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Study of Phylogeny by Multiple-Locus Variable-Number Tandem-Repeat for *Escherichia coli* Isolate from Patients with Diarrhea

A Thesis

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

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بسم الله الرحمن الرحيم

﴿ هَلۡ أَتَىٰ عَلَى ٱلۡإِنسَٰنِ حِينٞ مِّنَ ٱلدَّهۡرِ لَمۡ يَكُن شَيۡـٔٗا مَّذۡكُورًا ١ ﴾

صدق الله العلي العظيم

الانسان ايه (١)

Certification

We certify that this thesis entitled: (**Study of Phylogeny by Multiple-Locus Variable-Number Tandem-Repeat for *Escherichia coli* Isolate from Patients with Diarrhea**)was prepared under our supervision by "Zaid Kadhem Muhammad Ali Al-Khafaji" at the Department of Microbiology-College of Medicine/ University of Babylon, as a partial fulfillment for the requirements for the degree of Master of Science in Medical Microbiology and this work has never been published anywhere.

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Decision of Examination Committee

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Dedication

To …

My Mother and my Father.

My dear Wife, My Children and

My Friends.

To everyone who helps and supports me. I

Dedicate this work.

Zaid Kadhenm

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ZAID

Summary

A total from (150) stool specimens collected from patients having diarrhea infection , The results indicated that 22 (14.7%) of the isolates belonged to *Escherichia coli*,other bacterial isolates detect in (50%) while no growth detect in (35.3%) .These isolates were identified according to culturing characteristics ,morphological, biochemical, physiological, and VITEK -2 systems .

The distribution of isolates according to age were studed and it was found that (50%) of the samples belonged to patients between the ages one day to one year , (22.7%) belong to patients with an average age between (1-10) years, (9%) of the samples belong to patients with an average age between (11-20) years, (13.8%) of the samples belong to patients with an average age of (21-30) years. While (1%) of the samples were recorded for patients over 30 years old.

Antibiotics susceptibility test of *Escherichia coli* isolates showed It showed that all 22 isolates were 100% resistant to the three antibiotics (Ampicllin , Cefotaxime ,Ceftriaxone) while the results showed the least resistance to antibiotics Gentamicin 9 % and Norfloxacin 13.6 %.

MLVA were performed for all *E.coli* isolates when seven VNTR loci (seven repetitive element loci) were selected for genetic typing of *E. coli*.

PCR were carried out and the PCR products were electrophoresed on (1.5) % Agarose gel containing ethidium bromide and the allele profile were detected by MLVA . Analysis of MLVA profile showed that all *E.coli* isolate were grouped into (21) distinct MLVA type with (2) cluster (A) and (B).

TLR4 mRNA gene expression also study by quantitative Real- time PCR (Relative gene expression) in patients with diarrhea and the results show that the TLR4 was increase in diarrhea Patients with *E.coli* when compared with control groups. the expression of TLR4 is increased more than (%30) fold when compare with control group.

Studed showed The highest concentration of Ag Tio_2 after diluted for (1/2) made the highest inhibition effect for bacterial growth and this inhibition minimized the bacterial growth from (1.36) to (0.5) after 24hours of incubation while after 48 hours of incubation bacterial growth minimized from 1.29 to 0.22. While the lowest concentration of Ag Tio_2 that made by diluted the nanoparticles for (1/32) showed the lowest inhibition effect on *E.coli* growth after 24 and 48 hours of incubation.

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List of Abbreviation Expansion

Abbreviation	Expansion
Ag-TiO_2	Silver-doped titanium dioxide
AHL	Acyl homoserine lactones
ATP	Adenosine TriPhosphate
AMEs	Aminoglycoside modifying enzymes
API tests	Analytical Profile Index tests
CLSI	Clinical and Laboratory Standards Institute
CFU	Colony forming unit
dNTP	Deoxy Nucleotide Tri Phosphate
EDTA	Ethylene Diamine Tetra Acetate
UPEC	Uropathogenic *E.coli*
DMSO	Dimethyl Sulfoxide
DDT	Disc diffusion method
D.W	Distilled water
EHEC	Enterohemorrhagic *E.coli*
EIEC	Enteroinvasive *E.coli*
EPEC	Enteropathogenic *E.coli*
ETEC	Enterotoxigenic *E.coli*
µg/mL	microgram/milliliter
XDR	Extensively drug-resistant
EHEC	Enterohemorrhagic *E.coli*
MBL	Metallo-β-lactamases
MGEs	Mobile genetic elements
MDR	Multidrug drug-resistant
MLST	Multilocus sequence typing
NPs	Nanoparticles
PBS	Phosphate Buffer Saline

OD	Optical Dencity
OXA	Oxacillinases
PDR	Pan drug -resistant
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
EMB	Eosin Methylene Blue
Taq	Thermus aquaticus
ExPEC	Extra intestinal *E.coli*
TBE	Tris-Borate-EDTA buffer
TE buffer	Tris–EDTA buffer
ELISA	Enzyme-Linked ImmunoSorbent Assay
NMEC	Neonatal meningitis *E. coli*
DAEC	Diffusely adherent *E.coli*
VITEK 2 system	Vital Index of Traditional Environmental Knowledge
LB	Luria-Bertani
HUS	Hemorrhagic colitis
TLRs	Toll like Receptors
MLVA	multiple-locus variable-number tandem-repeat analysis
VNTR	variable-number tandem-repeat
PAMPs	pathogen-associated molecular patterns
BSI	bloodstream infections

