain (FimHPD). The interaction between these two terminal domains determines how affinitive FimH will be with the related molecule or receptor. FimH is in low affinity mode (T-state) but in the present of high amounts of stress, it can dynamically change into high affinity mode (R-state) (Kallas *et al.*, 2020; Sarshar *et al.*, 2020)

2.7.1.1.2 S fimbriae

S fimbriae is another type of fimbriae at the disposal of *E. coli* that is present in more than 22% of the uropathogenic type of *E. coli*. Encoded by the *sfa* gene, this type of fimbriae helps *E. coli* adhere to the urinary bladder and kidneys. S fimbriae is related genetically to F1C fimbriae, but only S fimbriae has been related to more

severe meningitis and sepsis infections caused by UPEC (Behzadi, 2018; O'Rouke and Cadieux, 2019).

2.7.1.1.3 P fimbriae

Recognized as important virulence factors for uropathogenic *E. coli* and its first recognized virulence factor, P fimbriae are encoded by the *pap* gene and are present in 70% of UPEC. These type of fimbriae are composed of PapA at the base, stretching out to form a stalk which is made of PapE and PapF, and found at the tip is the adhesion PapG. The receptor epitopes for P fimbriae are present on the entirety of the human urinary tract uroepithelial cells, including renal epithelial cells. Attachment to renal epithelial cells induces an inflammatory response which could lead to more tissue damage, opening more ways for UPEC to invade the urinary tract epithelial layer. It is also worth mentioning that P fimbriae expression is associated with inhibition or reduction of some immune functions of the host, such as the movement of IgA to the renal lumen and neutrophils bactericidal capabilities which in turn elevates UPEC chances of survival and causing infection (Lane and Mobley, 2007; Behzadi, 2018; O'Rouke and Cadieux, 2019).

2.7.2 A/E Lesions

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 the

main 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 (Gomes *et al.*, 2016; Yang *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 (Sperandio and Hovde, 2015). The intimin protein has an N-terminus and a C-terminus. 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 (Croxen *et al.*, 2013; Gomes *et al.*, 2016; Yang *et al.*, 2020).

2.7.3 Capsule

E. coli capsules are classified into 4 major groups based on the way their gene clusters are organized and the mechanism of their biosynthesis and assembly. Group 2 capsules includes many K antigens (K1, K2, K5, etc.). K1 is associated with meningitis. K2 is a polysaccharide complex made of repeating units of oligosaccharide that provide protection from the immune system by its anti-phagocytic property and serum resistance provided by the alteration of the classical complement pathway by the bacterial outer membrane protein A (OmpA). The genes responsible for the biosynthesis of the group 2 capsules are located on three different regions: Regions I (*kpsFEDUCS*) and III (*kpsMT*) encode a transmembrane complex involved in the export and the assembly of capsular polysaccharides. Region II presence is serotype-dependent and it encodes enzymes that synthesise the needed capsular polysaccharides (Frankel and Ron, 2018; Croxen and Finlay, 2010; Goh *et al.*, 2017).

2.7.4 Enzymes

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 cleaving

the 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 plasmid-mediated beta-lactams that work against carbapenems, ertapenem, meropenem, impenem, and doripenem (Frankel and Ron, 2018).

2.7.5 Toxins

Toxins are proteins or substances produced by some strains of uropathogenic *E. coli* that plays a vital role in causing UTIs. These toxins can modify cell signal pathways and modulate the host's inflammatory response. In 1987, cyclomodulin toxin (CDT) was first described as a virulent toxin in uropathogenic *E. coli* then other toxins were described like cytotoxic necrotizing factor 1 which is encoded by the *CNF1* gene, secreted autotransporter toxin (SAT), cytolysin A, plasmid-encoded toxin (PET), vacuolating autotransporter toxin (VAT), *Shigella* enterotoxin-1 (SHET-1) and α-hemolysin (*hly*A), which is the most important toxin. 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. When it attaches to red blood cells, this causes hemoglobin to be released into the environment which is then metabolized by the bacteria (Parvez and Rahman, 2018).

2.7.6 Siderophores

Bacteria need iron for growth, DNA synthesis, electron transport and metablosim but due to the fact that iron in the human body is mostly present as transferrin, bacteria secrete what is called 'siderophores' ¬¬ substances that chelate Fe and then attach to special receptor on the bacteria's surface. Utilization of iron acquired by this method is vital for the colonization process in UTI caused by UPEC. There are 4 systems of siderophores in *E. coli*: Yersiniabactin, aerobactin which is encoded by the *iuc* gene, enterobactin which is encoded by the *ent* gene, and salmochelin. Named "salmochelin" because it was first discovered in *Salmonella* species, it is a strongly hydrophilic siderophore encoded by the *iroBCDEN* gen cluster. These systems are expressed under low-iron conditions (Levinson *et al.*, 2018; Riedel *et al.*, 2019; Sarowksa *et al.*, 2019).

2.7.7 Outer Membrane Proteins (OMPs)

Bacteria that are Gram-negative are surrounded by 2 membranes: an inner membrane (IM) and an outer membrane (OM). The outer membrane's outer leaflet (outward facing side) is composed of lipopolysaccharides (LPS), a highly negatively charged molecule that protrudes into the bacteria's environment. The outer membrane's ability to stop hydrophobic molecules from entering the bacteria protecting the bacteria in environments such as the intestines but due to its lipid

nature, it also excludes hydrophilic substances. To overcome this, bacteria use special protein channels called pirons which allow low molecular weight hydrophilic substances into the cell (Riedel *et al.*, 2019).

Outer membrane proteins (OMPs) are an integral part of the outer membrane with many diverse roles, acting as adhesion factors, mediators for the uptake of nutrients, siderophore receptors and enzymes such as proteases and lipases, etc. LptD is a 26-stranded β-barrel which is responsible for the fundamental process of insertion of LPS into the outer leaflet of the OM, maintaining the asymmetry of the bilayer. β-barrel assembly machinery (BAM) complex is responsible for the insertion of β-barrel proteins (OMPs) into the OM (Rollauer *et al.*, 2015). The ompT protein is a 10-strand antiparallel β-barrel outer membrane protease inserted into the OM by BAM complex because it plays a role in the cleavage of antimicrobial peptides (AMPs) like colicin. AMPs are substances secreted by organisms into the extracellular environment due to their antimicrobial (bactericidal) activity (McCarter *et al.*, 2004; Urashima *et al.*, 2017).

2.8 Formation of the Biofilm

E. coli form a biofilm when the environmental is unfavorable, lack of nutrient, or a high density of cells in a specific location. The biofilm is made up of polysaccharides in addition to compounds from the bacterium's environment like

nutrient, minerals, amino acids, cell wall components, etc. (Billings *et al.*, 2015). First noted in 1933 by Henrici who found thick communities of bacteria grow on submerged slides in a number of different water sources (O'Toole and Wong, 2016). The process of making the biofilm is a multi-stage process, and it goes as follows:

1. Reversible attachment: The flagella and chemotaxis help the bacteria reach and attach to the surface of the host.
2. Irreversible attachment: The stage in which cells get into the stationary phase and the amount of elongation happening is reduced. During this stage type 1 pili, curli fibers and a type of antigen called antigen 43 are formed.
3. External matrix formation: The bacteria starts producing polysaccharides to ease the process of cell's attachment to surfaces. The matrix is composed of cellulose, polyglucosamine and colonic acid, in addition to other components such as nucleic acids, proteins, etc.
4. Formation of the biofilm in a three-dimensional structure: A stage that includes the making of bacterial colonies buried in a lot of materials such as nutrient, water and metabolism products.
5. Detachment: The stage at which the biofilm is formed and detached so the process can start all over again on another surface (Soto, 2014; Silva *et al.*, 2017) (Figure 2.1).

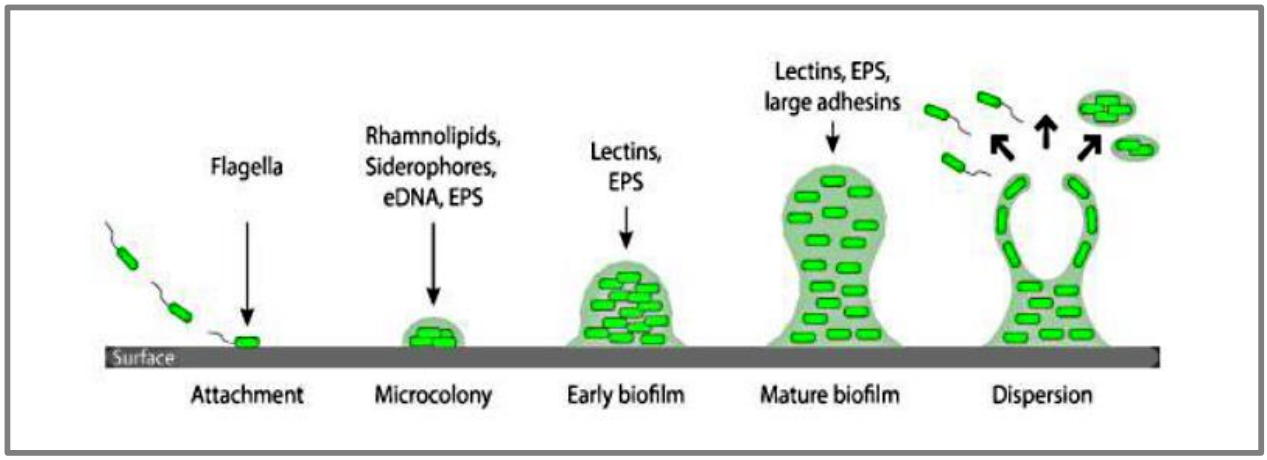

Figure 2.7 Stages of biofilm formation, from attachment to the formation of the extracellular matrix that'll keep growing till the detachment stage (Silva *et al.*, 2017).

Bacteria that grow together in complex multicellular communities are more likely to cause infection than bacteria that grow independently. Studies have shown that the biofilm is responsible for more than 80% of bacterial infections of the urinary tract in humans. It can be inhibited by many vegetarian extracts and chemical compounds, as their effectiveness shows in inhibition of the quorum sensing (QS) system of bacteria — a system in which bacteria can send chemical signals between each other. Antimicrobial resistance is higher in bacteria that produce biofilms than planktonic bacteria (Tajbakhish *et al.*, 2016; Poursina *et al.*, 2018).

2.9 Antibiotic Resistance

One of the biggest threats to global health, food safety and growth. According to World Health Organization (WHO), antimicrobial resistance is defined as the

ineffectiveness of drugs to treat health conditions caused by microorganisms, and it has led to the spread of many infections like UTIs, respiratory tract infections, and many other health conditions that are difficult to treat due to the progress these causative microorganism have made in their antimicrobial resistance abilities (Adhikari, 2020).

2.9.1 Evolution of Bacterial Resistance

Resistance can be gained through mutations, or from other organism through processes like conjugation, transduction (plasmids or transposons) or transformation. Mutations usually lead to a change in the antimicrobial's mode of action by modifying its target and thusly, reducing its effectiveness and the activation of efflux pumps or by shifting the metabolism by regulatory operons. Resistance can be classified into multiple levels, including: multi-drug resistant (MDR) (resistance to at least one antibiotic from 3 or more groups of antibiotics), extensively-drug resistant (XDR) (resistance to at least one antibiotic in 4 or more groups), and pan-drug resistant (PDR) (resistance to all antibiotics from all different groups) (Basak *et al.*, 2016; Munita and Arias, 2016).

The evolution and spread of antimicrobial resistance bacteria by different mechanisms and genes that are determinants of resistance is a major threat to public health globally (Tang *et al.*, 2014). It is estimated that more than 3 million

individuals will lose their lives by 2050 through MDR *E. coli* strains, especially carbapenem-resistant strains, which are already spreading across the globe, and the only treatment available for eradicating them (colistin) is already losing effectiveness (Vilalta, 2018).

2.9.2 Mechanisms of Resistance

Antibiotics are compounds that target specific locations in a bacterium and to be effective, it targets a vital operation in the bacterium leading to either a stop to the cell's growth (bacteriostatic) or its death (bactericidal). There are many antibiotics available differing from each other by principle and mechanic of action (Sebastian *et al.*, 2021).

One of the most important antibiotics in medicine are β-lactam antibiotics. It includes amoxicillin and cephalosporins. Used to treat UTI by interrupting the creation of the cell wall by attaching to the protein associated with penicillin, and thusly, inhibition of peptidoglycan formation for the bacterial cell wall (Ny, 2019). Many reports have documented the high rates of β-lactam antibiotics' resistance in *E. coli* and this is due to the production of β-lactamase, the most prevalent and evident resistance mechanism in *E. coli* (Furuya and Lowry, 2006; Pitout and Laupland, 2008; Tang *et al.*, 2014; Wright, 2011). There are other antibiotics which target the production of DNA or RNA and protein synthesis, and this includes

fluoroquinolone which inhibits duplication by attaching to DNA gyrase and topoisomerase IV which play a role in the uncoiling of DNA before duplication and induce double strand breaks before DNA polymerase (Ny, 2019). Fluoroquinolones have become the favorite antibiotics used in the treatment of upper and complicated UTIs due to their wide spectrum of effectiveness. Recently, it was shown that fluoroquinolones and quinolones antibiotics' resistance have increased between UPEC strains in the United States and Europe due to mutations or by acquiring plasmids that have the resistance determinant genes (Al-Ghoribi, 2015). Aminoglycosides target protein synthesis by attaching to 30S subunit which leads to misreading by mRNA which eventually leads to early termination of protein synthesis (Kapoor *et al.*, 2017). Resistance to aminoglycosides by *E. coli* has several causes and they include target modification (16S rRNA and ribosomal mutations), use of efflux pumps to reduce the intracellular concentration of the antibiotic and enzymatic drug modification (Kumar and Singh, 2013).

2.10 Polymerase-Chain Reaction (PCR)

The polymerase-chain reaction (PCR) is a technology used to multiply a specific part of DNA to be further studied instead of using the bacterium cloning method that is both complex and time-consuming. It is an enzyme-based reaction between a primer (a mix of DNA-polymerase enzymes with some buffer and magnesium

solution) and a target DNA. Primers are usually 18- to 24-nucleotide bp that surround the region that needs to be amplified (Biassoni and Raso, 2020; Disotell, 2017). This is controlled by a thermocycler that heats and cools the reaction tubes in three basic steps:

1. Denaturation: This step involves the denaturation of the double-stranded DNA into single-strands by reaching a temperature of usually around 94 – 98°C.
2. Annealing: In this step, the temperature of the reaction tubes is cooled down to allow the 2 primers to anneal the newly-formed single-stranded DNA.
3. Extension: The tube is heated again, this time to usually around 72°C. At this stage, dNTPs (deoxynucleotide triphosphates) is being added to the double-stranded DNA extending from the space between the primers and template DNA by the DNA-polymerase (Tisotell, 2017).

2.10.1 Multiplex PCR

Multiplex PCR allows the detection of multiple bacterial genes in a single tube simultaneously by having multiple primer pairs in the mixture for multiple target sequences. This process saves both time and reagents. Multiplex PCR is a highly sensitive, highly exclusive detection method for the detection and identification of different bacterial pathogens, including Enterobacteriaceae species (Nguyen *et al.*, 2016; Zhang *et al.*, 2019).

Chapter Three: Materials and Methods

3.1 Materials Used in the Study

3.1.1 Instruments and Apparatuses

The instruments and apparatuses used in this study are mentioned in table 3.1.

Table 3.1 Instruments and apparatuses and their corresponding manufacturers.

No.	Instrument or apparatus name	Manufacturer
1	Autoclave	Hirayama (Japan)
2	Centrifuge	Memmert (Germany)
3	Digital camera	ATTO (Japan)
4	DNA NanoDrop	Bioneer (South Korea)
5	Electrophoresis	Bioneer (South Korea)
6	Spectrophotometer	Kevn (Germany)
7	Hood	Labtech (South Korea)
8	Incubator	Memmert (Germany)
9	Light microscope	Kruss (Germany)
10	Millipore filters (µm)	Difco (USA)
11	Micro-titer plate	Holzel (Germany)
12	Oven	DLTG (China)
13	Refrigerator	Concord (Turkey)
14	Sensitive balance	Denver (Canada)
15	Thermal cycler	Techne (USA)
16	UV-tranilluminator	Scope-21 (Japan)
17	Vitek 2 Compact	BioMerieux (France)
18	Vortex	Bioneer (South Korea)
19	Water bath	GFL (Germany)

No.	Instrument or apparatus name	Manufacturer
20	Water distiller	GFL (Germany)

3.1.2 Chemical Compounds

The chemical and biological compounds mentioned in table 3.2 were used in the study.

Table 3.2 Chemicals and biological compounds and their corresponding manufacturers.

No.	Material	Manufacturer
1	Absolute ethanol 95%	Bioneer (South Korea)
2	Agarose gel	Promega (USA)
3	Ethylene Diamine Tetra Acetic acid (EDTA)	BDH (UK)
4	Glucose	BDH (UK)
5	Glycerol	BDH (UK)
6	Hydrogen peroxide	BDH (UK)
7	K_2HPo_4	BDH (UK)
8	Normal saline solution	Schuchard (Germany)
9	Nuclease-free water	Promega (USA)
10	Sodium chloride (NaCl)	BDH (UK)
11	Urea 40%	BDH (UK)

3.1.3 Stains and Reagents

The following stains and reagents which were used in the current study are mentioned in table 3.3.

Table 3.3 Stains and reagents used in the study.

No.	Stain	Manufacturer
1	Loading dye	Promega (USA)
2	Ethidium Bromide dye	Promega (USA)
3	Methyl Red	HiMedia (India)
4	Kovac's Indole Reagent	BioMerieux (France)
5	Voges-Proskauer (VP) Reagent: **1.** VP1: Potassium Hydroxide (KOH) and distilled water (40%) **2.** VP2: Alpha Naphthol and absolute ethanol (5%)	BDH (UK)
6	Catalase reagent	BDH (UK)
7	Nigrosin	BDH (UK)

3.1.4. The Culture Media

The following culture media were used, as explained in table 3.4. All of the culture media used in the study were prepared according to the manufacturer's instructions.

Table 3.4 Cultures used in the study and their corresponding manufacturers.