Chapter One

Introduction & Literature Review

1. Introduction

E. coli is one of the most prevalent hospital-acquired pathogens that can cause bloodstream infections (BSI), urinary tract infections (UTI), and stomach infections. Bacteremia is a leading cause of death with significant rises in incidence and mortality. In many parts of the world, *E. coli* ranks first as a cause of incidents that are obtained in the community and second as a cause of BSIs that are acquired in hospitals. (Dan Li *et al.*,2021).

In general, *E. coli* strains may be divided into three families: commensal organisms, which are normal inhabitants of the gastrointestinal system and do not cause disease, strains that induce diarrheal intestinal disease, and strains that typically cause disease outside the intestinal tract. Only 20% of the coding sequences in sample strains from each of these families are shared by all strains, with commensal and extra intestinal *E. coli* strains sharing 64% of the sequences. (Abdalhamid *et al.*, 2019 ; Díaz-Jiménez *et al.*, 2020).

For epidemiological investigations as well as the diagnosis and management of bacterial illnesses, it is crucial to type microbial pathogens or identify bacteria at the strain level. In the case of those bacteria kinds that are characterized by substantial genetic diversity, which results in significant variances in virulence potential, this is a particularly challenging but significant assignment.. (Kotłowski *et al.*,2020).

E. coli strains can also be classified based on the genetic substructures associated with different phylogeny. Scientific reports have noted that *E. coli* strains belonging to different phylogenetic groups (phylogroups) displayed different phenotypic and genotypic traits (Alfinete *et al.*,2022).

Multiple-locus VNTR analysis (MLVA), a quick and effective approach for genotyping E. coli and Shigella, has recently come into prominence. This technique involves doing PCRs with primers encircling each site, followed by electrophoresis, and is based on the polymorphism of the variable-number tandem repeats (VNTRs) found at numerous loci scattered across the bacterial chromosome. Even among highly clonal strains, several of these VNTRs exhibit a significant level of variability. (Caméléna *et al.*, 2019).

The primary pattern-recognition receptors for a wide range of microbial products, commonly known as pathogen-associated molecular patterns (PAMPs), are members of the transmembrane protein family (TLRs). In addition to recognizing PAMPs, certain TLRs have also been demonstrated to identify endogenous ligands linked to inflammation, known as danger-associated molecular patterns (DAMPs). A TLR's ligand selectivity, signal transduction pathways, and sub-cellular localization are its primary distinguishing characteristics. (Singh *et al.*, 2014).

Mammals have 10 functioning TLRs in humans and 12 in mice, totaling 13 TLRs. TLR1, TLR2, TLR4, TLR5, and TLR6 are found in the plasma membrane and are able to identify external ligands. While TLR11, TLR12, and TLR13 are absent from the human genome, TLR3, TLR7, TLR8, and TLR9 are intracellular and found in the endosomal membrane. (Keogh and Parker, 2011) .

TLR2, when dimerized with either TLR1 or TLR6, confers ligand specificity, and TLR1, TLR2, and TLR6 all detect bacterial lipoproteins. According to Goh and Midwood (2012), TLR5 detects flagellin, TLR9 identifies unmethylated C-phosphate-G (CpG) bacterial DNA, and TLR3, TLR7, and TLR8 bind to viral nucleic acids .

TLRs are widely dispersed in the body and include professional immune cells such dendritic cells (DCs), T cells, B cells, and macrophages.

1.1.1 aim of the study :

The current study aimed phylogenetic study of *E.coli* strains isolated from stool samples

The objective of this study include :-

1- Isolation and identification of *E.coli* from stool samples

2- Study the antibiotic susceptibility test and effect of nanoparticle on bacterial growth.

3- Extraction of bacterial DNA

4- Extraction of RNA from stool.

5- Genotyping method to *E.coli* by seven VNTR loci

6- Detection of the expression of TLR4 by using real- time PCR (Relative gene expression) in patients and control.

1.2 Literature review

1.2.1 *Escherichia coli*

1.2.2 General Characteristics of *E. coli*

Escherichia Coli is a Gram–negative bacterium belonging to Enterobacteriaceae, catalase positive, oxidase negative, non-spore-forming and facultative anaerobic, positive tests for indole, methyl red and produces acid and gas from glucose, mannitol, maltose, lactose, and sucrose while it gives a negative test for Vogues–Proskauer, urease, H_2S production, phenylalanine deaminase test, and gelatin liquefaction, and it is classified as part of the Enterobacteriaceae family of gamma-proteobacteria (Dielubanza and Schaeffer, 2011; Woodford and George, 2011).

The outer membrane of *E. coli* is covered by a polysaccharide capsule composed of K antigens. In other of polysaccharides the M antigens are synthesized under conditions of high osmolality, low temperature, and low humidity, typing strains based on differences in three structural antigens: O, H, and K, the O antigens (somatic or cell wall antigens) are found in the polysaccharide portion of the LPS, these antigens are heat-stable, and may be shared among different Enterobacteriaceae genera and commonly used to serologically type many of enteric Gram-negative rods, the H (flagellar) antigens are associated with flagella; therefore , only flagellated (motile) bacteria have H antigens, the K (capsular) antigens are most often associated with the capsule or, less commonly, with the fimbriae (Lillington *et al.,* 2014).

Taxonomically, *E. coli* belong to the Enterobacteriaceae family and are an important component of the intestinal microbiota, being involved in some essential metabolic processes such as the production of vitamin K and vitamin B12, *E. coli* also helps to maintain the anaerobic environment needed for most of the microbiota by consuming oxygen that enters the gut

and competitively exclude pathogens from the lower intestine of their hosts (Blount, 2015).

E. coli is a Gram-negative rod-shaped bacterium and a member of the family of Enterobacteriaceae, a large family which also includes *Salmonella, Klebsiella, Proteus, and Enterobacter spp. E. coli* are facultative anaerobes and display a wide diversity in phenotype and genotype. The *E. coli* genome undergoes constant change due to a continual flux of genetic insertions and deletions. The genome size, which can vary by up to 1.9 Mb, encodes approximately 4000–6500 proteins. The core genome comprises only 2000 genes, and the rest of the genome determines commensalism, species specificity, pathotype, and antibiotic susceptibility profile, the core genome contains mostly nonmobile elements, while strain-specific genes are more likely to be located on pathogenicity islands, which are large regions of the genome that contain virulence-related genes found in pathogenic strains but not commensal strains, and mobile elements including plasmids, bacteriophages, insertion sequence elements, transposons, integrons, and prophages, many of these mobile genetic elements are found in other pathogenic bacterial species suggesting a history of genetic transfer and exchange (Cao *et al.*, 2020).

1.2.3 Types of pathogenic *E. coli*

Based on the various human diseases which *E. coli* can cause, pathogenic *E. coli* have been broadly classified into two major categories: the diarrheagenic *E. coli* and the extraintestinal pathogenic *E. coli*, among the diarrheagenic *E. coli*, there are currently six categories: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E.coli* (EHEC)/Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusively adherent *E. coli* (DAEC) (Opintan *et al.*, 2010) .

Extra intestinal pathogenic *E. coli* is phylogenetically and epidemiologically distinct from diarrheagenic *E. coli*. They could colonize a variety of anatomical locations and cause various infections outside the gastrointestinal tract, *E. coli* is a major cause of nosocomial infections, including catheter-associated UTIs and ventilator-associated pneumonia (VAP). *E. coli* can also be found in soil, on vegetables, and in water, as well as in undercooked meats. Pathogenic strains cause intestinal illness in humans when ingested. (Mueller and Tainter,2022)

1.2.3.1 Diarrhoeagenic *E. coli* (DEC)

Although most *E. coli* strains live innocuously in the intestines and rarely cause intestinal disease in healthy individuals, some strains have evolved by acquiring virulence determinants that enable them to cause tissue damage in the intestinal tract, producing diarrhea illnesses known as diarrhoeagenic *E. coli* (DEC), the syndromes vary in clinical presentation, preferential host colonization sites and the distinctive virulence traits of the strains. *E. coli* is one of the main aetiological agents of diarrhoeal illnesses. The different intestinal *E. coli* pathotypes are classified according to the virulence-related properties they possess, which have several implications in their pathogenic pathways as well as in the treatment required, all the categories of diarrhoeagenic *E. coli* described have been shown to carry at least one virulence-related property upon a plasmid. EIEC, EHEC, EAEC, and EPEC strains typically harbor highly conserved plasmid families, each encoding multiple virulence factors (Gomes *et al.,* 2016).

1.2.3.2 Enteropathogenic *E. coli* (EPEC)

The term enteropathogenic *E. coli* (EPEC) is defined as *E. coli* strains that have the ability to cause diarrhea, to produce histopathology of the intestinal epithelium known as attaching and effacing (AE) lesions, and the

inability to produce Shiga toxins and heat-labile (LT) or heat stable (ST) enterotoxins, nowadays, EPEC strains are sub-classified into typical EPEC (tEPEC) and atypical EPEC (aEPEC) in accordance with the presence of a large virulent plasmid known as the EPEC adherence factor plasmid (pEAF), tEPEC strains harbour this virulence determinant which encodes for type IV fimbria called bundle forming pilus (BFP), while aEPEC strains do not possess the plasmid, the BFP enables a localized adherence pattern of the *E. coli* to the epithelial cells and other organ cultures in vitro, this fimbria also contributes to antigenicity, autoaggregation, and biofilm formation (Enserink *et al.*, 2014).