No.	Culture media	Purpose	Manu.	Reference
1	MacConkey agar	Used in the initial *E. coli* specimen collection and to determine whether the bacterium can ferment lactose	HiMedia (India)	Forbes *et al.*, 2007

No.	Culture media	Purpose	Manu.	Reference
2	Eosin Methylene Blue media	Used to differentiate *E. coli* from other Enterobacteriaceae members	HiMedia (India)	Forbes *et al.*, 2007
3	Blood base agar	Used to determine whether the bacteria can lyse blood cells and the lyse type	HiMedia (India)	Forbes *et al.*, 2007
4	Trypton soya broth	Used to test the organism's ability to form the biofilm	Oxoid (UK)	Ranjith *et al.*, 2017
5	Methyl Red (MR)/Voges-Proskauer (VP) media	Used to determine whether the bacteria can ferment lactose and if it can produce acetoin	Oxoid (UK)	Hemraj *et al.*, 2013
6	Urea agar base	Used to determine whether the bacteria can produce urease	HiMedia (India)	Snyder and Atlas, 2015
7	Muller-Hinton agar	Used to test susceptibility and beta-lactamase production	Oxoid (UK)	MacFaddin, 2000
8	Simmon citrate agar	Used to test the bacteria's ability to use citrate as the only source for carbon	HiMedia (India)	Hemraj *et al.*, 2013
9	Nutrient agar	Used to activate the isolates and as short-term storage	Oxoid (UK)	MacFaddin, 2000
10	Brain-heart infusion broth	Used as long-term storage after the addition of 15mL of glycerol to every 85mL of the broth	Oxoid (UK)	MacFaddin, 2000
11	Kligler's iron agar	Used to determine if the bacteria can ferment dextrose and lactose, and H_2S production	Merseyside (UK)	Forbes *et al.*, 2007
12	Sorbitol MacConkey agar	Used to determine if the isolates were strain O157:H7	HiMedia (India)	MacFaddin, 2000
13	Peptone water media	Used to check whether isolates could lyse tryptophan by producing tryptophanase, and to check for indole production	HiMedia (India)	Hemraj *et al.*, 2013

3.1.5 Laboratory Kits

The kits used in the current study are mentioned in table 3.5.

Table 3.5 Kits used in the study.

No.	Kit	Components	Manufacturer (Origin)
1	Gram stain	1. Crystal Violet 2. Iodine 3. Acetone 4. Sufranin	BDH (UK)
2	DNA Extraction kit	1. Nuclei lyses solution 2. RNase solution 3. Protein precipitation solution 4. Ethanol 70% 5. Isopropanol 6. TAE buffer 7. TBE buffer 8. CL buffer	Promega (USA)
3	Vitek 2 kit	1. Vitek strip 2. Normal saline 3. Vitek 2 GN card 4. Vortex 5. Vitek 2 Densichek	BioMerieux (France)

3.1.6. The Antibiotics

The antibiotics disc and their concentration which were used for determining the sensitivity of isolated bacteria are listed in table 3.6.

Table 3.6 Antibiotic discs and their related information.

Antibiotic disc (Symbol)	Conc. (mg /disc)	Inhibition zone diameter (mm)			Use	Manu.
		S	I	R		
B-lactam/carbapenem						HiMedia (India)
Meropenem (MEM)	10	≥23	20 – 22	19≤	Susceptibility testing	
Impenem (IMP)	10	≥23	20 – 22	19≤	Susceptibility testing	
Pencillin						

Antibiotic disc (Symbol)	Conc. (mg /disc)	Inhibition zone diameter (mm)			Use	Manu.
		S	I	R		
Piperacillin (PI)	100	≥21	18 – 20	17≤	Susceptibility testing	
B-lactam combinations						
Amoxicillin-clavulante (AMC)	30	≥18	14 – 17	13≤	Susceptibility and beta-lactamase enzyme testing	
Piperacillin-tazobactam (PIT)	30	≥21	18 – 20	17≤	Susceptibility testing	
Monocyclin						
Aztreonam (ATM)	30	≥21	18 – 20	17≤	Susceptibility and beta-lactamase enzyme testing	
Cephems (including cephalosporins)						
Cefotaxime (CTX)	30	≥26	23 – 25	22≤	Susceptibility and beta-lactamase enzyme testing	
Ceftriaxone (CRO)	30	≥23	20 – 22	19≤	Susceptibility and beta-lactamase enzyme testing	
Ceftazidime (CAZ)	30	≥21	18 – 20	17≤	Susceptibility and beta-lactamase enzyme testing	
Cefoxitins (FOX)	30	≥18	17 – 15	14≤	Susceptibility and beta-lactamase enzyme testing	
Cefepime (CPM)	30	≥25	19 – 24	18≤	Susceptibility testing, beta-lactamase enzyme testing and MIC determination.	
Fluroquinolone						
Ciprofloxacin (CIP)	5	≥26	22 – 25	21≤	Susceptibility testing	
Nalidixic acid (NA)	5	≥21	17 – 20	16≤	Susceptibility testing	
Levofloxacin (LVX)	30	≥19	14 – 18	13≤	Susceptibility testing	
Tetracycline						
Tetracycline (TE)	30	≥15	12 – 14	11≤	Susceptibility testing	
Phenicol						
Chloramphenicol (C)	30	≥18	13 – 17	12≤	Susceptibility testing	

3.1.7 Primer Sequences

The primers mentioned in table 3.7 were used in the current study for detection of some virulence factors.

Table 3.7 Primers used in the study.

No.	Primer Name	Sequence	Ann-ealing Temp. (°C)	Prod-uct size (bp)	Reference
1	*eae*-F *eae*-R	5`-CCCGAATTCGGCACAAGCATAAGC-3` 5`-CCCGGATCCGTCTCGCCAGTATTC-3`	52	881	Dung Tu, 2006
2	*sfa*-F *sfa*-R	5`-GTGGATACGACGATTACTGTG-3` 5`-CCGCCAGCATTCCCTGTATTC-3`	60	244	Zamani and Salehzadeh, 2018
3	*kpsMII*-F *kpsMII*-R	5`-AAGTCAAAGCAGGGGTTGCCCG-3` 5`-GACGCCGACATTAAGACGCAG-3`	65	668	Mostafavi *et al.*, 2018
4	*ompT*-F *ompT*-R	5`-ATCTAGCCGAAGAAGGAGGC-3` 5`-CCCGGGTCATAGTGTTCATC-3`	60	559	Adwan *et al.*, 2016
5	*pap*-F *pap*-R	5`-GACGGTGTACTGCAGGGTGTC-3` 5`-ATATCCTTTCTGCAGGGATGCAA-3`	54	328	Bahalo *et al.*, 2013
6	*fimH*-F *fimH*-R	5`-AACAGCGATGATTTCCAGTTTGTGTG-3 5`-TTGCGTACCAGCATTAGCAATGTCC-3`	54	465	Bahalo *et al.*, 2013
7	*bla* TEM-F *bla* TEM-R	5`-ATAAAATTCTTGAAGACGAAA-3` 5`-GACAGTTACCAATGCTTAATCA-3`	55	1080	Lalzampuia *et al.*, 2014

3.2 Methods

3.2.1 Sterilization Methods

Culture media and solutions were sterilized according to Brown and Smith, 2017 and as follows:

3.2.1.1 Sterilization by Heating

Most culture media and solutions that are not affected by high temperatures were sterilized using this method by utilizing an autoclave at 121°C with a pressure of 15lbs/in^2 for 15 minutes. As for glassware, they were sterilized using an electric oven at 180 °C for 2 hours.

3.2.1.2 Sterilization by Filtering

Solutions and materials that are affected by heat like sugars and urea were sterile using Millipore filters with varying pore diameters (0.22µm).

3.2.2 Preparation of Culture Media

3.2.2.1 Ready Prepared Culture Media

The cultures; MacConkey agar, eosin methylene blue, nutrient agar, peptone water, Simmon's citrate agar, Methyl Red-Voges-Proskauer (MR-VP), Muller Hinton agar, Trypton soya broth, Brian-Heart Infusion broth, Kligler's iron,

MacConkey Sorbitol agar were prepared each according to their manufacturer's instructions. Sterilization was done using the autoclave at 121 °C and a pressure of 15 lbs/in^2, then poured into sterile petri dishes and tubes and incubated at 37 °C for 24 hours to make sure the media weren't contaminated, then they were stored at 4 °C till needed.

3.2.2.2 Laboratory Prepared Media

1. Urea Agar Base Media

Urea agar base media was prepared according to the manufacturer's instructions by adding 24g of urea agar base media to 950mL of distilled water, then the mixture was sterilized by utilizing the autoclave. After sterilization, the mixture was left to cool until it reached 50 °C then 50mL of 40% urea solution — which was prepared by adding 40g of urea to 100mL of distilled water then filtered by Millipore filters with a pore diameter of 0.22µm was added to the mixture. It was then poured into sterile tubes and stored at 4 °C till needed. This media was used to determine whether the bacteria was capable of producing urease (Tille, 2017).

2. Blood Agar Base Media

This media was prepared according to the manufacturer's instructions then sterilized using the autoclave. After that, it was cooled to 45 °C then blood with a

concentration of 5% was added to the media, stirred carefully, poured into sterile petri dishes then left so that it can solidify (Forbes *et al.*, 2007).

3. Methyl Red (Voges-Proskauer Media)

Prepared according to the manufacturer's instructions found on the package by dissolving 7g of peptone, 5g of glucose and 5g of K_2HPo_4 in 950mL of distilled water then its pH was modified to 7.2 and its volume was completed to 1000mL. This was followed by sterilization of the media then it poured into sterile test tube, 5mL each (Hemraj *et al.*, 2013).

4. Semisolid Medium

For this medium, 1.3g of nutrient agar and 0.7g of agar-agar were dissolved in 100mL distilled water then 10mL of the resulting solution was poured into test tubes and autoclaved. After autoclaving was done, the tubes were left in vertical position. This medium was used for the motility test (Collee *et al.*, 1996).

3.2.2.3 Preparation of Solutions

1. Crystal Violet Solution

This solution was prepared by dissolving 0.1g of crystal violet powder in 10mL of distilled water then sterilized by Millipore filters of 0.22 pore diameter.

The filtered solution was then stored at 4 °C till needed. The solution was used to test the isolates ability to form the biofilm (Babapour *et al.*, 2016).

2. Tris-Boric-EDTA Buffer (TBE)

Prepared by the addition of 5mL of TBE to 95mL of distilled water. TBE was used in electrophoresis (Sambrools and Russell, 2001).

3. Antibiotic Stock Solutions

The antibiotic stock solution was prepared by dissolving 1g of antibiotic in 10mL distilled water then the volume was completed to 100mL. This was followed by sterilization by Millipore filter with a 0.22µm pore diameter and then they were stored in a refrigerator. The final concentration of this stock solution was 100000µg/mL as primarily stock. Stock 2 was prepared with a concentration of 2048µg/mL from the primarily stock solution (100000µg/mL) using the formula below (Weinstein *et al.*, 2020):

$$C_1 * V_1 = C_2 * V_2$$

C_1: Concentration of stock 1 (10^5), V_1: volume of stock 1 (unknown), C_2: concentration of stock 2 (2048), V_2: Volume of stock 2 (100)

3.2.2.4 Preparation of the Reagents

The reagents used in the study were prepared according to manufacturing instructions companies as mentioned in table 3.8.

Table 3.8 Reagents used in the study.

No.	Reagent	Purpose	Reference
1	Oxidase reagent	Used to perform the oxidase test on the isolates	Forbes *et al.*, 2007
2	Catalase reagent	Used to perform the catalase test on the isolates	Tadesse and Alem, 2006
3	Kovac's reagent	Used to perform the indole test on the isolates	Koneman *et al.*, 1992
4	Methylen red	Used in the methylene red test	Brown, 2007
5	Voges-Proskauer reagents (VP1, VP2)	Used to determine whether the isolates could produce acetoin or not	Brown, 2007

3.2.3 Sample Collection

Two hundred of urine samples were collected from women suffering from symptoms of urinary tract infections (UTIs) from different hospitals around Diyala Province (Al-Sadiyah General Hospital, Jalawla General Hospital and Khanaqin General Hospital) from September 2020 to November 2020. The women were chosen based on their general urine examination results. Samples were placed in sterile urine collection tubes then cultured on MacConkey agar using the streaking method, then they were incubated at 37 °C for 24 hours.

3.2.4 Isolation and Identification of the Bacterial Isolates

3.2.4.1. Characteristic of Cultural Isolation

Samples were cultured and diagnosed using the appropriate culture media then the right characteristics of *E. coli* grown colonies were noticed, on MacConkey agar, shiny pink colonies were noted (Rajeshwari *et al.*, 2010). Pure colonies were taken and cultured on EMB (Eosin Methylene Blue) which is a differentiating media for *E. coli*. The grown of the bacteria gives colonies with a green metallic sheen (Forbes *et al.*, 2007).

3.2.4.2 Microscopic Identification

Small amount of the grown bacterial colonies was taken and spreaded on microscopic slides mixed with a drop of distilled water, the smear then stained with Gram stain and checked under light microscope, observing the bacteria's color, and under oil immersion, cells' shape and arrangement were observed (Mahon *et al.*, 2018).

3.2.4.3 Biochemical Tests

1. Oxidase Test

Grown colonies on MacConkey agar were transported onto filter papers using sterile wooden sticks, then a drop of oxidase reagent was added. Change of color to

purple in 20 – 30 seconds results in a positive reaction, and indicates the production of oxidase enzyme (Biswas and Rather, 2019).

2. Catalase Test

Grown colonies were transferred onto sterile microscopic slides by sterile wooden sticks, then a drop of 3% catalase reagent was added. The appearance of bubbles on the surface of the slides indicates a positive result (Biswas and Rather, 2019).

3. Urease Production Test

Stabbing was used to inoculate the bacteria into slant urea agar media. The tubes were incubated at 37 °C for 24 hours. The change of color from yellow to pink indicates a positive result; the bacterial isolates produced urease and lysed urea (Snyder and Atlas, 2015).

4. Kligler's Iron Agar Test

Slant Kligler's iron agar was inoculated with the bacteria by streaking and stabbing, then incubated at 37 °C for 24 hours. The changing of color from red to yellow due to the shift of pH to lower value (acidic medium) indicates a positive result, and no black sediment as explained in table 3.9 (Forbes *et al.*, 2007).

Table 3.9 Kligler's iron agar test results interpretation*.

No.	Slant's Color	Bottom's Color	Interpretation
1	Red	Yellow	Lactose fermentation
2	Yellow	Yellow	Lactose and glucose fermentation
3	Red	Red	No fermentation
H_2S production:			
4	Black sediment (H_2S produced)		No black sediment (No H_2S produced)

*Medium remains red in alkaline environment and yellow in acidic environment.

5. Indole Production Test

This test was done by inoculated the isolated bacteria in a tubes containing peptone water then incubated at 37 °C for 24 hours. After that, 10 drops of Kovac's reagent were added, presence of the indole ring after a few seconds indicates a positive result (Hemraj *et al.*, 2013).

6. Methyl Red Test

Tubes containing methyl red-Voges-Proskauer media were inoculated with bacterial colonies then incubated at 37 °C for 24 hours, after that 4 – 5 drops of methyl red reagent were added. Shift of color to red indicates a positive result (Hemraj *et al.*, 2013).

7. Voges-Proskauer's Test

Tubes with Methyl Red-Voges-Proskauer's (MR-VP) media were inoculated with bacterial colonies then incubated at 37 °C for 24 hours. After that, 10 drops of VP1 (alpha-naphthol) were added then 5 drops of VP2 (KOH) followed up by a vigorous shaking of the tubes. The tubes then were observed for color shifting within 30 minutes. The shift of color from yellow to red was an indicator of a positive result. This test was used to test the bacteria's ability to produce acetoin as result of sugars' fermentation (Brown and Smith, 2017).

8. Citrate Utilization Test

Tubes containing Simmon's citrate media were inoculated with bacterial colonies by streaking and stabbing followed by incubation for 24 hours at 37 °C. The shift of color from green to blue is an indicator of a positive result. This test was done to test the bacteria's capability to utilize citrate as its only source for carbon and ammonium salts as a source for nitrogen (Brown and Smith, 2017).

3.2.5 Vitek 2 System

Vitek 2 System was used to confirm *E. coli* from a diagnostic group specific to the system, and this requires a diagnostic card specific to Gram-negative bacteria containing 64 slots and in each slot, a dried color-indicator. These indicators react to the sample given, and the System records these changes that were happening due

to bacterial growth on the slots. According to the given changes in color, the System identifies the bacterial sample according to the guidance given by bioMerieux (Pincus, 2011).

3.2.6 Preservation of Bacterial Isolates

3.2.6.1 Short-term Preservation

Tubes containing slant nutrient agar were inoculated by streaking then they were incubated at 37 °C for 24 hours and then at 4 °C till needed. Every month, isolates were continued and cultured on nutrient agar and then on slant nutrient agar again (MacFaddin, 2000).

3.2.6.2 Long-term Preservation

A media to preserve isolates for long-term was prepared by adding 15mL of glycerol to 85mL of brain-heart infusion broth which was then spread across multiple sterile tubes that were not affected by heat to then be sterilized by incubation. After being left to cool down to room temperature, the tubes were inoculated with colonies grown on nutrient agar and stored at 37 °C for 24 hours followed by storage at -20 °C, knowing that the isolates could survive for about 6 – 8 months (McFadden, 2000).

3.2.7 Antibiotic Susceptibility Testing

3.2.7.1 Antibiotic Disks

The susceptibility of the isolates to selected antibiotics was tested according to the guides of CLSI (2020) using Kirby-Bauer method and as follows (Vandepitte *et al.*, 2003):

- Three to five colonies grown on nutrient agar were transported to tubes contain normal saline till the turbidity of MacFarland's standard ($1.5x10^8$ cell/mL) was achieved.
- A sterile cotton swab was inserted into a tube containing bacterial suspension then it was rotated around and pressed against the inner walls of the tube to remove excess feed, then spread across petri dishes containing Muller-Hinton agar, in different directions to ensure a homogenous growth.
- Antibiotic disks used in the study mentioned in table 3.6 were placed on the surface of the cultured media and with equal distance from each other and were pressed in using sterile forceps then stored at 37 °C for 24 hours.

Results were read by measuring the diameter of the inhibition zone around the antibiotic disks then comparing them to the standards mentioned in CLSI, 2020.

3.2.7.2 Minimum Inhibitory Concentration (MIC) Determination

MIC for cefepime and cefetazidime to each isolate was determined using the following steps (Boer *et al.*, 2015):

- Two to four bacterial colonies that were grown on nutrient agar incubated for 24 hours then transferred to tubes containing 5mL of normal saline to obtain a turbidity equal to MacFarland's standard turbidity ($1.5x10^8$ cell/mL).
- A series of concentrations (4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048 µg/mL) of the antibiotics powder (cefepime and cefetazidime) were prepared and added to sterilized Muller-Hinton agar. It was then poured into clean and sterile petri dishes and left to solidify, and stored till needed.
- A sterile cotton swab was rinsed into the bacterial suspension then spreaded across Muller-Hinton dishes with different concentrations of the used antibiotics (cefepime and ceftazidime),
- The dishes were left for 5 minutes to dry then incubated for 18 – 24 hours at 37 °C.