Typical EPEC strains also present 1. large surface protein, lymphocyte inhibitory factor A (LifA), which inhibits the expression of multiple lymphokines and lymphocyte proliferation , 2. Intimin, a 94- kDa protein encoded by the eae gene and required for intimate adherence of EPEC to host cells at the sites of AE lesions, the eae gene is used for the detection of EPEC by DNA probes or PCR assays (Abdalhamid *et al.*, 2019).

1.2.3.3 Enterohaemorrhagic (or Shiga toxin producing) *E.coli* (EHEC/ STEC)

Enterohaemorrhagic (Shiga toxin producing) *E. coli* constitutes a well-known group of foodborne pathogens distributed worldwide, the ability to produce one or more of the Shiga toxin (Stx) family cytotoxins is the main virulence attribute of this pathotype of *E. coli*, EHEC/STEC can cause a wide variety of infections ranging from almost unapparent diarrhea to more serious manifestations such as hemorrhagic colitis (HC) and the development of a life-threatening syndrome known as a hemolytic uremic syndrome (HUS), which mainly affects infants and children (Branchu *et al.*, 2014).

The stx operon is usually found within the sequence for an inducible, lysogenic, lambda-like bacteriophage , stx toxins inhibit protein synthesis as well as act in cell signal transduction and immune modulation causing proinflammatory and proapoptotic responses, the ability to adhere, colonies and form biofilm in food and on several types of surfaces may be an important source and/or vehicle of EHEC/STEC transmission, In addition, its biofilm-forming capacity may also act as bacterial protection against adverse environmental conditions (Gomes *et al.,* 2016).

1.2.3.4 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* strains are currently defined as *E. coli* strains that adhere in a stacked brick arrangement to the surface of epithelial cells and also to the coverslip between cells, they produce a characteristic histopathologic lesion in the intestine and several specific virulence factors, EAEC strains characteristically enhance mucus secretion by the mucosa, with trapping of the bacteria in a bacterium mucus biofilm, Thus, diarrhea produced bacteria in a bacterium mucus biofilm, Thus, the diarrhea produced is predominantly watery, often mucoid, with or without blood and inducing abdominal pain, vomiting, and low fever, Acute self-limiting diarrhea is the usual pathology, but some patients may develop protracted diarrhea, lasting up to more than 14 days, the formation of a heavy biofilm may be related to its ability to cause persistent colonization and diarrhea (Herzog *et al.*, 2014).

1.2.3.5 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* strains are characterized by the production of colonization factors (CFs) and at least one of the following two enterotoxins: heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST), which are located in transmissible plasmids, these enterotoxins are

produced after the adhesion of the ETEC strains to the intestinal mucosa and, throughout the activation of the adenylate and guanylate cyclase's for LT and ST, respectively, cause deregulation of membrane ion channels in the epithelial membrane, leading to a massive loss of ions and water, on the other hand, the main groups of CFs harbored in genetic mobile elements of ETEC strains are (Nada *et al.,* 2011):

- Colonization factor antigen I (CFA/1)-like group, the most clinically relevant being CS1, CS2, CS4, CS14, CS17 and CS19.
- *E.Coli* surface antigen 5 (CS5)-like group, mainly harboring CS5 and CS7 in clinical strains.
- The class 1b group, the most important being the CS12, CS18, CS20, CS26-28, and CS30 types

The ETEC group represents an epidemiologically highly diverse pathotype of diarrhoeagenic *E. coli*, this diversity has been evaluated by both phenotypic and molecular approaches such as serotyping, multilocus sequence typing (MLST), phylogrouping and whole genome sequencing. These methods demonstrate that ETEC strains can belong to more than 100 somatic serogroups (O) and at least 34 flagellar types (H), although only a limited number of O: H serotypes are associated with infections, The 5 major phylogenetic groups are represented in the ETEC group, as well as several distinct sequence types (ST). However, some clonally related ETEC lineages of the worldwide distribution share the same serotype, CFs, and toxin profiles (Von Mentzer *et al.*, 2014).

ETEC represents one of the most common causes of diarrhea in children in LMIC and travelers to these regions, with infection being more frequent during warm periods of the year, the clinical features of ETEC-infected patients are a watery stool often accompanied by vomiting without fever. Massive loss of fluids and electrolytes can lead to rapid dehydration, the diarrhea is usually self-limited to 3-4 days, but in some cases it can

persist and cause other complications , it is estimated that ETEC causes more than 200 million diarrheal episodes annually, and approximately 75,000 deaths due to dehydration, mainly among infants and children in tropical areas with poor sanitary conditions (Fekete *et al*., 2013).

1.2.3.6 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* are one of the most common causes (together with Shigella spp.) of dysentery in humans, especially in LMIC, causing fever, abdominal cramps and diarrhea containing blood and mucous , EIEC are characterized by their capacity of invasion, penetration and multiplication in the enterocytes , leading to their subsequent destruction and an important proinflammatory response , the bacteria specifically bind to the large intestine mucosa and invade the cells by endocytosis , this invasive capacity is dependent on the presence of large plasmids (approximately 220 kb) known as pInv, encoding the production of several outer membrane proteins involved in invasiveness by cell spreading (encoded by the ics A and ics B genes), inhibition of autophagy, regulation of host immune response (encoded by the osp gene), and the type II secretion system (TTSS) (Levine, 2016).

EIEC often resemble Shigella by being non motile and unable to ferment lactose, and the proteins involved in invasiveness are antigenically closely related (in some cases identical), but the infectious dose of EIEC needed to cause dysentery is much higher than that of *Shigella*, and it appears to be a milder and self-limiting form of the disease. This *E. coli* diarrhoeagenic pathotype belongs to well defined serotypes (such as O28ac:H-, O124:H30, O136:H- or O167:H-) that have produced large outbreaks worldwide since its first description in 1947 , although there are few reports on the routes of transmission and distribution in nature, water

and milk products as well as direct person to person transmission has been described as potential sources of transmission (Harris *et al.*, 2013).

1.2.3.7 Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* are characterized by adhering to monolayers of epithelial cells HEp- 2 in a diffused pattern. This group has been reported to cause diarrhea in children older than 12 months of age by inducing a cytopathic effect long cellular extensions are developed and wrap around the bacteria adhered , approximately 75% of DAEC strains produce a fimbria adhesion called F1845, belonging to the family of adhesins , This adhesin binds to an epithelial cellular receptor called DAF enhancing the cytopathic effect , infection by DAEC can be proinflammatory, inducing inflammatory bowel diseases. However, this pathotype requires further epidemiological studies and is difficult to identify and classify (Martinez-Medina *et al.*, 2014).

1.2.4 Virulence factors of *E. coli*

Virulence is the pathogenic ability to cause damage in a host and is mediated by the expression of combined virulence factors, *E. coli* have many virulence-associated factors, including adhesins, toxins, iron acquisition factors, lipopolysaccharides, polysaccharide capsules, and invasions', which are usually encoded on pathogenicity islands (PAIs), plasmids, and other mobile genetic elements (Köhler and Dobrindt, 2011).

Virulence factors are specific determinants that contribute to pathogen virulence , there are several categories of virulence factors including adhesins, toxins, invasion's, secretion systems, iron uptake systems or siderophores which are codified by virulence factor genes (VFGs) and are not specific for syndromes, hosts or strains , a combination of factors determines whether a bacterium can cause infection since the

presence of a single VF rarely makes an organism virulent , moreover, the combinations of VFGs that each bacteria possesses for causing infections at a specific site are very diverse , these genes can be located in different parts of the bacterial genome including chromosomal DNA, though they are mostly located on mobile genetic elements such as bacteriophages , plasmids and genomic islands (GEIs) ,in the *E. coli* K12 model strain, 18% of the genome has been estimated to represent horizontally acquired DNA, elucidating the high capacity to retain foreign information, and thus, the genomic plasticity of the species (Van Elsas *et al.,* 2011).

1.2.5 Pathogenicity of *E. coli*

Bacterial pathogenicity is defined as the genetic capacity of the bacterium to cause disease, based on the virulence and resistance traits it possesses. Unfortunately, *E.coli* can easily acquire this capacity and is considered a major cause of human infectious disease worldwide. In order to better understand the double component of the pathogenicity of *E.coli*, it is important to accurately define two concepts: virulence and resistance , *E. coli* is a ubiquitous commensal of food-producing animals and humans . Most strains of this enterobacterial species are harmless commensals that live in a mutually beneficial association with their hosts and seldom cause disease , *E. coli* is, however, a particularly complex species, having diversified into pathogenic strains , based on the type of virulence factor present , and the host's clinical symptoms *E. coli* strains are classified into pathotypes of zoonotic intestinal pathogenic *E. coli* (IPEC) or extraintestinal pathogenic *E. coli* (ExPEC) (Lindstedt *et al.,* 2018).

E. coli follows the typical Gram-negative pattern, with two membranes – an inner (cytoplasmic) membrane and an outer membrane (OM), separated by periplasmic space. The cell wall is a monomolecular murein sacculus, composed of β-1→4 linearly linked N-acetylglucosamine

and N-acetylmuramic acid monomers, cross-linked by L-Ala-D-Glu-L-meso-DAP-D-Ala peptides, located on the outside surface of the inner membrane (Zlatkov,2019) .

The outer membrane contains different outer membrane proteins (OMPs), most of them functioning as porins allowing passive diffusion of different metabolites, ions and other compounds. Linked to the extracellular surface of the OM is the lipopolysaccharide (LPS) molecular complex. It is composed of a hydrophobic anchor (lipid A) and a core domain, i.e., polysaccharide that connects lipid A to a glycan polymer - the third element in the structure. The glycan polymer is exposed directly to the extracellular milieu and it can vary in number and composition of its sugar residues. This variation of modified simple sugar monomers gives the bacterial LPS strong antigenic properties referred to as the O antigen .Most strains of *E. coli* are motile by means of peritrichous flagella. Each flagellum is composed of a basal body, a hook and a filament consisting of a single protein called flagellin (encoded by the fliC gene). Flagellin is also known as the H antigen ,since it can also vary antigenically due to changes in the amino acid composition of its central region or due to posttranscriptional modifications (such as acetylation) (Vila *et al.*,2016).