MIC, which is the minimum concentration that allows bacterial growth, was measured for each isolate.

3.2.8 Detection of Virulence Factors

3.2.8.1 Biofilm Formation

3.2.8.1.1 Tube Method (Qualitative Method)

- Two to four isolates grown on nutrient agar were inoculated to trypton soya broth with 1% glucose prepared beforehand, and were incubated for 24 hrs at 37 °C.
- The bacterial growth was removed after incubation then the tubes were washed by distilled water then left to dry.
- 5mL of crystal violet with a concentration of 0.5% was added to each tube. After 10 – 15 minutes, they were washed with distilled water then were left upside down to dry.

Results were considered positive as visible rings were observed around the tubes' inner walls and bottom. If no rings were observed, the test is considered negative (Ibrahim *et al.*, 2014).

3.2.8.1.2 Micro-Titer Plate Method (MTP)

- Two to four isolates grown on nutrient agar were transported to nutrient broth and its turbidity was compared to MacFarland's standard turbidity.

- 200µL of bacterial suspension was injected to a polystyrene plate containing 96 well. This was repeated 3 times for each isolates, and nutrient broth containing no bacteria was used as a negative control. The plate was covered and incubated for 24 hours at 37 °C.
- After incubation period was over, the cover was removed and the bacterial suspension was carefully removed and each well washed three times with 0.9% NaCl.
- 200µL of methanol was added and left for 10 minutes to fix the cells in the wells.
- 200µL of crystal violet with a concentration of 0.5% was added to each well and left for 15 minutes so the biofilms get stained. The stain was then removed by washing with distilled water 2 – 3 times.
- 200µL of 95% ethanol was added to each well for a duration of 10 minutes to remove the excess stain from the cells.

Absorption for each well was measured using a spectrophotometer at a wavelength of 630nm and the readings were compared with zero control, and as follows (Shrestha *et al.*, 2019) (table 3.10):

Table 3.10 Formula for interpreting the absorption results for MTP.

No.	Absorption value	Interpretation
1	$OD_C \geq OD_I$	Incapable of forming biofilm
2	$2OD_C < OD_I$	Strong biofilm producer
3	$OD_C < OD_I \leq 2OD_C$	Intermediate biofilm producer
Note: OD_I = Optical density of isolates OD_C = Optical density of control		

3.2.8.2 Hemolysis Test

Isolates were cultured by streaking on the surface of blood agar plates then incubated for 24 hours at 37 °C. Hemolysis types was observed then and as follows:

- α-hemolysis: Colonies will be surrounded by a green hemolysis area.
- β-hemolysis: Area surrounding colonies are clear.
- ɣ-hemolysis: No lyse is observed around colonies.

3.2.8.3 Beta-lactamase Enzyme Production Testing

3.2.8.3.1 Extended Spectrum Beta-lactamase (ESBL)

Double disk synergy disk method was implemented and as follows (Saha and Jhora, 2018):

- Bacterial suspension was created by mixing the bacterial colonies with 15mL of normal saline and the turbidity was corresponded to MacFarland's standard solution.
- A sterile cotton swab was rinsed into the bacterial suspension then spread across petri dishes with a diameter of 8.5cm containing Muller-Hinton agar to obtain a homogenous growth. The dishes were left for 5 minutes to dry and absorb the cultured bacteria.
- Amoxicillin/clavulinic acid antibiotic disc (20/10mg) with a concentration mentioned in Table 3.6 was placed in the center of the petri dish, and the antibiotic discs: aztreonam (30mg), ceftazidim (30mg), cefotaxime (30mg), and ceftriaxone (30mg), were placed around the amoxicillin/calvulinic acid disc and apart from each other by 20mm.

Then the inhibition zones were observed, and it was found that the inhibition zone of each disc of the 4 discs has been enhanced towards the amoxicillin/clavulinic acid central disc, and this means a positive result (produce ESBL).

3.2.8.4 Capsule

Negative staining method was implemented by using a small amount of bacterial growth which was mixed with nigrosine stain using a wooden stick. A smear was made from the mixture on a slide, and the slide was left to dry.

Microscopic examination was done and clear halos were observed surrounding the bacterial cells indicating that the isolates were capable of producing capsules (Atlas *et al.*, 1995).

3.2.8.5 Motility Test

Tubes containing motility test media were inoculated with bacterial growth by stabbing till 3cm deep, paying attention not to touch the tubes' bottom. The tubes were then incubated for 24 hours at 37 °C. The presence of cloudy shapes around the stabbing area indicates a positive result.

3.2.9 Molecular Examination

3.2.9.1 DNA Extraction

Genomic DNA was extracted from bacterial growth according to the protocol of Wizard Genomic DNA Purification Kit, Promega as the following steps:

- For pellet cells, 1ml of overnight culture was centrifuged for 2 minutes at 13000 RPM. Supernatant was then discarded.
- 500µL of Nuclei Lysis Solution was added and pipetted gently for mixing.
- All mixes were incubated for 5 minutes at 80 °C, then cooled to room temperature.

- For RNA lysis, 3µL of RNase Solution was mixed, then incubated at 37°C for 15 minutes.
- For Protein Precipitation, 200µL of Protein Precipitation Solution was added to cell lysate. Then mixed well by vortexing. Then incubated in deep freeze (-30 °C). After that, centrifuged at 13,000 RPM for 5 minutes.
- Diluted DNA was transferred to a clean tube containing 500µL of room temperature isopropanol. After mixing gently, centrifuge as in "Pellet Cells" above, and supernatant was decanted.
- Under room temperature, 500µL of 70% ethanol was added then centrifuged for 2 minutes at 13,000 RPM, then aspirated and air-dried the pellet.
- DNA pellet was rehydrated in 100µL of Rehydration Solution for 1 hour at 65 °C.

3.2.9.2 Quantitation of extracted DNA

Quantus Fluorometer was used in order to determine the quality of the DNA extraction for downstream applications. For 1 µL of DNA, 199 µL of diluted Quantifluor Dye was mixed. After 5 minutes of incubation at room temperature, DNA concentration values were detected.

3.2.9.3 Primers Preparation

All primers used in this study were supplied by Macrogen in a lyophilized form. All lyophilized primers as mentioned in table 3.7 was 300µL of nuclease-free water to obtain a final concentration of 100pmol/µL as a stock solution. A working solution was prepared by adding 10µL of primer stock solution that was stored in a freeze at -20 °C to 90µL of nuclease-free water to finally have a working primer stock solution with a concentration of 10pmol/µL.

3.2.9.4 PCR Mixture Preparation

The DNA that was extracted from the all *Escherichia coli* isolates examined under a PCR procedure to different target genes in the study. The final volume of each PCR reaction was 20µL, and all the genes targeted and their appropriate size were appeared in table 3.11. The PCR mixture was composed of GoTag Green Master Mix (2x) solution that was melted at room temperature and mixed by using a vortex for homogenizing before use. The primer's solutions were also mixed by utilizing a vortex before use.

Table 3.11 Primers and their proper volumes for PCR mixture.

No.	Gene	PCR type	Vol. of master mix (2x)	Primer	N.F.W.	Vol. of template DNA	Total volume
1	*eae*	Uniplex	10	2	5	3	20
2	*kpsMII*	Uniplex	10	2	5	3	20

No.	Gene	PCR type	Vol. of master mix (2x)	Primer	N.F.W.	Vol. of template DNA	Total volume
3	*bla-TEM*	Unpliex	10	2	5	3	20
4	*fimH*	Multiplex	10	2	3	3	20
5	*pap*	Multiplex	10	2	3	3	20
6	*ompT*	Multiplex	10	2	3	3	20
7	*sfa*	Multiplex	10	2	3	3	20
Aliquot per single RXN			17µL of Master mix per tube and add 3µL of Template				

3.2.9.5 Thermal Cycling Condition

The components of each PCR mixture were mixed in the Eppendorf tube by vortex before being set into the thermocycler. The reaction was done in a PCR thermal cycler apparatus and according to the manufacturer's guide. The process starts with the initial denaturation step at 94 – 95 °C for different durations (minutes) followed by repeated cycles which consist of the denaturation step (94 – 95 °C), the annealing step at a temperature that's dependent on the primer going through the process, the extension step done at 72 °C followed by the final extension step which was done at 72 °C as shown in table 3.12.

Table 3.12 Genes and their thermal cycling conditions.

No.	Gene	Initial denatu. (°C/min)	Denatu. (°C/s)	Anne. (°C/s)	Exten. (°C/s)	Final exten. (°C/min)	Cycle	Amplicon size (bp)
1	*fimH*	94/30s	94/30	54/30	72/60	72/8	32	465

No.	Gene	Initial denatu. (°C/min)	Denatu. (°C/s)	Anne. (°C/s)	Exten. (°C/s)	Final exten. (°C/min)	Cycle	Amplicon size (bp)
2	*pap*	94/30s	94/30	54/30	72/60	72/8	32	328
3	*sfa*	95/4	94/30	60/45	72/60	72/8	33	244
4	*ompT*	94/4	94/30	60/30	72/60	72/8	25	559
5	*kpsMII*	94/4	94/60	65/60	72/60	72/10	25	668
6	*eae*	95/1	95/60	52/60	72/60	72/10	30	881
7	*bla-TEM*	94/5	94/30	60/30	72/50	72/5	30	848

3.2.9.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis was implemented after PCR amplification to confirm the presence of amplification.

3.2.9.6.1 Preparation of Agarose

- 100mL of 1X TAE was poured into a beaker.
- 1.5gm agarose was added to the buffer to achieve a concentration of 1.5%.
- The solution was then heated to boiling (using Microwave) until all the gel particles were dissolved.
- 1µL of Ethidium Bromide (10mg/ml) was added to the agarose.
- The agarose was stirred in order to get mixed and to avoid bubbles.
- The solution was left to cool down at 50-60C°.

3.2.9.6.2 Casting of the Horizontal Agarose Gel

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel was placed in the gel tray. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5mm over the surface of the gel.

3.2.9.6.3 DNA Loading

PCR products were loaded directly. For PCR product, 5µl was directly loaded to well. Electrical power was turned on at 100v/mAmp for 75 minutes. DNA moves from Cathode to plus Anode poles. The Ethidium bromide-stained bands in gel were visualized using Gel imaging system.

3.2.10 Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 3.06. Standard deviations are plotted as error bars for the data points on all figures. *P value* is as follows: P value < 0.01. Hierarchical cluster analysis based on similarity coefficient was used in this study to identify the relatively homogenous bacterial groups of based on antibiogram results using PRIMER-E7 software package (http://www.primer-e.com/) (Clarke et al., 2014). The correlation matrix between virulence genes and susceptibility agents was estimated by using Pearson's

correlation coefficient analysis. Correlation coefficients were considered significant at P values less than 0.05 by using GraphPad Prism version 3.06.

Chapter Four: Results and Discussion

4.1 *E. coli* Isolation

Two hundred urine samples were collected from women suffering from urinary tract infection (UTI) symptoms from September 2020 to November 2020 from Al-Sa'dyia General Hospital, Jalwla General Hospital and Khanaqin General Hospital. The women had an age range between 15 – 45 years. The positive growth from all samples was 125 (62.5%) samples with the remaining 75 (37.5%) being negative growth. After morphological, microscopic and biochemical tests were done and Vitek 2 System implementation was done, 30 *E. coli* isolates were obtained from 125 positive growths (24% of the total growth obtained) (Figure 4.1).

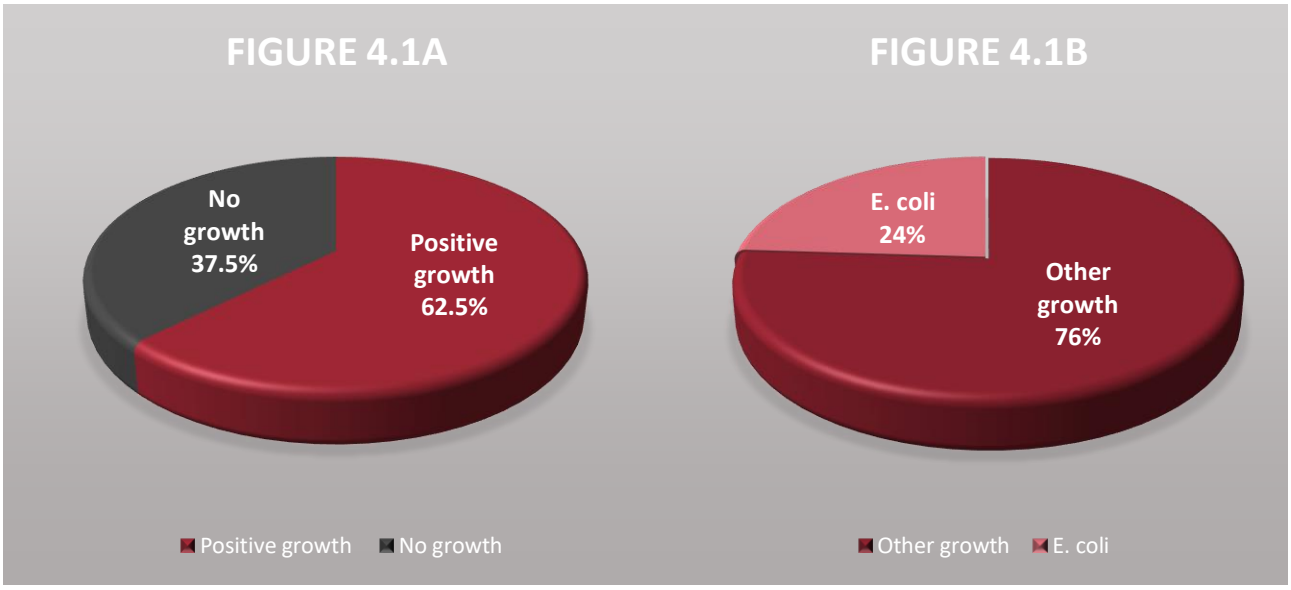

Figure 4.1 Growth percentage from all samples collected. Figure 4.1A: Positive growth from all samples shown as a chart. Figure 4.1B: Percentage of *E. coli* from all 125 positive growths.

4.1.1 Morphological Identification

E. coli isolates were identified depending on their physical characteristics. The isolate appeared as bright pink colonies when cultured on MacConkey agar and the colonies appeared green metallic sheen on EMB media. This results due to MacConkey agar containing crystal violet and bile salts which allows Gram-negative bacteria to grow while inhibiting Gram-positive bacteria growth. It was found that the bacteria were sugar fermenters as shown in Figure 4.2A, on Eosin Methylene Blue (EMB), which it was differential medium for *E. coli* used to differentiate it from other Enterobacteriaceae members. Sheen green metallic colonies were observed, which means that the colonies produced organic acids due to glucose and

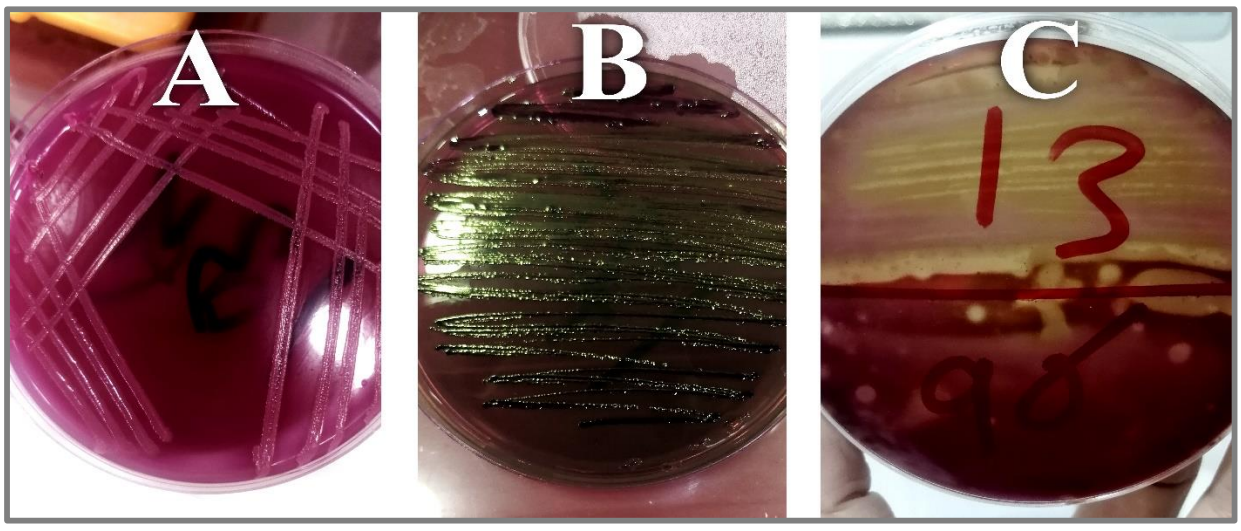

Figure 4.2 Bacterial growth on 3 different mediums. A: Bacterial colonies on MacConkey agar, notice the bright pink colonies. B: Sheen green metallic colonies on EMB media. C: Hemolysis on blood agar plates, circular, convex and smooth colonies.

lactose fermentation which in the presence of eosin and methylene gives a sheen green metallic color as shown in Figure 4.2B (Singha and Prakash, 2008). The isolates were also cultured on sheep blood agar to test their ability to lyse red blood cells, and as shown in Figure 4.2C, the isolates did lyse red blood cells and cause hemolysis.

4.1.2 Microscopic Examination

A smear was made from a colony grown on MacConkey agar and it was stained with Gram's stain. The bacteria appeared short rods, negative to Gram's stain, and not spore forming and this is what was mentioned in Levinson *et al.* (2018).

4.1.3 Biochemical Tests

Biochemical tests were carried out for all the isolates and the results are shown in table 4.1 and appendices 1-A and 1-B. The isolates gave a positive result to catalase test as they dissolved the reagent into water and oxygen. Oxidase test was also done on the isolates and all gave a negative results as there was no color shifting to burgundy which indicates the isolates don't have cytochrome oxidase as a hydrogen receptor. Urease test was also done and all gave a negative result since the isolates don't have urease enzyme and can't lyse urea.

Table 4.1 Identification of *E. coli* by biochemical tests.

Isolate	Biochemical test								
	Lactose fermentation	Catalase	Oxidase	Urease	KIA	Indole	MR	VP	Simmon's citrate
E. coli	+	+	-	-	A/A, H_2S	+	+	-	-

As for IMVIC tests, the bacterial isolates showed a positive result to indole test; red ring was observed on the surface of media resulting from the lysis of triptophane by tryptophase enzyme. This is considered an important test for differentiating *E. coli* from other members of Enterobacteriaceae. The isolates gave a positive result to methyl red test as a color shift to red was observed due to the fact that the bacteria consumed glucose and peptose, and the fermentation of these sugars caused a reduction in the pH of the medium. When it comes down to Voges-Proskauer, the isolates gave negative result since the media appeared to be yellow-brown because the bacteria didn't convert the provided glucose to acetoin which meant no reaction from VP1 and VP2. Regarding citrate test, the media had no color shift to green-blue which indicates the bacteria couldn't use citrate as their only source for carbon since they don't have citrate permease enzyme and since no citric acid was produced, there was no decrease in the media's pH and thus, no color

change. And these results are in agreement to what's mention in each of Tille (2017), Brown and Smith (2017), and Sharmin *et al.* (2010).

4.1.4 Vitek 2 System

To confirm our identification of the bacterial isolates, Vitek 2 System was utilized. A GNID card was used for the Gram-negative bacteria. The System provides 64 tests during a 5 – 8 hours period which provides a practical time period to identify the isolates without any mutations happening and with high accuracy (about 99%) and very low chance of any error (Pincus, 2011). Table 4.2 shows the results of Vitek 2 System and appendix 2 is the Vitek 2 report.

Table 4.2 Vitek 2 System results.

Test	Result	Test	Result	Test	Result	Test	Result	Test	Result	Test	Result
APPA	-	ADO	-	PyrA	-	IARL	-	dCEL	-	BGAL	+
H2S	-	BNAG	-	AGLTp	-	dGLU	+	GGT	-	OFF	+
BGLU	-	dMAL	+	dMAN	+	dMNE	+	BXYL	-	BAlap	-
ProA	-	LIP	-	PLE	-	TyrA	-	URE	-	dSOR	+
SAC	+	dTAG	-	dTRE	+	CIT	-	MNT	-	5KG	-
ILATk	-	AGLU	-	SUCT	-	NAGA	-	AGAL	-	PHOS	-

Test	Result	Test	Result	Test	Result	Test	Result	Test	Result	Test	Result
GlyA	-	ODC	+	LDC	+	IHISa	-	CMT	+	BGUR	+
O129R	+	GGAA	-	IMLTa	-	ELLM	-	ILATa	-		

4.2 Prevalence of *E. coli* According to Collected Samples

The results appeared as shown in table 4.3, 30 (15%) isolates of *E. coli* were obtained from 200 samples, distributed as 60 (30%) pregnant women samples and 140 non-pregnant women samples. Non-pregnant samples gave 101 positive growths of which 19 (18.8%) were identified as *E. coli* while pregnant women had 24 positive growths which provided 11 (45.8%) isolates of *E. coli*.

Table 4.3 Distribution of *E. coli* isolates according to collected samples*.

Age group	Pregnant women samples	*E. coli* isolates	Non-pregnant women samples	*E. coli* isolates	Total isolates
15 – 19	11	1	14	1	2
20 – 24	16	3	51	7	10
25 – 29	22	4	19	1	5
30 – 34	5	2	23	6	8
35 – 40	6	1	33	4	5
Total	60 (30%)	**11 (45.8%)**	140 (70%)	**19 (18.8%)**	30
Chi-square	**32.2**				
P-value	**0.01**				

*P value < 0.05 is significant, P value > 0.05 is insignificant.

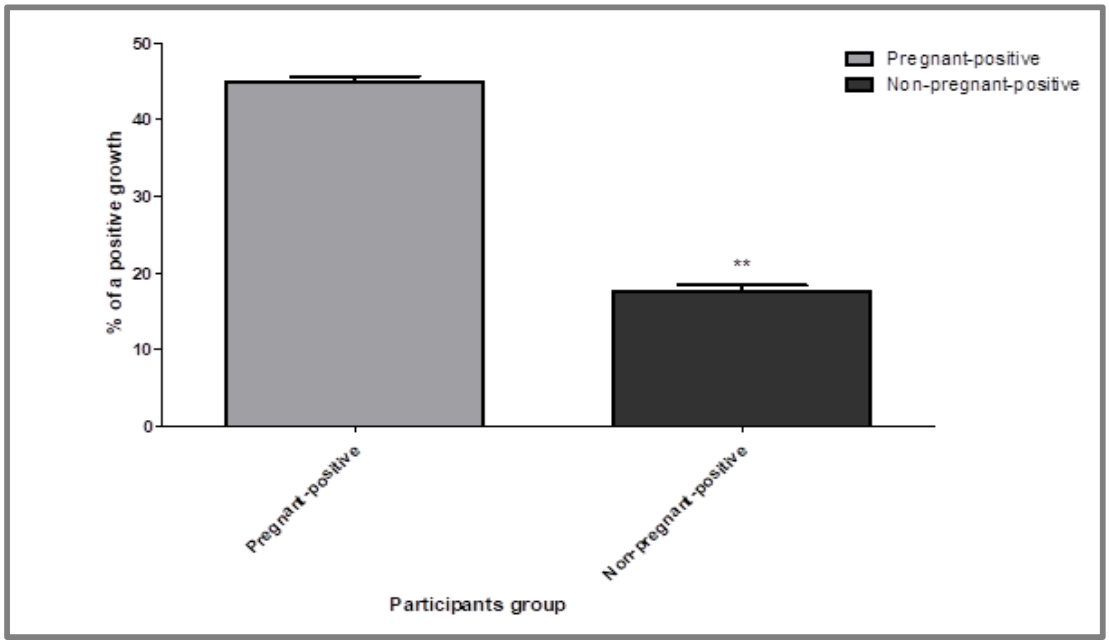

Figure 4.3 The percentage of pregnant and non-pregnant women having UTI caused by *E. coli*. The X^2 test shows the significant difference ($X^2 = 0.5$, $p \leq 0.01$) between the two tested groups according to the positive growth of *E. coli*.

The results as they appeared in table 4.3 and figure 4.3 show that *E. coli* was present more frequently in pregnant women than non-pregnant women significantly, being highest (4 isolates) in the age group of 25 – 29 years for pregnant women while being highest (7 isolates) in the age group of 20 – 24 of non-pregnant women. A study by Ahmed and Yosry (2021) in Egypt explains that *E. coli* was present with the highest frequency in pregnant women in the age group of 21 – 30 years, the higher presence in this age group could be due to the fact that women are more sexually active and intensive intercourse could lead to minor urethral damage and also transfer bacteria from the perineum to the urethra and bladder which made them

more susceptible to UTIs (Derese *et al.*, 2016). Statistical analysis was done using Graphpad Prism version 3.06. Results show that UTIs are more present in pregnant women than non-pregnant women with a significant different (P-value = 0.01) as shown in table 4.3.

A study done by Almukhtar (2018) in Kirkuk, Iraq from 450 urine samples found that *E. coli* accounted for 11.7% for pregnant women while it accounted for 21% and 44% for married and single women, respectively. Another study done by Al-Nasrawi and Al-Hashimy (2020) in Al-Najaf, Iraq from 500 urine samples, 27.82% of it define as *E. coli*. Al-Kashif (2019) detected in a study in Saudi Arabia on 303 pregnant women, *E. coli* accounted for 37% while Azami (2020) in Iran found that *E. coli* was present in 63.3% in pregnant women.

It is apparent from the studies mentioned above and this study that *E. coli*'s presence in pregnant women is high and this could be due to physiological reasons, pregnancy's effects on the woman's body which could make her more susceptible to infectious microorganisms, or the high presence of albumin and other amino acids in a pregnant woman's body which makes her a suitable environment for microorganisms capable of causing UTI (Almukhtar, 2018). The weakening of the immune system and the incapability of a pregnant woman to take antibiotics during

the first trimester increase a pregnant woman's risk of getting a UTI (Raza *et al.*, 2011; Geraldo *et al.*, 2012).

On the other hand, Odongo *et al.* (2020) in Uganda noticed that *E. coli* was found in 10% of their samples, and that 52% of these *E. coli* samples were isolated from women compared to 48% men. The reason for these difference in *E. coli*'s prevalence could be due to geographical, health reasons, social habits and economic state of the community, awareness and hygiene, period when samples were collected and their amount in addition to taking antibiotics before sample collection (Al-Shoyaikh and Jasim, 2016; Almukhar, 2018). *E. coli*'s higher presence in women than men could be caused by the close proximity of the anus to the urethral tube. Also, the urethral tube of the female body is shorter than that of men which shortens the distance microorganisms have to travel to get to the bladder (Odongo *et al.* 2020). *E. coli*'s presence might be contributed to the microorganism's adaptation to the harsh environment of the urinary tract. The high number of virulence factors help the bacteria not only to survive but also cause infection and disease, and the capability of the bacteria to travel from the anus, which is its natural habitat, to the urethral tube due to the short distance these two are from each other could be one of the most important reasons for UTIs (Kandekar and Sekaran, 2015; Raeispour and Ranjbar, 2018).

4.2.1 Isolation and Identification of EHEC

After identification of bacteria was done using suitable culture media, biochemical tests and Vitek 2, the isolates were cultured on Sorbitol MacConkey agar (SMAC), which is a differentiating media used to differentiate EHEC from other serotypes of *E. coli* containing 1% D-sorbitol instead of lactose, and pale colonies were noticed on the media which indicates that the bacteria in question was EHEC indeed (Figure 4.3). Three isolates (E3, E6, E19) from the total 30 isolates of *E. coli* (10%) were showing a conversion in color on media and thusly, 3 EHEC isolates were obtained.

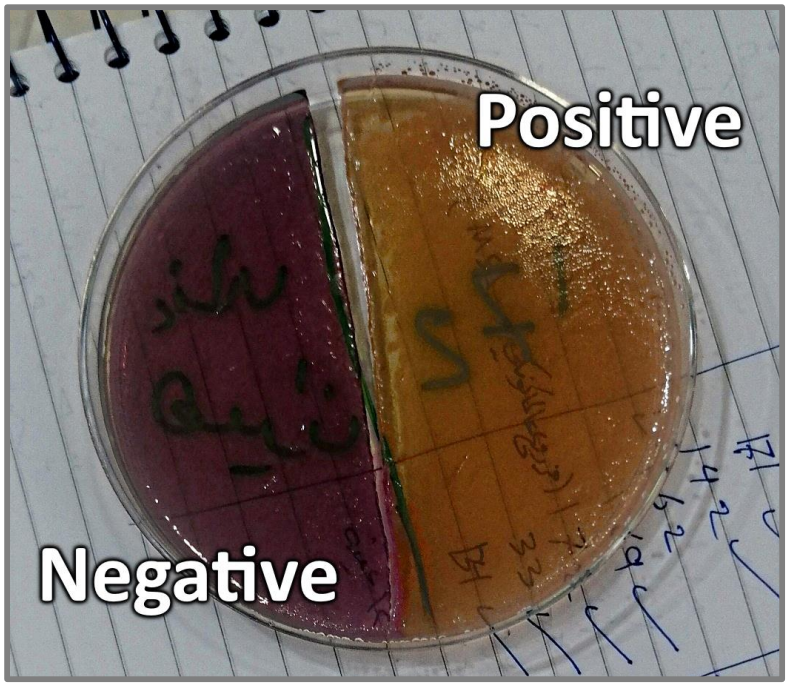

Figure 4.3 *E. coli* growth on Sorbitol MacConkey agar (SMAC).

Sheep blood agar was used to find out whether EHEC isolates were capable of lysing red blood cells — produce hemolysin, the results showed that all 3 isolates were capable of lysing red blood cells, 100% of which were hemolysin producers. Enterohemolysin is a toxin produced by EHEC and lysing red blood cells is a visual characteristic of EHEC (Toval *et al.*, 2014), as well as motility which was tested using semi-solid medium and it was noticed that all isolates were motile which meant EHEC isolates were flagellated. Table 4.4 demonstrates the unique characteristics of EHEC. Further confirmation was carried out in the form of a genetic confirmation by looking for *eae*, a defining gene for EHEC.

Table 4.4 The differentiating characteristics of EHEC isolates.

Isolates	Characteristic test	Result
E3, E6, E19	SMAC fermentation	-
	Motility	+
	Hemolysin	+
	eae gene	+

The detection of EHEC poses a challenge for clinical microbiology laboratories. Key issues concering their detection include the need to distinguish EHEC from other types. The currently accepted method for detection is direct planning on SMAC, which is a simple detection method for EHEC O157. Usage of hemolysin (encoded by *hly* gene) and *eae* gene is advantageous for rapid detection,

and is highly recommended (Selim *et al.*, 2014). A genetic confirmation was carried out to fully confirm whether the isolates in question were EHEC by looking for *eae* gene which is a defining gene for EHEC. This is explained in detail in section 4.7.2.2.

Enterohemorrhagic *E. coli* is a serotype of *E. coli* that secrete shiga toxin (stx) which has a special receptor found on intestinal and renal epithelial cells. Attachment of EHEC to epithelial cells of the intestine and kidneys can lead to hemolytic uremic syndrome (HUS). Intimin, which is a protein encoded by *eae* gene, is expressed by some EHEC strains which can promote actin accumulation in eukaryotic cells (Derakhshan *et al.*, 2019). UTI can be caused by strains of EHEC that carry specific virulence genes that gives them the ability to colonize the perineum, which in turn, allows them to climb up the urinary tract and colonize causing disease .

This study showed that EHEC is present in patients with UTI, which was also noticed by a study in Iraq by Al-wgaa and Alwan (2014) that isolated EHEC from human urine in 3.5% of the samples, and demonstrate this serotype may has critical role in UTI. Another study in Uruguay (Gadea *et al.*, 2012) that found that EHEC was present in women suffering from UTIs, and they suggested that it could be due to the strain being able to move from the ascending digestive system to urinary tract.

Derakhshan *et al.* (2019) found that the strain was present in 110 isolates of *E. coli* collected from patients suffering from UTIs, and that they accounted for 6.7% of the total isolate count.

4.3 Bacterial Resistance to Antibiotics

Thirty *E. coli* isolates were tested for 16 antibiotic discs from different kinds of antibiotics using disc diffusion method, and it was found that isolates had varying levels of resistance to different antibiotics (Figure 4.4).

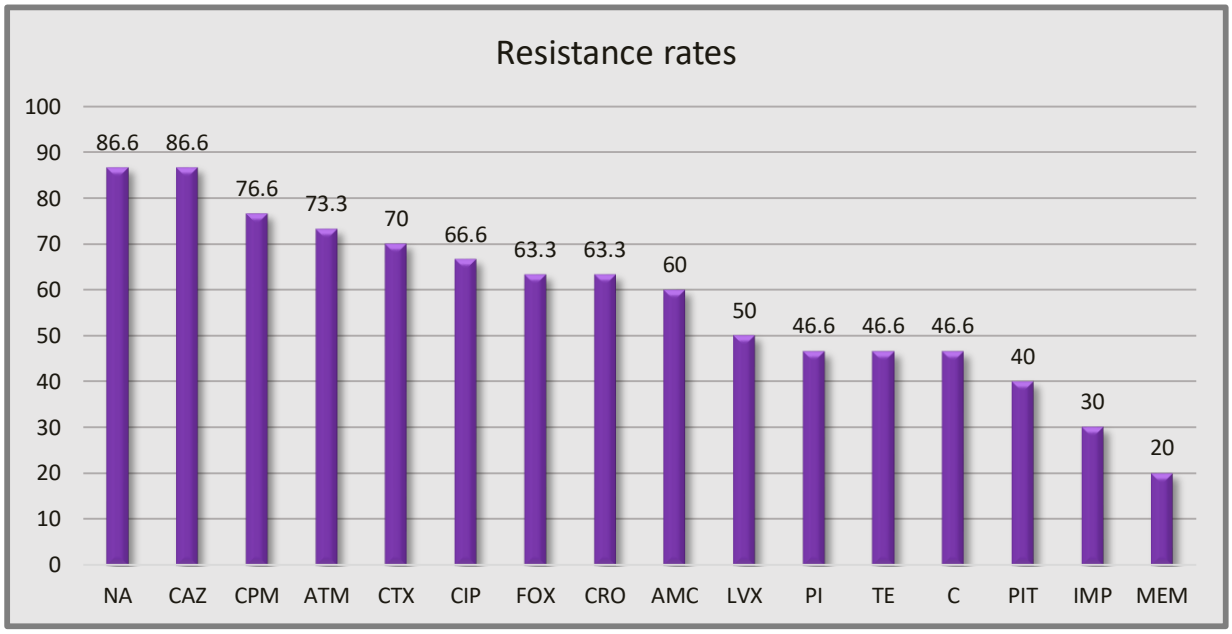

Figure 4.4 Resistance rates of the isolates. NA: Nalidixic acid, CAZ: Ceftazidime, CPM: Cefepime, ATM: Azetreonam, CTX: Cefotaxime, CIP: Ciprofloxacin, FOX: Cefoxitins, CRO: Ceftriaxone, AMC: Amoxicillin-clavulante (augmentin), LVX: Levofloxacin, PI: Piperacillin, TE: Tetracycline, C: Chloramphenicol, PIT: Piperacillin-tazobactam, IMP: Impenem, MEM: Meropenem.

The study showed that the isolates' resistance to quinolounes antibiotics, which includes nalidixic acid, ciprofloxacin and levofloxacin was at 88.6%, 66.6% and 50%, respectively. Cephalosporins II, III, IV, which includes cefoxitin, cefotaxime, ceftazidime, cefepime and ceftriaxone had a resistance of 63.3%, 70%, 86.6%, 76.6%, and 63.3%, respectively for each aforementioned antibiotic. The isolates' resistance to carbapenems, which includes meropenem and impenem, was at 20% and 30%, respectively. As for β-lactam combinations, which includes augmentin and piperacillin-tazobactam, the resistance was found to be at 60% and 40%. And as for pencillin group of antibiotics that includes piperacillin, the resistance was found to be 46.6%. Tetracyclin from the antibiotic group of the same name and chloramphenicol from the antibiotic group phenicol both had a resistance rate of 46.6%. Azetreonam from the antibiotic group monocycline, resistance was at 73.3%. Table 4.5 and appendix 3 details the resistance rates and the number of isolates that were resistant.

Table 4.5 Resistance percentage and the number of isolates that were resistant to each antibiotic tested.