In addition to the flagellar machinery, *E coli* cells possess extracellular attachment organelles, called fimbriae (or pili), via which bacteria adhere to different surfaces. *E. coli* bacteria can express more than 30 fimbriae 6,9. Each 13 organelle is composed of linearly polymerized fimbria subunits protruding outside of the bacterial cell, ending with a lectin-like adhesion molecule, i.e., adhesion, on its tip via which bacteria can selectively recognize and adhere to different surfaces, including host tissues. One of the most abundant types of fimbriae belongs to the group of the chaperone/usher pathway (CUP) family to which the virulence-associated (e.g., P- and S-fimbriae) and the common type-1 fimbriae

belong . Once the fimbria subunits are made, they are secreted to the periplasmic space, bound to a chaperone that preserves them partially unfolded guiding them to the usher – a big proteinaceous platform where the assembly of the fimbria subunits happens in a step-wise linear manner (Puorger *et al.*, 2011).

Once guided to the usher, the chaperone subsequently leaves the fimbrial subunit, exposing the N-terminal sequence of the guided structural monomer 10- 12. Thus, the sequence becomes a donor for the C-terminal incomplete immunoglobulin fold of the last structural unit of the growing filament. The most common type of fimbriae are the type-1 fimbriae (encoded by the fim gene cluster) produced by 80% of all *E. coli* strains via which bacteria can selectively adhere to mannosylated glycoconjugate's . Even though the type-1 fimbriae are not considered virulence factors, since they are also produced by the commensal bacteria, they can assist in the colonization by the uropathogenic, neuroinvasive, and adherent-invasive *E. coli* strains,

The pathogenic *E. coli* species comprise a very versatile group with numerous virulence determinants (virulence factors) including adhesins, invasion's, toxins and secretion systems that allow them act as causative agents in both human and veterinary medicine,

In humans, these pathogens are responsible for three main types of clinical infections: (i) enteric or diarrheal diseases, (ii) urinary tract infections, and (iii) meningitis/septicemia. Based on their distinct virulence properties and clinical symptoms of the host, pathogenic *E. coli* strains are divided into numerous categories or pathotypes Diarrheagenic *Escherichia coli* (DEC); Uropathogenic *Escherichia coli* (UPEC); Neonatal meningitis/sepsis associated *Escherichia coli* (MNEC)(Belete *et al.*,2022)

Diarrheal illnesses are a severe public health problem and a major cause of morbidity and mortality in infants and young children. Low- and

middle-income countries in Africa, Asia and Latin America are the most affected regions with diarrheal diseases occurring more often with lethal outcomes mainly due to poor living conditions (inadequate water supplies, poor environmental hygiene and sanitation, and insufficient education (Gomes *et al.*, 2016)

The prevalence of diarrheagenic *E. coli* was 42%, and rotavirus had a prevalence of 18%, while parasites accounted for 38%. *E. coli* is a normal flora of the gastrointestinal tract (GIT) of humans and other mammals, *E. coli* pathogenic tendencies lie in its ability to express genetic flexibility through the acquisition and/or transfer of virulence genes by both vertical and horizontal gene transfer mechanisms (Halawani ,2010).

1.2.6 Antibiotic Susceptibility of *E.coli*

Gram-negative bacteria are intrinsically resistant to more antibiotics than are Gram-positives. This is due to the low permeability of the outer membrane coupled with the presence of membrane spanning efflux pumps. These two mechanisms prevent entry and promote extrusion of antibiotics respectively, decreasing intracellular concentrations (Albrecht,2018)

Escherichia coli though a commensal bacterium of the intestinal flora of humans and animals, they are commonly used as indicator organisms for antimicrobial resistance , This is because they are extensively distributed in the gut and easily acquire those genes that encode antimicrobial resistance due to their genomic plasticity, Therefore, it serves as a reservoir for lifelong antimicrobial resistance genes and exerts pressure on the intestinal flora of the organism exposed to the pressures applied on the gut flora of the organism (Azabo *et al.*, 2022).

The ability of *E. coli* to colonize different environments, including the gut of humans and animals, has provided this organism with the evolutionary advantage to acquire antibiotic resistance traits from other

bacteria within its environment, as well as to be easily transmitted via the fecal-oral route. The gut microbiota of humans can harbor more than 1000 different antibiotic-resistant genes and transmission of these traits among gut commensals is a constant phenomenon (Hu *et al.,* 2013).