Antibiotic group	Antibiotics (mg/dL)	No. of resistant isolates	Resistance percentage
β-lactam carabapenem	Meropenem (10)	6	20%
	Impenem (10)	9	30%

Antibiotic group	Antibiotics (mg/dL)	No. of resistant isolates	Resistance percentage
β-lactam combinations	Amoxillin-clavulnate (30)	18	60%
	Piperacillin-tazobactam (30)	12	40%
Monocyclin	Azetronam (30)	22	73.3%
Cephems (including cephalosporins II, III, IV)	Cefotaxime (30)	21	70%
	Cefoxitin (30)	19	63.3%
	Ceftazidime (30)	26	86.6%
	Cefepime (30)	23	76.6%
	Ceftriaxone (30)	19	63.3%
Fluroquinolones	Nalidixic acid (30)	26	86.6%
	Levofloxacin (5)	15	50%
	Cirpofloaxacin (5)	20	66.6%
Tetracycline	Tetracycline (30)	14	46.6%
Pencillin	Piperacillin (100)	14	46.6%
Phenicol	Chloramphenicol (30)	14	46.6%

This study showed a resistance to the antibiotics of nalidixic acid, ceftazidime and augmentin (amoxillin/clavulanic acid) that was relatively close to what was found by Al-No'aemy (2018) when he noticed that the isolates were resistance to nalidixic acid, ceftizidime and augmentin at 80%, 85% and 52%, respectively, but the resistance to levofloxacin and impinem was different from the current study at 73% and 4.7%, respectively. He further explains that resistance to augmentin was caused by beta-lactamase enzymes TEM-1 and SHV-5, and AMP enzymes which has a vital role in clavulanic acid resistance. A study done by Salman *et al.* (2013) showed that resistance to cefotaxime and cefepime was at 78% and levofloxacin was at 55.5%, but it was also found that cefoxitin was at 7% only. The current study's results differ from what was found by Maleki *et al.* (2017) in Iran where they found the resistance to ceftazidime had reached 26.1% and cefotaxime was at 30%. They then explain that the cause to the increase in the resistance to these antibiotics were the efflux pumps.

The current study's findings corresponds with what was found by Shah *et al.* (2019) when they found that resistance to meropenem was at 26% but they also found that cefotriaxone was 49%.

The increase in resistance to β-lactam antibiotics is due to the production of β-lactamase, which includes cephalopsorinase and pencillinase, which work on

breaking the beta-lactam ring which inhibits the antibiotics belonging to the groups pencillins and cephalosporins (Paltansing, 2015; Al-Shoyaikh, 2016).

The reason to the increase in resistance and the increased appearance of MDR UPEC isolates in women is the excessive use of broad spectrum antibiotics the likes of floraquinolones and cephalosporins (Bortoletti *et al.*, 2016). A study done by Polse *et al.* (2016) found different findings from the current study results when it comes to azetronam resistance which was found to be at 100%, but their findings corresponds to the current study when it comes to ceftriaxone resistance which was at 55%. Polse *et al.* later explains that the reason behind the high resistance shown to azetronam by the isolates was due to the random and uncontrolled use of the antibiotic. This study showed low resistance to impenem and meropenem which could be due to their stability against ESBL enzyme and their effectiveness in treating infections caused by ESBL-producing bacteria.

The current study corresponds with what Tajbakhsh *et al.* (2016) found in Iran when they found that resistance to ciprofloxacin was 56.25%. It was shown that the gained resistance to ciprofloxacin was the production of the biofilm by bacteria. The study findings also agree with what was found by Ramirez-Castillo *et al.* (2018). They found that resistance to the aforementioned antibiotic was at 47.3% and they explain that it could be caused by the excessive use of quinolones to treat UTI, as

they're the first choice to treating UTIs. The reasons behind *E. coli*'s resistance to quinolones group of antibiotics are possibly target modification, change in outer membrane permeability (Paltansing, 2015). Also, the activation of DNA production by DNA gyrase or by mutation is one of the most important causes of resistance to quinolones (Zaman *et al.*, 2017)

This study showed that resistance to each of tetracycline and chloramphenicol was at 46.6% while Mohammed (2018) in Iraq found that resistance to the same antibiotics was at 88% each and Abdelhamid and Abozahra (2017) in Egypt found that resistance to tetracycline was 64.3% by *E. coli*. This could be due to the bacteria having efflux pumps and modifying its outer membrane permeability. Resistance to chloramphenicol could be caused by the inactivation of chloramphenol acetyl transferase (CAT) by an enzyme that introduces new acetyl groups into chloramphenicol antibiotics, and thus, modifying the antibiotic and increasing resistance to it. The reduced drug uptake by Gram-negative bacteria could also be a factor helping increase resistance to this antibiotic (Hasan and Alharmoosh, 2020). Also the resistance to pipracillin-tazobactam in the current study was 40%, this disagrees with Zaman *et al.* (2019) who found the resistance to this antibiotic was at 8%. This shows that this antibiotic was effective against *E. coli* once but due to self-medication and overuse of antibiotics — which is a quite common in this region — resistance has seen an increase.

4.3.1 Statistical Analysis of Isolates' Resistance Towards Antibiotics

Statistical analysis for the resistance of the isolates to the antibiotics tested was done using Primer E7 to figure out similarities between the isolates during antibiotic resistance testing. An obtained dendogram shows 4 main groups: A, B, C, D. As shown in Figure 4.5, group A consists of E2, E11, E26, E27, E29, E30 which showed similarities ranging from 51 – 83%. Group B is made of E1, E4, E7, E12, E14, E16, E22, E28 with a similarity between 63 – 79%. Group C consists of 2

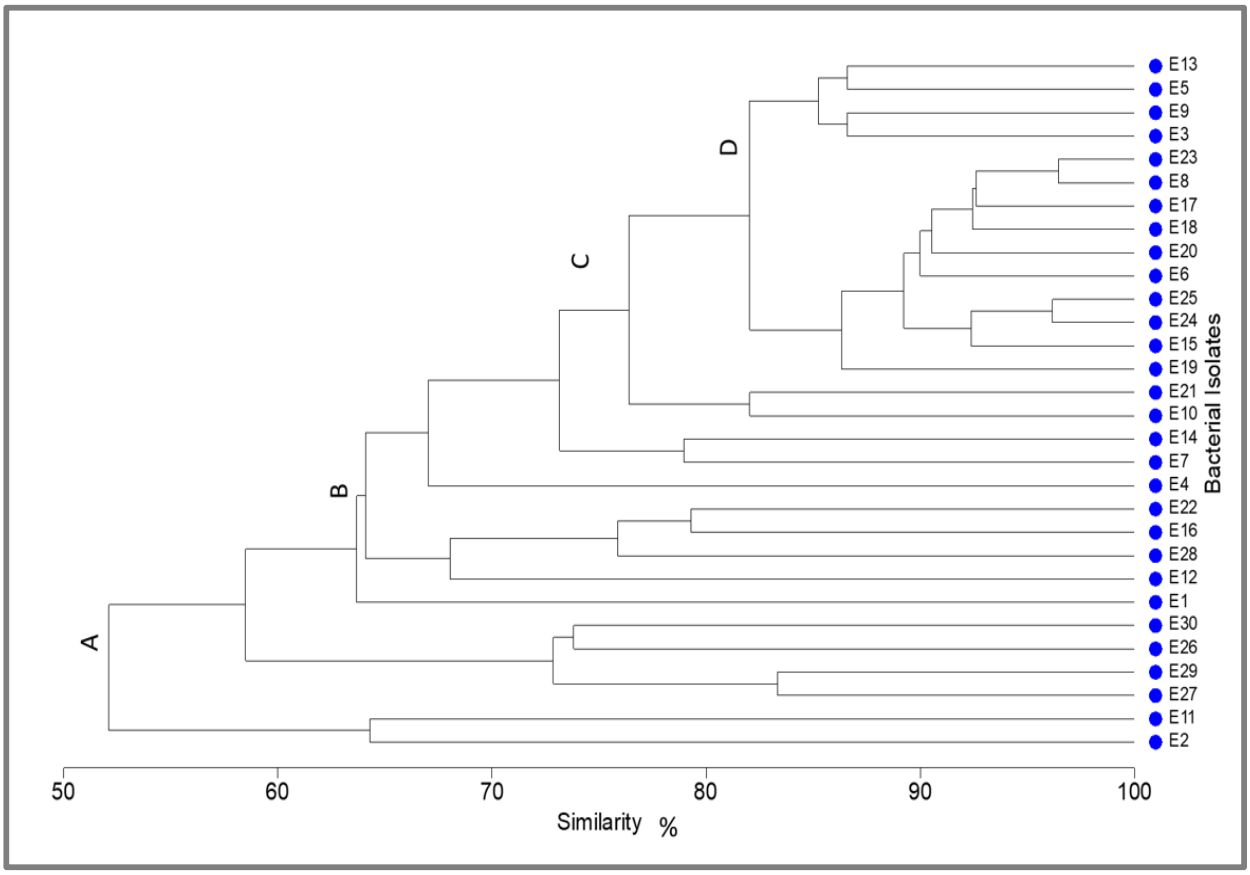

Figure 4.5 Dendogram of phylogenetic comparison of *E. coli* isolates based on antibiogram results.

isolates, E10, E21, which had a similarity rate of 82%. Group D had two subgroups: D1 consisting of E6, E8, E15, E17, E18, E19, E20, E23, E24 which showed a similarity rate of 85 – 95%; D2 consisting of E3, E5, E9, E13 showing a similarity rate of 87%.

4.4 Patterns of Isolates' Multi-drug Resistance

All isolates in the study showed a multi-drug resistance (MDR, XDR) level of resistance to the tested antibiotics, with a rate of 100%. Results showed that isolates E1, E4, E7, E11, E16, E28 and E29 were resistant to 3 antibiotic groups — the lowest number of groups of antibiotics resisted by all isolates — while another group of isolates consisting of E2, E14, E26 and E27 had resistance to 4 antibiotic groups. E5, E12, E13, E22 and E30 were resistance to 5 antibiotic groups. One isolate was found to be resistant to the highest number of antibiotic groups, resisting 8 groups and it was isolate E23. Isolates E3, E9, E15, E17, E19, E21 and E25 were found to be resistant to 6 antibiotic groups each. Another group of isolates that's made of E6, E8, E10, E18, E20 and E24 were showing resistance towards 7 groups of antibiotics (table 4.6).

Table 4.6 Resistance type for each isolate.

No. of isolates	Isolates	No. of antibiotic groups resisted	Resistance type
7	E1, E4, E7, E11, E16, E28, E29	3	MDR

No. of isolates	Isolates	No. of antibiotic groups resisted	Resistance type
4	E2, E14, E26, E27	4	XDR
5	E5, E12, E13, E22, E30	5	XDR
7	E3, E9, E15, E17, E19, E21, E25	6	XDR
6	E6, E8, E10, E18, E20, E24	7	XDR
1	E23	8	XDR

The study's results showed that the isolates were all MDR and this could be due to a mutation or the acquiring of a plasmid which gives the high level of resistance to the cell. The widespread and excessive use of broad spectrum antibiotics has also lead to the emerging of many multi-drug resistant *E. coli* (Gawad *et al.*, 2018). This high magnitude of antibiotic resistance is considered one of the most important health problems and especially *E. coli* which causes UTIs — one of the biggest health issues around the globe (Polse *et al.*, 2016).

4.5 Minimum Inhibition Concentration

MIC was done on all isolates using cefepime (1g) and cefotazidime (1g). MIC was determined using serial dilutions on MHA medium and the dilutions were 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048. MIC was done to find out how resistance the isolates were to the 2 antibiotics tested with high precision. Table 4.7 contains all

details on the MIC test carried on the isolates from the checkpoint that was ≥16µg/mL which means the isolates are resistant according to CLSI (2020). The results show that MIC for cefepime was 1024µg/mL and cefotazidime was 512µg/mL.

Table 4.7 MIC results for cefotazidime and cefepime.

MIC (µg/mL)	CAZ-resistant isolates	CPM-resistant isolates
16	—	—
32	E8, E16, E17, E28, E30	E9, E16, E26
64	E12, E13, E19, E21	E14
128	E7, E9, E10, E15, E22, E23, E25	E11, E13, E17, E21, E22
256	E1, E2, E3, E4, E5, E6, E11, E14, E24, E27, E29	E10, E12, E15, E23, E25, E28
512	E18, E20, E26	E2, E7, E20, E27, E29
1024	—	E1, E3, E4, E5,E6, E8, E18, E19, E24, E30
2048	—	—

4.6 Phenotypic Detection of Virulence Factors of *Escherichia coli*

A number of virulence factors harbored by *E. coli* were investigated and the following sections detail all aspects of the phenotypic investigation done on the isolates.

4.6.1 Biofilm Detection

4.6.1.1 Tube method (TM)

Biofilm formation capabilities of the isolates were investigated using tube method and it was found that 19 isolates (63.3%) were biofilm producers while 11 isolates (36.6%) were not as shown in table 4.8.

Table 4.8 Phenotypic test of biofilm production

Biofilm production	Isolates	Percentage
+	E2, E3, E5, E6, E7, E8, E9, E10, E11, E12, E13, E15, E17, E19, E20, E21, E23, E24, E25	63.3%
-	E1, E4, E14, E16, E22, E26, E27, E28, E29, E29, E30	36.6%

This is a qualitative assay that depends on now sturdy and thick the biofilm is for the bacteria in question in relation to the inner wall of the test tube. The appearance of a purple ring around the inner wall of the test tube indicates a positive result.

A prior study done by Abdullah (2020) on 20 isolates has showed that 8 isolates (40%) were biofilm producers while Sabah (2018) has found that all isolates of *E. coli* in their study were biofilm producers.

4.6.1.2 Micro titer plate (MTP) method

In addition to the aforementioned method, micro titer plate (MTP) was also used to detect biofilm production in the isolates, and it was found that most of the isolates were capable of producing biofilms; 25 isolates (83.3%) were biofilm producers out of 30 isolates, 6 isolates accounting for 24% were strong biofilm producers while 19 isolates (76%) were moderate biofilm producers. The remaining 5 isolates accounting for 16.7% of the total isolates were weak biofilm producers as shown in appendix 4 and Figure 4.6 shows a chart of MTP results.

MTP is a quantitative method using 96 wells to determine the strength of the biofilm produced by the isolates using spectrophotometer at a wavelength of 630nm to give a final digital value representing the quantity of biofilms produced by the bacterial suspension in the wells.

Prior studies have shown a similar result when using MTP to investigate biofilm production, as was found by Gawad *et al.* (2018) in Egypt when they found that 76.5% of their isolates were biofilm producers and Zaman *et al.* (2019) found that 68% of their isolates were actually biofilm producers.

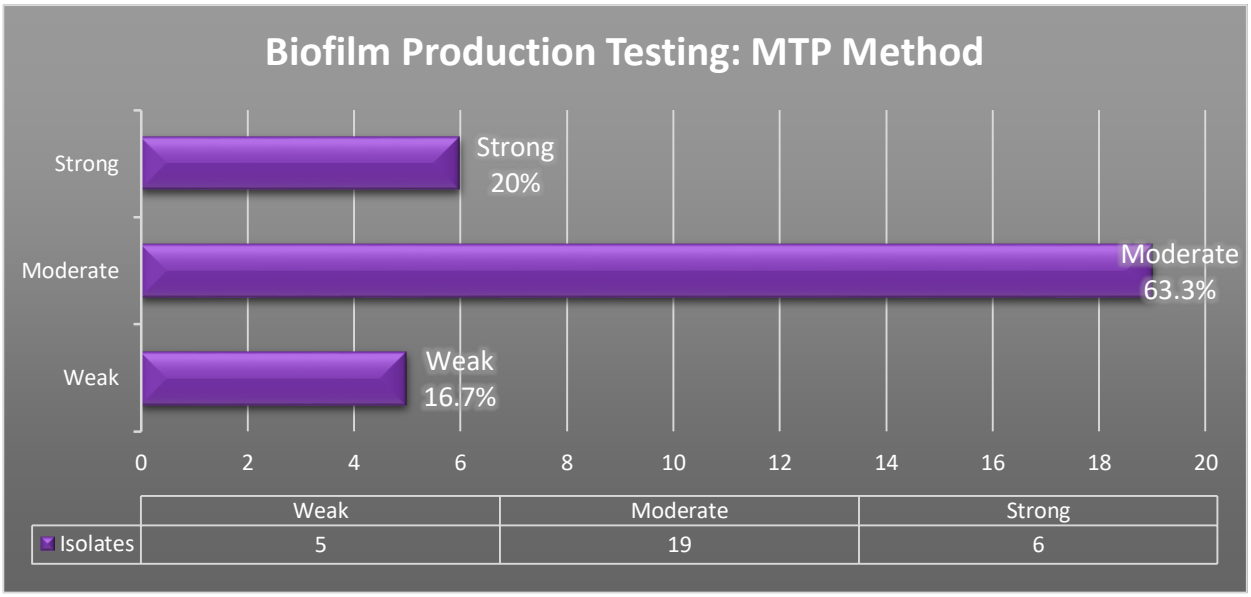

Figure 4.6 Biofilm production testing: MTP method results.

The difference between the two methods could be a result of the two different media used in both methods (trypto soya broth for MT method and nutrient broth for MTP) and the difference in bacteria concentration and incubation period could also affect results (Wanger *et al.*, 2017).

A biofilm is a microbial community enclosed by a layer of extracellular polymeric matrix which allows them to adhere to different surfaces (Hall and Mah, 2017). Recent studies have shown biofilm production in *E. coli* is mediated by the expression of curli and cellulose and that it helps UPEC survive for long periods in the urethra by cover whole communities of the bacteria with a hydrophobic, extracellular matrix (Kudinha, 2017).

Biofilm formation has an effect on both the effectiveness of antimicrobials and the triggering of host immune systems, which contributes to the presence of UPEC in the urinary tract and the consequential severe symptoms of UTI and antibiotic resistance (Asadi-Karam *et al.*, 2018).

4.6.2 Extended Spectrum Beta-lactamase (ESBL) Production

The isolates were tested for ESBL enzyme production using the double disk synergy test (DDST) (Figure 4.7). Results showed that 19 (63.3%) were ESBL producers while 11 isolates did not produce the enzyme, accounting for 36.6% as shown in appendix 5. A study done in Nepal (Pandit *et al.*, 2020) found that ESBL production was at 40.3% while Hassuna *et al.* (2020) in Egypt found that 59.7% of their isolates were capable of producing ESBL and another study carried out in Ethiopia (Belete, 2020) found that 66.7% of their isolates were ESBL producers.

The difference in ESBL production between this study could be caused by the difference in the isolates, difference in local antibiotic usage in each country in addition to the excessive useage of broad spectrum antibiotics especially the 3rd generation of cephalosporins and the magnitude of drug-resistance the pathogens in the local area of the study have (Pandit *et al.*, 2020). β-lactamase is one of the most important virulence factors which helps in destroying the β-lactam ring in certain antibiotics and thus, increasing antibiotic resistance and virulence of *E. coli*.

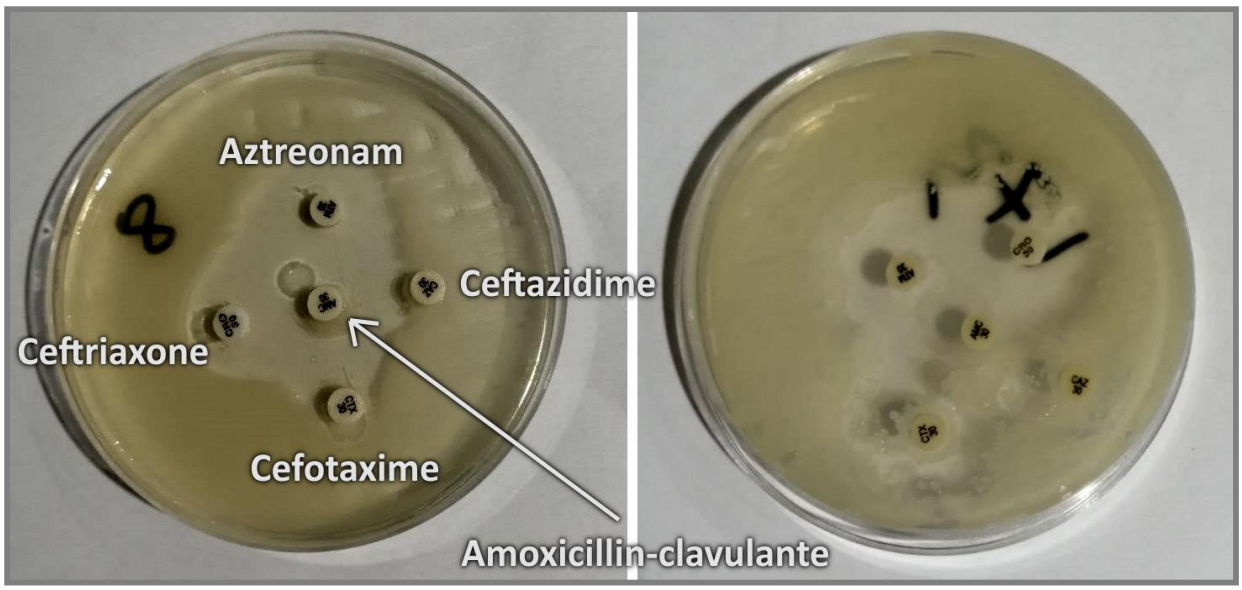

Figure 4.7 Double disk synergy test (DDST) results for ESBL production test.

4.6.3 Hemolysin Production

Sheep blood agar was used to test isolates' ability to lyse red blood cells; produce hemolysin. It was found that 12 (40%) isolates out of the 30 total were capable of lysing red blood cells with varying degrees while the remaining 18 (60%) were not capable (ɣ-hemoysis). The 12 isolates could further be divided into 2 groups: the first group consists of 3 isolates that showed β-hemolysis while the other group consists of 9 isolates showing α-hemolysis as shown in table 4.9.

Table 4.9 Phenotypic test of hemolysin production.

Hemolysis type	Isolates
β-hemolysis	E3, E10, E23
α-hemolysis	E6, E8, E17, E18, E19, E20, E24, E25, E30

Hemolysis type	Isolates
ɣ-hemolysis	E1, E2, E4, E5, E7, E9, E11, E12, E13, E14, E15, E16, E21, E22, E26, E27, E28, E29

Mohammed *et al.* (2018) did a study in Iraq on 25 *E. coli* isolates and they found that 10 of them showed α-hemolysis while 2 were β-hemolytic and 13 were ɣ-hemolytic. On the other hand, Bhattacharyya *et al.* (2015) in India found that only 10% of their *E. coli* isolates that were taken from UTI samples were hemolytic.

E. coli produces exotoxins like hemolysin which helps the virulence of the bacteria and increases its potential in causing and spreading UTIs. hlyA is a protein that has a pore-forming activity which causes the death of host's nucleated cells in addition to red blood cells. Plus, it can initiate apoptosis of urinary tract epithelium and cells associated with host's immune system (Bien *et al.*, 2012; Lee *et al.*, 2016).

4.6.4 Capsule Formation

Isolates were investigated for capsules using microscopic examination after staining with nigrosin. It was observed that 23 (76.7%) of the isolates had capsules indicated by a clear halo surrounding the dyed cells while 7 (23.3%) did not have any halos and were considered not being able to produce a capsule.

K antigen is one of the virulence factors at the disposal of *E. coli* that allows the bacterium evade host's immune system (Whitefield, 2006). Capsules help *E. coli*

by defending it from phagocytosis by the host's immune cells. This is done by preventing the activation of the phagocytic process through the lowering of opsonins binding and the masking of ligands for phagocytic cell attachment (Maruvada *et al.*, 2008). Another function the capsule can serve is in adhesion and biofilm formation (Fleitas and France, 2016).

4.6.5 Motility

Motility of the isolates was tested using semi-solid medium and it was found that 100% of the isolates were motile.

4.6.6 Statistical Analysis of Phenotypic Characteristics

An analysis was carried on the results of the phenotypic tests based on the similarities between the isolates and a dendogram was obtained which is shown in Figure 4.8.

Group A were the smallest group consisting of E1, E26, E29 which showed a similarity of 30%; these isolates only had the flagella from of the tested characteristics. B group was the largest group which further divided into B1 (E5, E9, E12, E13, E15, E21) which showed a similarity of 79%; B1 were capsulated, flagellated, biofilm-producers, and capable of producing ESBL but not hemolysin. The other B group, B2, consists of E3, E6, E8, E10, E17, E18, E19, E20, E23, E24 which had all the phenotypic characteristics and E30 was also phenotypically

identical to B2 with a similarity rate of 80%. Group C was also divided into 2 subgroups: C1 consists of E7, E11, E14, E16, E27 which had in common being moderate biofilm producers and flagellated with a similarity rate between them of 57% while C2 includes E2, E4, E22, E28 which each isolate had more than 1 characteristic.

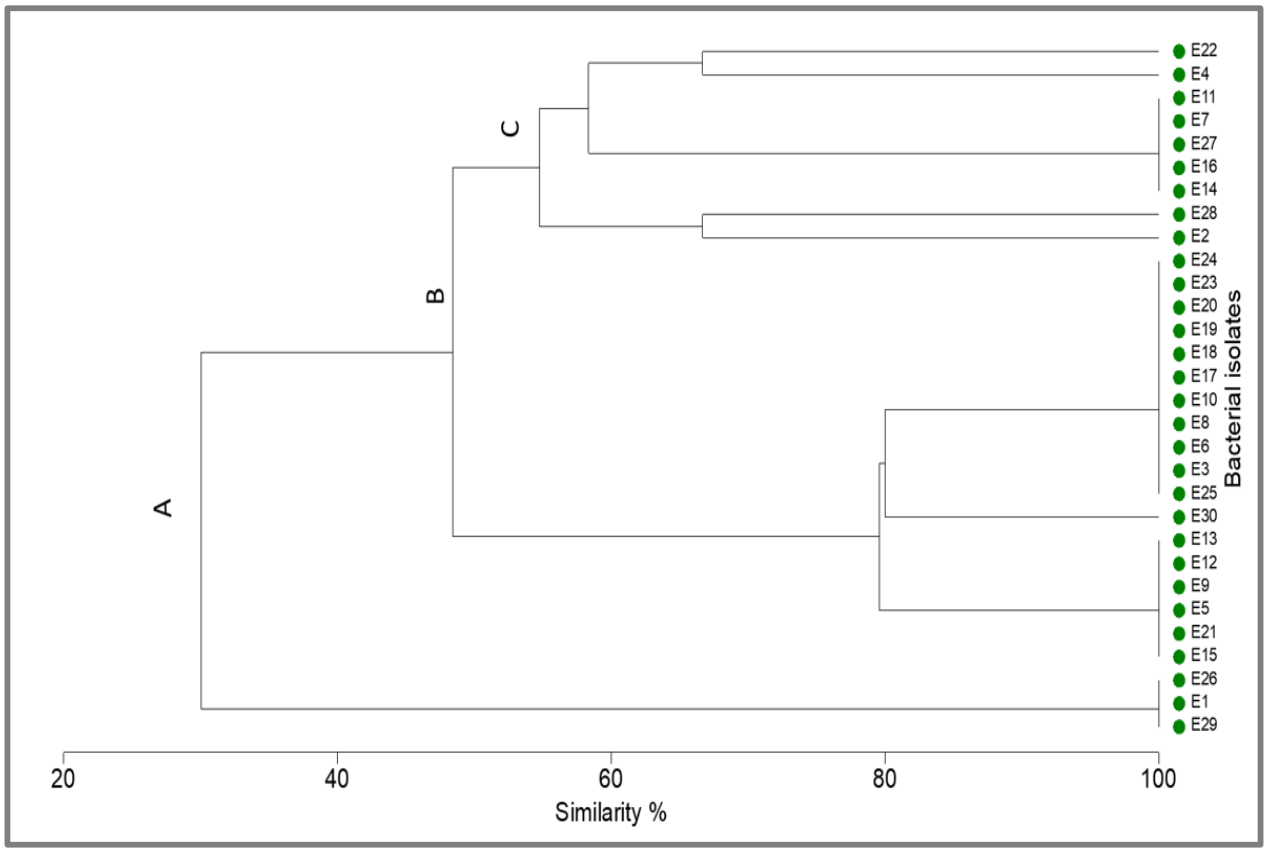

Figure 4.8 Dendogram of phylogenetic comparison of *E. coli* isolates based on phenotype results

4.7 Association between Antibiotic Resistance and Virulence Factors

The relationship between virulence factor and antibiotic resistance has developed over a long period. This strong relationship depends on the bacterial species, specific mechanism of resistance and virulence factor, the ecological niche and environmental condition and immune system of the host. This is in addition to the age and host susceptibility to infections (Cepas and Soto, 2020).

Results of this study showed that all isolates were MDR which is resistance towards 2 or more antibiotics from 2 different groups of antibiotics. Results also show that there is a relationship between the isolates' resistance and biofilm production as it was observed that isolates which were biofilm producers were 1000 times more resistance to antibiotics than isolates that were not (Soto, 2014). Biofilms can have a strong effect on the bacteria's antimicrobial tolerance and it can allow the bacteria to dodge urinary tract defenses which helps the MDR strains to evolve even further and cause more severe UTIs (Mittal *et al.*, 2015).

The main source of resistance of *E. coli* to 3rd and 4th generation cephalosporins is ESBL enzyme which has developed due to a mutation in the genes *bla-TEM1*, *bla-TEM2*, and *bla-SHV-1* (Kot, 2019). ESBL is carried on plasmid which is also responsible for encoding resistance to other antibiotics, such as ampicillin,

floroquinolones, aminioglycosides, macrolides and chloramphenicol (Mishra *et al.*, 2019).

The current study also showed that hemolysin was present in isolates with the highest resistance. This is in accordance to what was found by Mare *et al.* (2020) when they found that hemolysin was present in all MDR isolates and what Yazdanpour *et al.* (2020) noticed in their study in Iran where they mentioned that hemolysin was more present in isolates with resistance to all antibiotics tested than isolates with low resistance.

The capsule's role in bacteria's defense mechanisms has already been explored before. Some serotypes of capsule have even been linked to certain multi-drug resistance in addition to the capsule's role in survival and immune system escape mechanism, which some studies even found that switching capsule locus between different serotypes of bacteria suggested that the ability to escape from macrophages was strongly associated with their capsule serotype (Buffet *et al.*, 2021; Jochum *et al.*, 2021).

Table 4.10 Association between phenotypic features and antibiotic resistance of the isolates.

Isolate	No. of antibiotics resisted (Res. type)	Phenotypic tests				
		Biofilm	ESBL	Hemolysin	Capsule	Motility
E1	3 (MDR)	-	-	-	-	+

Isolate	No. of antibiotics resisted (Res. type)	Phenotypic tests				
		Biofilm	ESBL	Hemolysin	Capsule	Motility
E2	8 (XDR)	+	+	-	-	+
E3	12 (XDR)	++	+	+	+	+
E4	5 (MDR)	-	-	-	+	+
E5	10 (XDR)	+	+	-	+	+
E6	13 (XDR)	++	+	+	+	+
E7	6 (MDR)	+	-	-	-	+
E8	13 (XDR)	++	+	+	+	+
E9	11 (XDR)	+	+	-	+	+
E10	13 (XDR)	+	+	+	+	+
E11	5 (MDR)	+	-	-	-	+
E12	6 (XDR)	+	+	-	+	+
E13	10 (XDR)	+	+	-	+	+
E14	7 (MDR)	+	-	-	-	+
E15	11 (XDR)	++	+	-	+	+
E16	4 (MDR)	+	-	-	-	+
E17	13 (XDR)	+	+	+	+	+
E18	14 (XDR)	+	+	+	+	+
E19	11 (XDR)	+	+	+	+	+
E20	14 (XDR)	+	+	+	+	+
E21	10 (XDR)	+	+	-	+	+
E22	9 (XDR)	+	-	-	+	+
E23	14 (XDR)	++	+	+	+	+
E24	12 (XDR)	++	+	+	+	+
E25	14 (XDR)	+	+	+	+	+
E26	6 (XDR)	-	-	-	-	+

Isolate	No. of antibiotics resisted (Res. type)	Phenotypic tests				
		Biofilm	ESBL	Hemolysin	Capsule	Motility
E27	6 (MDR)	+	-	-	-	+
E28	6 (MDR)	-	+	-	-	+
E29	5 (MDR)	-	-	-	-	+
E30	7 (XDR)	+	-	+	+	+
Percentage	100% MDR	83.3%	63.3%	40%	76.6%	100%

4.8 Genotype and Molecular Detection of *E. coli*

4.8.1 DNA Extraction

15 DNA extracts were prepared from 30 isolates of *E. coli* based on their antibiotic resistance (MDR), biofilm formation, ESBL and capsule production. Concentration of DNA extract was measured using Quantus Florurometer and the ideal concentration was between 15 – 30ng/mL as shown in table 4.11. Purity of DNA extracts was measured using spectrophotometer and it was found to be at 1.6 – 1.8. Purity of the extracts is crucial for PCR as it is required to obtain ideal results when making multiple copies of DNA template.

Table 4.11 DNA extract concentration for the isolates in the study.

PCR no.	Isolate	Conc. (ng/mL)	PCR no.	Isolate	Conc. (ng/mL)
1	E23	18	9	E3	30
2	E19	20	10	E5	26
3	E18	23	11	E25	17

PCR no.	Isolate	Conc. (ng/mL)	PCR no.	Isolate	Conc. (ng/mL)
4	E20	28	12	E17	20
5	E6	15	13	E15	24
6	E8	19	14	E9	28
7	E10	22	15	E21	18
8	E24	24			

4.8.2 Molecular Detection of Virulence Genes

Some virulence genes for *E. coli* isolates were investigated using multi- and uni-plex PCR by utilizing a thermocycler to confirm the presence of the genes in question in the isolates and by using a primer specific to each gene.

4.8.2.1 Molecular detection of *fimH*, *pap*, *sfa*, *ompT*

Multiplex PCR was used to detect the genes of *fimH* measuring 465bp and *pap* measuring 328bp. Results showed that all the isolates, numbering 15, had *fimH* (100%) and *pap* was present in 12 isolates (80% of the total isolates). As for *sfa* and *ompT* genes, measuring 244bp and 559bp respectively, results showed that 7 isolates had *sfa* while 10 isolates showed *ompT*, accounting for 46.6% and 66.6% of total isolates respectively, as shown in Figure 4.9.

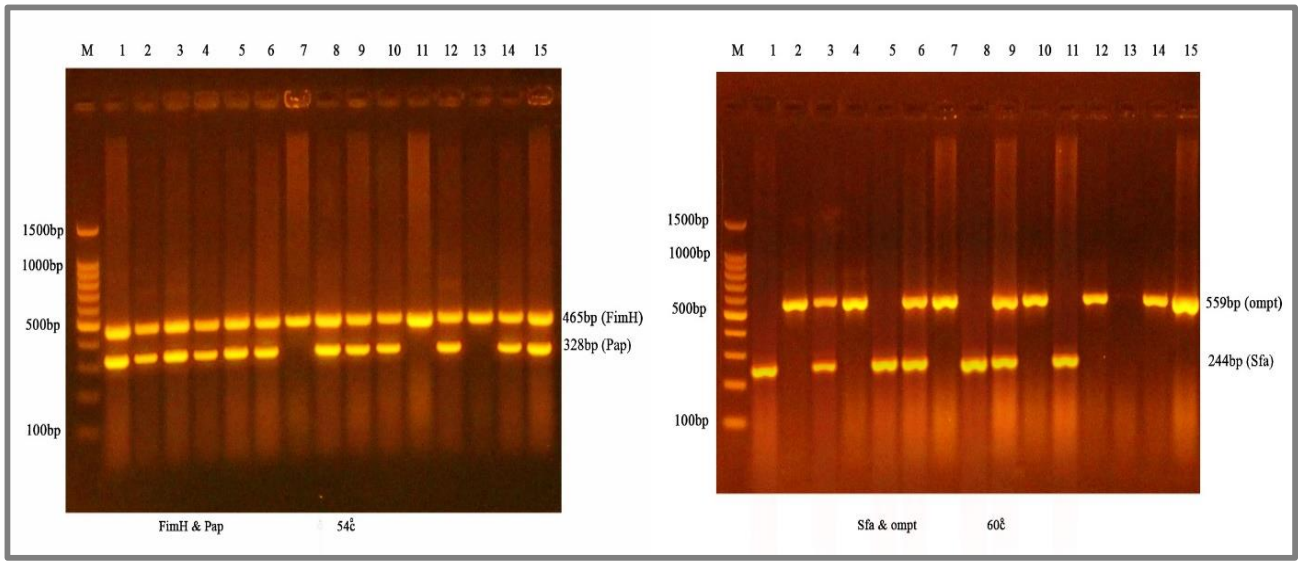

Figure 4.9 Multiplex PCR amplification of *fimH*, *pap*, *ompT*, and *sfa* genes of *Escherichia coli* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 on each image resemble 465bp (*fimH*), 328bp (*pap*), 559bp (*ompT*), and 244bp (*sfa*) PCR products for each gene.

Researchers such as Salih *et al.* (2015) in Iraq found that *fimH* was present in 91%, *sfa* was present in 75% and *pap* was in 51.7% of their *E. coli* isolates. On the other hand, Ibrahim *et al.* (2020) found that *sfa* was present in 17.5% in their study. This study had results that were close to what was found by Yazdi *et al.* (2020) in Iran where they found that *fim*, *pap* and *sfa* were present at 100%, 78.8% and 69.2%, respectively. The study's results disagrees with what Dadi *et al.* (2020) found in Ethiopia, in which they explain that *fim* was present in 82% of their isolates and that *pap* and *sfa* were present at 29.5% and 25%, respectively. The current study comes into a disagreement with what was found by Dehkordi *et al.* (2020) in Iran where

they found *ompT* to be at 29.54% but it corresponds to the 66.8% presence rate of *ompT* gene and the 96.8% rate of *fimH* of Baldiris-Avila *et al.* (2020) in Cambodia but differ on *pap* which was found to be at 26% in the last study.