As many antibiotic resistance genes are associated with elements such as plasmids or transposons, and while the transfer of these elements may also occur through transformation or transduction, conjugation is often considered as the most likely responsible mechanism for the transmission of these traits (Von Wintersdorff *el at.,* 2016)

Carbapenems represent in many cases the last option for effective treatment against these infections. Nevertheless, with an increasing consumption of these agents, Carbapenem-resistant strains, particularly *Klebsiella spp*. and in a lesser degree *E. coli*, have become a public health concern, particularly in the hospital setting. Carbapenems bind to penicillin-binding proteins and induce spheroplast formation and cell lysis without filament formation. The carbapenems include four agents: imipenem, meropenem, ertapenem and doripenem.(Nordmann el at.,2011)

The reported carbapenem's in *E.coli* primarily include *Klebsiella* pneumoniae carbapenem's (KPC), metallo-β-lactamases (MBL), including the VIM, IMP, GIM and NDM type, and oxacillin-hydrolyzing metallo-β-lactamases (OXA). (Nordmann *el at.*,2011)

Although the production of class A extended spectrum β-lactamases is the most common mechanism of resistance in *E. coli* against β-lactam agents, class C β-lactamases, or AmpC, can also confer those strains that produce them the ability to inactivate some of these compounds. Similar to ESBL, AmpC-producing organisms hydrolyze amino- and ureidopenicillins, oxyimino-β-lactams such as ceftazidime, ceftiofur, and aztreonam, but contrary to the former enzymes, AmpC also inactivates broad and extended-spectrum cephalosporins such as

cephamycin's (cefoxitin) and are not inhibited by β-lactamase inhibitors such as clavulanic acid. Neither ESBL nor AmpC confer bacteria resistance to carbapenem (Knackstedt *et al.,* 2009).

Bacterial resistance to β-lactams is a major public health problem around the world. Although ESBL production clearly exceeds AmpC production as the major cause of β-lactam resistance, the later enzymes are now being recognized as a growing problem in different members of the Enterobacteriaceae, including *E. coli*, as evidenced by the increasing number of these strains being reported across the globe. Sources of AmpC-producing *E. coli* strains include livestock (Vounba *el at.*,2019), the environment (Sen K *el at.*,2019), as colonizers of the human gut (Nakayama *el at.*,2019) and as cause of human infections

On the other hand, Cefotaxim and Ceftriaxone, are a broad spectrum, antibiotics useful for the treatment of a number of bacterial infections. Belong to 'third generation' cephalosporins, these drugs have bactericidal activity by interfering with bacterial cell wall synthesis and inhibiting cross-linking of the peptidoglycan (Knackstedt *et al.,* 2009). The cephalosporins are also thought to play a role in the activation of bacterial cell autolysins which may contribute to bacterial cell lysis (Heinz *et al.,* 2000).

1.2.7 Molecular typing of *E. coli*

Typing of microbial pathogens or identifying bacteria at the strain level is important for epidemiological studies but also the diagnosis and treatment of bacterial infections. This is a particularly difficult but important challenge in the case of those types of bacteria that are characterized by high genetic variability which leads to important differences in virulence potential. (Kotłowski *et al.*,2020)

Multi-locus sequence typing is thought to be the 'gold standard' for typing *E. coli* (Larsen *et al.*,2012). MLST schemes use different combinations of genes to assign a sequence type (ST) to a strain (Clermont *et al.,* 2015).

Each different nucleotide sequence of an allele of a gene is assigned a unique number, and the resultant combination of the allele numbers defines a particular ST *E. coli* strains . (Alfinete *et al.*,2022).

Several methods have been described to determine *E. coli* phylogroups. These include polymerase chain reaction (PCR)-based assays, multi-locus sequence typing (MLST), ribotyping and 16S rRNA gene sequencing (Logue *et al.,* 2017).

The PCR-based assay developed by (Clermont *et al.*,2000) is a rapid tool for the classification of *E. coli* strains into the main phylogroups A, B1, B2 and D, referred to as the Clermont triplex PCR phylogroup method. This method targeted three *E. coli* genes: (i) yjaA gene encoding hypothetical protein, identified in the genome of *E. coli* K-12; (ii) chuA gene, an outer membrane gene required for haem transport in *E. coli* O157:H7; and (iii) TspE4.C2, a DNA sequence situated within a gene encoding a putative lipase esterase (Doumith *et al.,* 2012).

The Clermont triplex PCR method has been applied successfully to determine *E. coli* phylogroups in humans, animals and the environment (Lamprecht *et al.,* 2014) .

Electrophoretic techniques such as pulse-field gel electrophoresis (PFGE) or repetitive element sequence-based PCR (rep-PCR) have been considered the gold standard methods for bacterial strain tracking for many years. Although PFGE is a highly discriminatory and robust method, its disadvantages such as extreme time and labor demands combined with potential resolution issues and inconsistencies in interpretation make it difficult to standardize between laboratories.

Whole genome sequencing (WGS) has been introduced and has become a widely used sequence-based genotyping tool. Along with the rapid development, WGS technology has also become more accessible for routine applications. However, it is still impractical for prospective typing and/or screening typing larger numbers of strains. In particular, data processing and their evaluation are currently WGS' main disadvantage, demanding both extra computational and human resources (Bezdicek *et al.*,2021).

Multi Locus VNTR Analysis (MLVA) is a molecular typing method to subtype microbial isolates based upon the Variable copy Numbers of Tandem Repeats (VNTR). A VNTR typically exhibits a large range of copy numbers, even among highly related bacterial strains. For a selected set of tandem repeats, copy number analysis reveals insights about the relationships at a micro-evolutionary level. VNTR loci are selected that are sufficiently and complementary discriminatory for the organisms studied, and conserved primers are designed outside the tandem repeat for each VNTR. Thus, the size in base pairs of each PCR-amplicon is the sum of the size of the tandem repeat plus the offsets at both ends.