The genes responsible for adhesion proteins encoding are the most frequently present genes in UPEC. *fimH* encodes fimbriae H which bind with uroepithelial protein α-D-mannosylated, stabilizing adhesion to the host's uroepithelial under extreme pressure which in turn could potentially lead to urosepsis while *pap* encodes P fimbriae which is associated with cystitis and pyelonephritis cause it binds to α-D-galactopyronosol-(1-4)-β-D-galactopyronoside-containing receptor found on upper urinary tract. The gene *sfa* encodes S fimbriae, a type of fimbriae that is also associated with bacterial pathogenicity in human (Malekzadegan *et al.*, 2018; Li *et al.*, 2020). The wide variety of virulence factors encoded by associated genes present in *E. coli* are acquired via DNA transfer to plasmid, transposon, bacteriophage, and pathogenicity island (PAI) help it in overcoming host's defenses and causing UTIs (Foxman, 2010).

Detection of *ompT*, in addition to aforementioned genes, is crucial for the detection of virulent clinical UPEC (Johnson *et al.*, 2001; Najafi *et al.*, 2018). Having a role in invasion, adhesion and/or inactivation, ompT is often associated with cystitis and prostatitis. It is also mentioned that ompT is used as a bacterial

escape mechanism by giving the bacteria the ability to resist the cationic peptides found in the urinary tract which in turn helps the bacteria survive longer and increase its potential to cause UTI (Sun *et al.*, 2020).

4.8.2.2 Molecular detection of *kpsMII*, *bla-TEM*, *eae*

The genes *kpsMII*, *bla-TEM* and *eae*, measuring 668bp, 1080bp and 881bp respectively, were investigated using uniplex PCR. Figures 4.10, 4.11 and 4.12 show the multiplex PCR results for *kpsMII*, *bla-TEM* and *eae*, respectively. As seen in the figures, *kpsMII* was found in 8 (53.3%) isolates, *bla-TEM* was found in 9 (60%) and *eae* was found in 4 (26.6%).

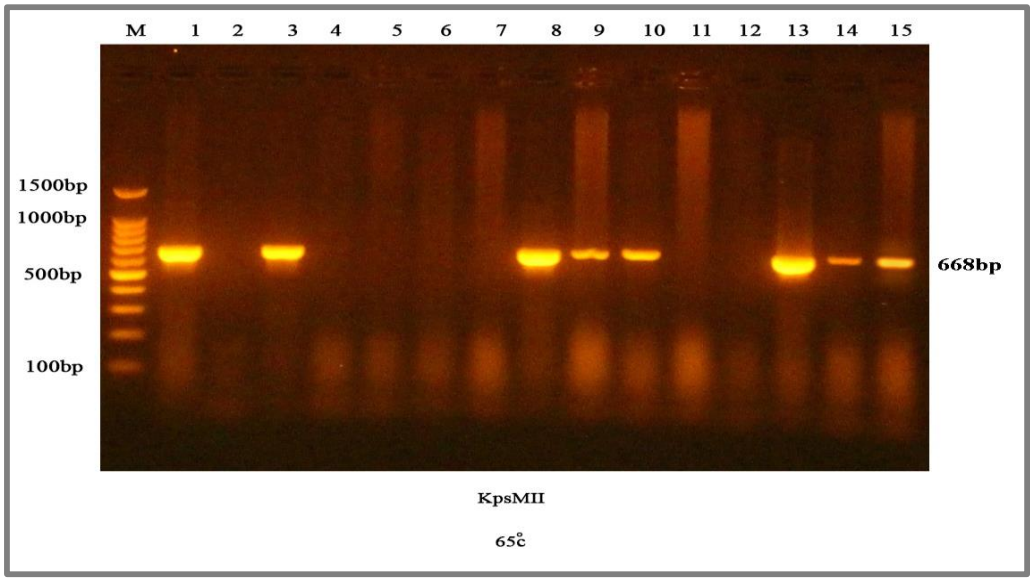

Figure 4.10 Uniplex PCR amplification of *kpsMII* gene of *Escherichia coli* samples was fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 resemble 668bp PCR product.

Ahmed *et al.* (2019) in Iraq reported that they found *kpsMII* in 40% of their isolates. In Iran, Haghighatpanah and Mojtahdi (2019) and Sheikh *et al.* (2019) found the same gene to be at 76% and 23%, respectively. Also Baldiris-Avila *et al.* (2020) in Cambodia found it to be at 66.8%.

kpsMII encodes for the production of the capsule — one of the most important virulence factors for *E. coli* as it prevents phagocytosis and the immune system's complement system by providing the bacteria with an extra layer of protection made of polysaccharides. The capsule enhances the chances of *E. coli* to cause UTI by providing extra protection (Sarkar *et al.*, 2019).

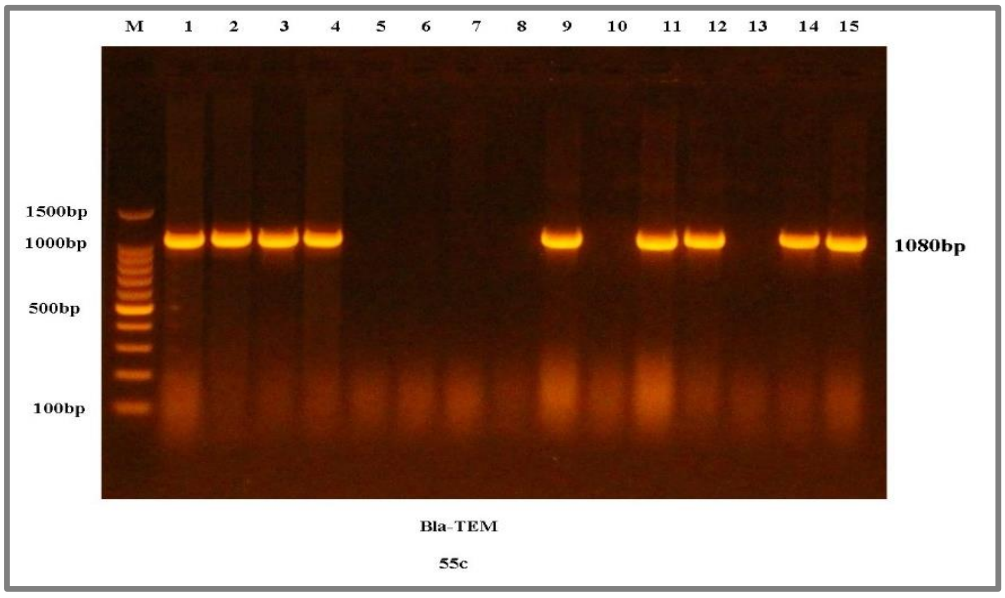

Figure 4.11 Uniplex PCR amplification of *bla-TEM* gene of *Escherichia coli* samples was fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 resemble 1080bp PCR product.

The study showed a presence rate of *bla-TEM* that was 60% (9 isolates) which doesn't correspond with another local study done by Al-Kudhairy and Alshammari (2019) which they found at 28.6%. Mirkalantari *et al.* (2020) found the same gene at 47.7% in Iran while Hassuna *et al.* (2020) found it at 75% in Egypt.

ESBL enzymes are encoded by *bla-TEM* and they greatly enhance *E. coli*'s chance to cause UTI by providing an outstanding range of resistance to different antibiotics. The enzymes are encoded by a plasmid gene probably due to a mutation in *bla-TEM* (Temoberia) (Goudarzi *et al.*, 2013).

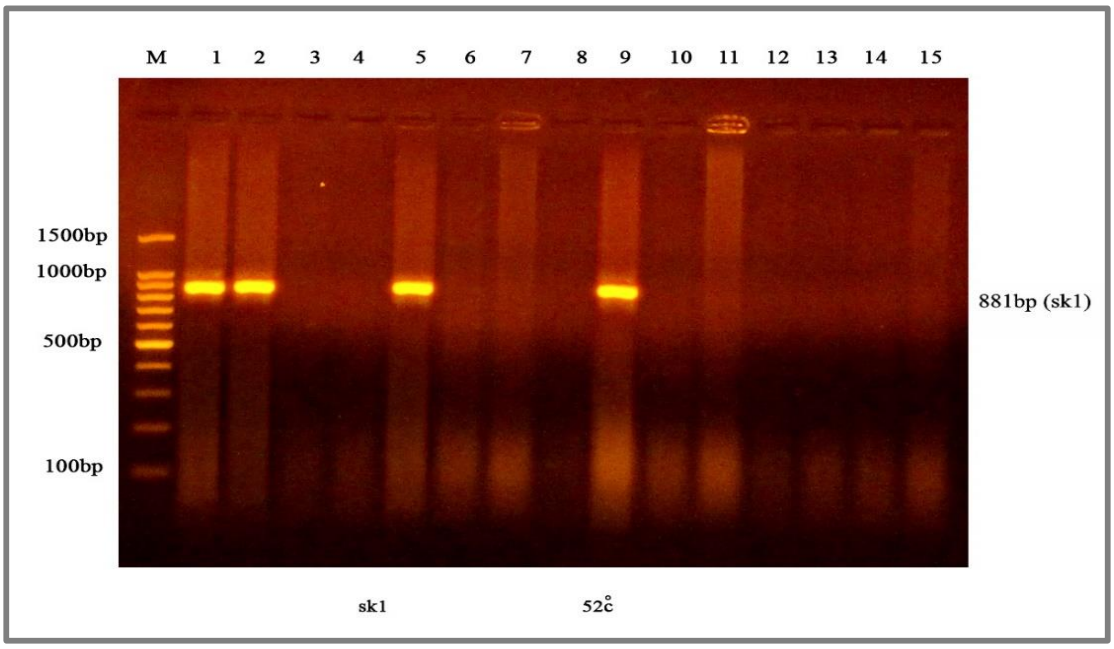

Figure 4.12 Uniplex PCR amplification of *eae* gene of *Escherichia coli* samples was fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 resemble 881bp PCR product

When it comes down to *eae* gene, 3 isolates carrying this gene were hemolytic, capable of shifting SMAC color, and motile on semi-solid media which indicates that these isolates could be EHEC (Selim *et al.*, 2014). The fourth isolate (E23) had the same characteristics but was not capable of shifting colors on SMAC. This study showed that *eae* was present in only 1 (8.3%) isolate from the 12 UPEC isolates. This is in correlation with Derakhshan *et al.* (2019) findings in Iran when they found *eae* to be present in 5.4% of the isolates but Yilmaz and Aslantas (2020) in Turkey found it in 0.7% of their isolates. The presence of *eae* in EPEC is not out of the ordinary but its presence in UPEC is not as common as other types of *E. coli* which could indicate that the obtained isolates were a new hybrid *E. coli* strain resulting from the elasticity of the bacterial genome (Ramirez-Castillo *et al.*, 2018). Appendix 6 contains all the details about the genetic tests carried out on the isolates.

4.8.3 Genetic Patterns

PCR results showed that 2 isolates (E10, E15) had 2 virulence genes, 1 isolate (E25) had 3 genes, 6 isolates (E5, E6, E8, E17, E20, E24) had 4 genes, 3 isolates (E9, E19, E21) had 5 genes, 2 isolates (E18, E23) had 6 genes and one isolates (E3) had all of the virulence genes looked for by PCR, as shown in table 4.12.

The difference in the distribution of virulence genes could be a result of differences in geographical location or the climate of said locations, habits, public health, how clean the food sources are and difference in UPEC strains genetics (Ibrahim *et al.*, 2020).

Table 4.12 Genetic patterns among the isolates in the study.

Isolate	No. of genes	Genes detected
E10	2	*fimH, ompT*
E15	2	*fimH, kpsMII*
E25	3	*fimH, sfa, bla-TEM*
E6	4	*fimH, pap, sfa, eae*
E5	4	*fimH, pap, sfa, ompT*
E8	4	*fimH, pap, ompT, kpsMII*
E17	4	*fimH, pap, ompT, bla-TEM*
E20	3	*fimH, pap, ompT, bla-TEM*
E24	4	*fimH, pap, sfa, kpsMII*
E9	5	*fimH, pap, ompT, kpsMII, bla-TEM*
E19	5	*fimH, pap, ompT, bla-TEM, eae*
E21	5	*fimH, pap, ompT, kpsMII, bla-TEM*
E18	6	*fimH, pap, sfa, ompT, kpsMII, bla-TEM*
E23	6	*fimdH, pap, sfa, kpsMII, bla-TEM, eae*
E3	7	*fimH, pap, sfa, ompT, kpsMII, bla-TEM, eae*

4.8.4 Relationship of Genes and Antibiotic Susceptibility

Many studies have shown a relationship between the genes of UPEC and sensitivity towards certain antibiotics, and the current study demonstrated the same finding.

It was noted that 3 isolates (E10, E15, E25) which didn't have the *pap* gene were susceptible to cefoxitin, meropenem and ceftriaxone while another 5 isolates (E6, E15, E23, E24, E25) which were missing *ompT* were found to be susceptible to piperacillin, meropenem and ceftriaxone, and that 8 isolates (E5, E9, E10, E15, E17, E19, E20, E21) were missing *sfa* and were susceptible to chloramphenicol, piperacillin-tazobactam, cefotaxime, cefotixin, meropenem, augmentin, azterinam and cefotazidime. The gene *kpsMII* was missing in 7 isolates (E6, E8, E10, E17, E19, E20, E25) and they were sensitive to chloramphenicol, pipracillin, piperacillin-tazobactam, cefotixin, cefotaxim and meropenem. The gene *bla-TEM* was missing in 6 isolates (E5, E6, E8, E10, E15, E24) which showed sensitivity towards piperacillin, cefotixin, meropenem and ceftriaxone. And as for *eae*, it wasn't found in most isolates and they showed different degrees of resistant to different antibiotics. Appendix 7 details the isolates' missing genes and their antibiotic susceptibility.

Miranda-Estrada *et al.* (2017) in Mexico explained that there is a relationship between antibiotics resistance and virulence genes. Ghavidel *et al.* (2020) did a study in Iran in which they explain that there's a relationship between *pap* and resistance to fluoroquinolones and cephalosporins, and that *sfa* has a relationship with flouroquinolones. Another study done in Iran by Yazdanpour *et al.* (2020) found that *fim* and *sfa* were missing in isolates resistant to impenem and this is in accordance with the current study's finding which shows that *sfa* was missing in isolates resistant to impenem. Spearman's correlation test was performed to establish the linear association between the absence of certain genes relative to the antibiotic resistance. Table 4.13 showed clear linear correlation between some pairs of genes and antibiotic. Results showed a significant correlation between *eae* with meropenem and chloramphenicol and a relationship between *bla-TEM* and ceftriaxone (P value of ≤ 0.04). The gene *pap* had a significant correlation with the cefotixin while *ompT* had a significant correlation with ceftriaxone, both having a P value of ≤ 0.05. Also, *sfa* correlated with meropenem significantly (P value = 0.02). The results also show some other correlations between some virulence genes and some antibiotics but they're not significant.

Table 4.13 Relationship between virulence genes and antibiotics susceptibility.

Antibiotic	Genes					
	bla-TEM	*eae*	*pap*	*sfa*	*ompT*	*kpsMII*
MEM	0.43	**0.03***	0.47	**0.02***	0.50	0.22
PIP	0.24	0.06	0.48	0.12	0.62	0.93
CTX	0.43	0.57	0.63	0.37	0.50	0.30
CAZ	0.23	0.57	0.63	0.37	0.50	0.37
FOX	0.43	0.57	**0.04***	0.37	0.50	0.30
C	0.10	**0.04***	0.37	0.63	0.20	0.63
AMC	0.23	0.57	0.63	0.37	0.50	0.37
ATM	0.23	0.57	0.63	0.37	0.50	0.37
PIT	0.23	0.10	0.63	0.37	0.50	0.30
CRO	**0.04***	0.40	0.29	0.93	**0.03***	0.18

*P value < 0.05 is significant, P value > 0.05 is insignificant.

4.8.5 Relationship between Genes and Virulence Factors

Virulence genes were investigated in 15 isolates which were all biofilm producers. The current study showed that the genes most prevalence were *fim*, *pap* and *ompT* and with a presence percentage of 100%, 80% and 66%, respectively while the genes which were rarest among the isolates were *sfa* and *eae* which were at 40% and 24%, respectively. Prior studies like Tewawong *et al.* (2020) in Thailand have showed that isolates which were biofilm producers had *fim* and *pap* at the highest frequencies while having *sfa* at the lowest frequencies. Zamani and Salehzadeh (2018) did a study in Iran and found that *fim*, *pap* and *sfa* were the most

frequent genes among biofilm-producing isolates and Baldiris-Avila *et al.* (2020) in Cambodia found that *ompT* and *kpsMII* were the most prevalent genes in isolates which were capable of producing biofilms. The importance of biofilms and some other virulence factors in UPEC have been explained before (Rahdar *et al.*, 2015; Tajbakhsh *et al.*, 2016).

The 15 isolates used in the PCR test were divided into 2 groups: Non-hemolytic isolates (4) all had *kpsMII* which puts it at a rate of 100% in the non-hemolytic isolates while the other, hemolytic isolates (11) had a presence rate for *kpsMII* of 36.3%. *ompT* was found to be at 75% and 63.3% for hemolysin-negative and -positive isolates, respectively. *Bla-TEM* was found to be higher in hemolytic isolates appearing in 63.3% compared to non-hemolytic isolates which had the gene at 50%. Table 4.14 contains the linear correlation between virulence genes and phenotypes in the isolates. Examining the table, it is apparent that hemolysis has a significant correlation with the genes *sfa*, *fimH*, and *kpsMII* (P value ≤ 0.03) while the capsule had a correlation with *kpsMII* only (P value = 0.03). Biofilm production and *eae*, *sfa* and *fimH* showed significant correlation (P value ≤ 0.04).

Table 4.14 Association between virulence genes and phenotypes in *E. coli* isolates.

Phenotype	Genes						
	eae	*sfa*	*pap*	*fimH*	*ompT*	*kpsMII*	*bla-TEM*
Biofilm	**0.01***	**0.04***	0.20	**0.02***	0.10	0.70	0.30

Phenotype	Genes						
	eae	*sfa*	*pap*	*fimH*	*ompT*	*kpsMII*	*bla-TEM*
ESBL	0.10	0.50	0.20	0.30	0.20	0.20	0.50
Hemolysis	0.20	**0.03***	0.80	**0.02***	0.70	**0.02***	0.70
Capsule	0.30	0.40	0.50	0.30	0.40	**0.03***	0.80
Motility	0.70	0.90	070	0.20	0.10	0.20	0.10

*P value < 0.05 is significant, P value > 0.05 is insignificant.

Conclusions and Recommendations

Conclusions

1. The current study provides a basic database for UPEC's spread, virulence factors and genes in Diyala Province and shows that an epidemiological program is necessary to monitor the spread of UPEC and its virulence genes in the local area.
2. *E. coli* is prevalent in women in eastern Diyala Province and it's more common in pregnant women than non-pregnant women.
3. The detection of EHEC in the urine samples of women with UTIs.
4. In this study, *E. coli* isolates showed many virulence factors which could lead to chronic and severe cases of UTIs in women.
5. In this study, *E. coli* isolates showed a high rate of resistance to different antibiotics (MDR, XDR). Ceftazidim and nalidixic acid were the most resisted antibiotics while meropenem was the most effective.
6. The genes *fimH*, *pap*, *ompT* were the most prevalent in the study's UPEC isolates and the gene that was least common among the UPEC isolates was *sfa*.

Recommendations

1. The spread of *E. coli* in the area, and especially in women, to be studied further to obtain a better image of how widespread the bacteria is in the province and what serotypes are the most prevalence.
2. The virulence factors and genes of *E. coli* to be further studied to better understand the virulence of *E. coli* currently spread across Diyala Province and the utilization of sequencing analysis to see if any mutations has occurred.
3. Increase the awareness of what excessive and random usage of antibiotics has caused and could lead to in antibiotic resistance that has been gradually getting worse for all mankind and for prescriptions to take into account what resistance of bacteria to antibiotics could mean in the future.
4. Study gene expression for virulence genes using real-time PCR.
5. Further study of *fimH* in pathogenic *E. coli* to see if it can be used to identify *E. coli*.

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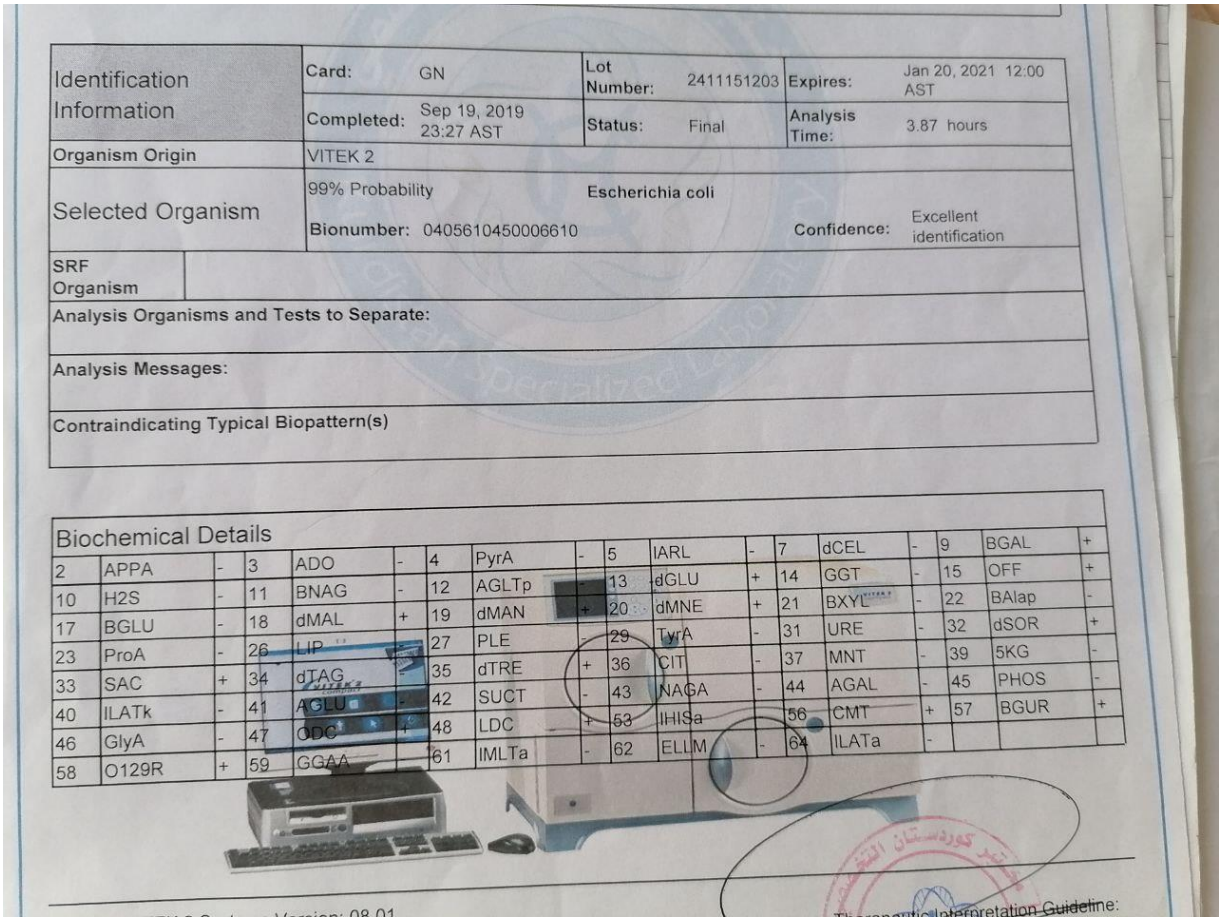
Appendices

Appendix 1-A: Biochemical tests results.

Appendix 1-B: Biochemical tests done on all isolates.

Appendix 2: Vitek 2 System report.



Identification Information	Card: GN	Lot Number: 2411151203	Expires: Jan 20, 2021 12:00 AST
	Completed: Sep 19, 2019 23:27 AST	Status: Final	Analysis Time: 3.87 hours
Organism Origin	VITEK 2		
Selected Organism	99% Probability Escherichia coli		
	Bionumber: 0405610450006610		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	-			

Appendix 3 Table detailing antibiotic resistance test results.

Isolate	ATM	AMC	C	TE	CPM	IMP	FOX	CIP	NA	CAZ	LVX	CTX	PIP	MEM	PIT	CRO
E1	R	S	S	R	I	I	I	I	R	I	S	S	I	S	I	S
E2	S	S	R	S	R	I	I	R	R	R	S	R	S	S	R	R
E3	R	R	R	R	R	S	R	R	R	R	R	R	S	S	I	R
E4	I	I	I	S	I	R	S	I	R	S	R	R	I	S	I	D
E5	I	R	R	I	R	I	I	R	R	R	R	R	R	S	I	R
E6	R	R	R	R	R	I	R	R	R	R	R	R	S	R	R	I

Isolate	ATM	AMC	C	TE	CPM	IMP	FOX	CIP	NA	CAZ	LVX	CTX	PIP	MEM	PIT	CRO
E7	S	R	I	S	R	I	R	S	R	R	I	R	I	I	I	I
E8	R	R	R	R	R	R	R	R	R	R	R	R	I	I	I	R
E9	R	S	R	R	R	I	I	R	R	R	R	R	I	S	R	R
E10	I	R	R	R	R	R	S	R	R	S	R	R	R	I	R	R
E11	S	S	S	S	S	S	R	R	R	R	S	R	I	S	R	S
E12	R	R	S	S	S	I	S	I	R	R	S	R	R	I	I	S
E13	R	I	R	I	R	I	R	R	R	R	S	R	R	S	I	R
E14	R	S	I	I	R	I	R	I	R	R	I	R	I	I	R	R
E15	R	R	R	R	R	I	R	R	R	R	I	R	I	S	R	S
E16	I	I	S	S	I	I	R	R	R	R	S	S	I	R	S	S
E17	R	R	I	R	R	I	R	R	R	R	R	R	R	R	R	R
E18	R	R	S	R	R	R	R	R	R	R	R	R	R	I	R	R
E19	R	R	S	I	I	R	R	R	R	R	R	R	R	R	S	I
E20	R	R	I	R	R	R	R	R	R	R	R	S	R	R	R	R
E21	S	R	S	R	I	R	R	R	R	S	R	R	R	R	I	S
E22	R	R	S	S	R	I	R	R	R	R	I	S	R	I	I	R
E23	R	R	R	R	R	R	R	R	R	R	R	R	R	I	I	R
E24	R	R	R	R	R	I	R	R	R	R	R	R	R	S	I	S
E25	R	R	I	R	R	I	R	R	R	R	R	R	R	S	R	R
E26	R	I	R	I	R	S	S	I	R	R	S	S	I	S	I	R
E27	R	I	I	S	R	I	S	S	I	R	S	S	S	R	R	R
E28	R	R	S	S	R	I	R	S	I	R	S	S	I	I	I	R
E29	R	I	R	S	R	I	S	I	I	R	S	S	S	S	I	R
E30	R	S	R	S	R	R	S	I	I	R	S	S	R	I	S	R

Appendix 4 Table detailing micro-titer plate (MTP) results for the isolates and their absorbance values when read at 630nm.

Isolate	Absorbance reading		Median value	Biofilm production
	Reading 1	Reading 2		
Control	0.040	0.044	0.042	
E1	0.034	0.050	0.042	Weak
E2	0.055	0.067	0.061	Intermediate
E3	0.092	0.084	0.088	Strong
E4	0.042	0.040	0.041	Weak
E5	0.055	0.058	0.056	Intermediate
E6	0.088	0.098	0.093	Strong
E7	0.058	0.066	0.062	Intermediate
E8	0.089	0.099	0.094	Strong
E9	0.054	0.072	0.063	Intermediate
E10	0.043	0.063	0.051	Intermediate
E11	0.060	0.066	0.063	Intermediate
E12	0.062	0.082	0.072	Intermediate
E13	0.072	0.078	0.075	Intermediate
E14	0.042	0.052	0.046	Weak
E15	0.090	0.085	0.088	Strong
E16	0.060	0.044	0.052	Intermediate
E17	0.066	0.070	0.068	Intermediate
E18	0.036	0.055	0.046	Weak
E19	0.064	0.058	0.061	Intermediate
E20	0.058	0.066	0.062	Intermediate
E21	0.076	0.060	0.068	Intermediate
E22	0.058	0.065	0.060	Intermediate
E23	0.080	0.088	0.084	Strong

Isolate	Absorbance reading		Median value	Biofilm production
	Reading 1	Reading 2		
E24	0.089	0.090	0.089	Strong
E25	0.063	0.057	0.060	Intermediate
E26	0.047	0.033	0.040	Weak
E27	0.051	0.046	0.048	Intermediate
E28	0.036	0.040	0.038	Weak
E29	0.038	0.036	0.037	Weak
E30	0.045	0.053	0.049	Intermediate

Appendix 5 Table detailing which isolates produced ESBL and which ones did not.

Isolate	ESBL-production
E1	-
E2	+
E3	+
E4	-
E5	+
E6	+
E7	-
E8	+
E9	+
E10	+
E11	-
E12	+
E13	+
E14	-

Isolate	ESBL-production
E15	+
E16	-
E17	+
E18	+
E19	+
E20	+
E21	+
E22	-
E23	+
E24	+
E25	+
E26	-
E27	-
E28	+
E29	-
E30	-

Appendix 6 Table detailing PCR results for each gene done on the 15 isolates.

Isolate	PCR column	*fimH*	*pap*	*sfa*	*ompT*	*kpsMII*	*bla-TEM*	*eae*
E23	1	+	+	+	-	+	+	+
E19	2	+	+	-	+	-	+	+
E18	3	+	+	+	+	+	+	-
E20	4	+	+	-	+	-	+	-
E6	5	+	+	+	-	-	-	+
E8	6	+	+	+	+	-	-	-

Isolate	PCR column	*fimH*	*pap*	*sfa*	*ompT*	*kpsMII*	*bla-TEM*	*eae*
E10	7	+	-	-	+	-	-	-
E24	8	+	+	+	-	+	-	-
E3	9	+	+	+	+	+	+	+
E5	10	+	-	-	+	+	-	-
E25	11	+	+	+	-	-	+	-
E17	12	+	+	-	+	-	+	-
E15	13	+	-	-	-	+	-	-
E9	14	+	+	-	+	+	+	-
E21	15	+	+	-	+	+	+	-
Total (%)		15 (100%)	12 (80%)	7 (46%)	10 (66%)	8 (53%)	9 (60%)	4 (26%)

Appendix 7 Gene absence and antibiotic susceptibility patterns.

Gene	Isolates missing the gene	Antibiotics isolates were susceptible to
pap	E10	FOX
	E25	MEM
	E15	MEM, CRO
sfa	E19	C, PIT
	E20	CTX
	E10	FOX
	E5	MEM
	E17	MEM
	E15	MEM, CRO
	E9	MEME, AMC
	E21	CAZ, CH, ATM
ompT	E23	—
	E6	PIP
	E24	MEM, CRO
	E25	MEM

Gene	Isolates missing the gene	Antibiotics isolates were susceptible to
	E15	MEM, CRO
kpsMII	E19	C, PIT
	E20	CTX
	E6	PIP
	E8	—
	E10	FOX
	E25	MEM
	E17	MEM

Appendix 8 Patient's information form.

Sample No.:

Patient name:

Patient age:

Gender:

Address:

Sample type:

Isolation place:

Collection date:

وزارة التعليم العالي والبحث العلمي
جامعة ديالى
كلية العلوم
قسم علوم الحياة

الكشف الجزيئي لبعض جينات الفوعة في ايشريشيا القولونية المعزولة من نساء مصابات بألتهابات المجارية البولية

رسالة مقدمة الى
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الخلاصة

بكتريا الايشريشيا القولونية احد اهم مسببات االتهابات المجاري البولية في النساء والتي لها قدرة عالية على مقاومة المضادات الحيوية المختلفة ويرجع ذلك لامتلاكها العديد من عوامل الضراوة التي تمكنها من احداث الإصابة الشديدة والمزمنة في النساء. تم عزل 30 عزلة من بكتريا الايشريشيا القولونية من مجموع 200 عينة ادرار وبنسبة (15%) من نساء تعاني من اخماج المجاري البولية في مستشفى السعدية العام , ومستشفى جلولاء العام, ومستشفى خانقين العام, شملت نساء حوامل وغير حوامل ولاعمار تتراوح من (15-40) سنة ومن مناطق شرق محافظة ديالى خلال الفترة من 2020/9/1 ولغاية 2020/11/1. تم التشخيص الاولي لعزلات *E. coli* باستخدام الأوساط الزرعية الملائمة (وسط الماكونكي اكار, وسط والايوسين المثيلين الأزرق, وسط اكار الدم) واستخدم وسط السوربيتول ماكونكي اكار لتشخيص EHEC من العزلات. استخدمت الاختبارات البيوكيميائية وجهاز Vitek 2 System لتاكيد التشخيص النهائي للعزلات. كان العدد الأكبر للعزلات البكتريا من النساء الحوامل (11) عزلة من (24) نمو موجب وبنسبة (45.8%) و(19) عزلة من (101)نمو موجب وبنسبة (18.8%) وقد تم العثور على 3 عزلات EHEC و بنسبة 10%.

تم اجراء فحص الحساسية للعزلات باستخدام مضادات حيوية مختلفة وكانت نتائج المقاومة كما يلي: nalidixic acid (86%), cefepime (76%), azetreonam (73%), cefotaxime (70%), ceftrazidime (70%), ciprofloxacin (66%), ceftriaxone (63%), cefoxitin (63%), amoxicillin-clavulate (60%), tetracycline (46%), chloramphenicol (46%), piracillin (46%), pipracillin-tazobactam (40%), impenem (30%), meropenem (20%).

اختلفت العزلات في مقاومتها للمضادات الحيوية واظهرت نمطين من المقاومة: MDR والتي تشمل العزلات التي قاومت ثلاث مجاميع من المضادات الحيوية ونمط XDR التي قاومت اربع او خمس مجاميع من

المضادات الحيوية المختلفة. تم قياس MIC لجمع العزلات البكتيرية لمضادين هما ceftazidime و cefepime باستعمال وسط مولر هنتون اكار والتخافيف المتسلسلة من المضادات وكانت نتيجة MIC 512 و 1024عالتوالي .

شملت الدراسة أيضا الكشف المضهري لعوامل الضراوة التي تملكها بكتريا ال E.coli لمعرفة شدة امراضيتها ,حيث كشف عن قابلية انتاج الغشاء الحيوي بطريقتين الأولى طريقة الانبوب (MT) والطريقة الثانية الصفيحة المعيارية (MTP) واضهرت النتائج ان 19 عزلة (63.3%) مكونة للغشاء بالطريقة الأولى و25 عزلة (83.3%) مكونة للغشاء بالطريقة الثانية . كما كشف مضهريا عن قابلية العزلات على انتاج انزيمات البيتا لاكتام الواسعة الطيف ESBLs باستخدام طريقة Double-Disk Synergy Test وكانت النتائج 19 عزلة منتجة للانزيم و(11) غير منتجة . اما قابلية العزلات على انتاج الهيمولايسين فقد كشف عنها مضهريا باستخدام وسط Sheep blood agar وكانت النتيجة كما يلي: (3) عزلات منتجة تحلل من نوع بيتا و(9) عزلات تحلل من نوع الفا (18) عزلة تحللها كاما. اما احتواء العزلات على الكبسولة فقد كشف عنها مضهريا في هذه الدراسة باستخدام صبغة النكروسين والفحص المجهري المباشر واضهرت النتائج ان 23 عزلة (76.6%) منتجة للكبسولة . كما كشف مضهريا عن قابلية العزلات البكتيرية على الحركة واحتوائها على الاسواط باستخدام وسط Semi-solid media وكانت النتيجة ان جميع العزلات وبنسبة 100% متحركة.

تضمنت الدراسة الحالية الكشف الجزيئي لجينات الضراوة في 15 عزلة بكتيرية من مجموع 30 .استخدمت تقنية Multiplex PCR في الكشف عن جينات الالتصاق (*fimH, pap, sfa*) وعن جين (*ompT*)الذي له دور مهم في انتاج الغشاء الحيوي واضهرت النتائج ان 15 عزلة وبنسبة 100% حاوية على جين *fim* و12 عزلة وبنسبة 80% حاوية على جين *pap* و10 عزلات وبنسبة 66% حاوية على جين *ompT*اما جين *sfa* فقد ظهر في 7 عزلات وبنسبة 46%. كما كشف عن جين *kpsMII* المسؤول عن انتاج

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الكبسولة باستخدام تقنية Uniplex PCR وكانت نسبة وجود هذا الجين في 8 عزلات 53% ,استخدمت نفس التقنية في الكشف عن جين *bla-TEM* المسؤول عن تشفير انزيمات ESBLs وعن جين *eae* المستخدم في الكشف عنEHEC والمسؤول عن تشفير بروتين الانتيمبن واضهرت النتائج ان 9 عزلات وبنسبة 60% حاوية على جين ال *bla-TEM* و 4 عزلات وبنسبة 26.6% حاوية على جين *eae*, 3 منها عائدة الى EHEC.