VNTR are tandem repeats of sequences ranging from 5 to 100 bp; they have been used more and more for genotyping due to their portability between different laboratories, their reproducibility, and their high level of polymorphism on monomorphic bacteria. Multilocus VNTR analysis (MLVA) has been used widely in the medical field, especially to monitor epidemics of monomorphic bacteria, such as *Bacillus anthracis, Yersinia pestis .,Mycobacterium tuberculosis, Burkholderia pseudomallei*, and methicillin-resistant *Staphylococcus aureus*, and to assess their evolutionary history on different time and space scales'.(Guinard *et al.*,2017).

1.2.8 Toll like Receptors (TLRs)

TLRs are a type I transmembrane protein family that are the main receptors for pattern recognition that binds to a variety of microbial products, also referred to as pathogen-associated molecular patterns (PAMPs). Several TLRs have also been shown to recognize endogenous ligands associated with inflammation, which have been called danger-associated molecular patterns (DAMPs), in addition to them identification of PAMPs. The main features that distinguish different TLRs are ligand specificity, signal transduction pathways, and sub-cellular localization (Singh *et al.*, 2014).

TLRs with 20 to 27 extracellular leucine rich repeats (LRR) for the recognition of PAMP/DAMP, transmembrane domains, and intracellular toll interleukin-1 (IL1) receptor (TIR) domains required for the activation of downstream signal transduction pathways (Gao *et al.*, 2017).

The extracellular domains of TLR contain glycan moieties that serve as binding sites for ligands (Isaza Correa *etal*.,2014).

There are 13 TLRs identified in mammals (10 functional receptors in humans and 12 in mice). In the plasma membrane, TLR1, TLR2, TLR4, TLR5 and TLR6 reside and recognize extracellular ligands. TLR3, TLR7, TLR8 and TLR9 are intracellular and located in the endosomal membrane, while the human genome does not contain TLR11, TLR12 and TLR13 (Keogh and Parker, 2011).

TLR1, TLR2 and TLR6 detect bacterial lipoproteins and dimerization of TLR2 with either TLR1 or TLR6 confers ligand specificity. TLR5 senses flagellin, TLR9 recognizes unmethylated C-phosphate-G (CpG) bacterial DNA and TLR3, TLR7 and TLR8 bind to viral nucleic acids (Goh and Midwood, 2012) .

TLRs, including professional immune cells such as dendritic cells (DCs),Tcells, B cells and macrophages, are commonly distributed in the body.

Interestingly, TLR expression differs within subsets of these forms of immune cells. DCs vary in their levels of TLR expression, for instance. Myeloid DCs usually express TLRs 1-9, while TLRs 7 and 9 are typically strongly expressed by plasmacytoid DCs, whereas TLRs 2 and 4 are only weakly expressed (Keogh and Parker, 2011) .

TLR-4 Genes Also known as (TOLL, CD284, TLR-4, ARMD10) this gene is a locus in chromosome number 9 (9q33.1) and has 4 exons. The protein encoded by this gene is a member of the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity. TLRs are highly conserved from Drosophila to humans and share structural and functional similarities. This receptor has also been implicated in signal transduction events induced by lipopolysaccharide (LPS) found in most gram-negative bacteria. Multiple transcript variants encoding different isoforms have been found for this gene (Firmal *et al.*, 2020).

1.2.9 The Role of Nanoparticles against *E.coli*

Nanoscience and nanotechnology have attracted a great interest over the last few years due to its potential impact on many scientific areas such as energy, medicine, pharmaceutical industries, electronics, and space industries. This technology deals with small structures and small-sized materials of dimensions in the range of few nanometers to less than 100 nanometers. Nanoparticles (NPs) show unique and considerably changed chemical, physical, and biological properties compared to bulk of the same chemical composition, due to their high surface-to-volume ratio. NPs

exhibit size and shape-dependent properties which are of interest for applications ranging from biosensing and catalysts to optics, antimicrobial activity, computer transistors, electrometers, chemical sensors, and wireless electronic logic and memory schemes. These particles also have many applications in different fields such as medical imaging, nanocomposites, filters, drug delivery, and hyperthermia of tumors (Bayda *et al.*, 2019).

An important area of research in nanoscience deals with the synthesis of nanometer-size particles of different morphologies, sizes, and monodispersity . In this regard, there is a growing need to develop reliable, nontoxic, clean, ecofriendly, and green experimental protocols for the synthesis of NPs (Iravani *et al.*, 2014).

The antimicrobial activity of NPs depends on several physicochemical properties, such as their size, shape, solubility, and ability to form free biocidal metal ions (Gold *et al.*, 2018).

Generally, smaller NPs show increased antibacterial activity compared to larger NPs (Abdussalam-Mohammed, 2020).

Gram-positive and Gram -negative bacteria differ in terms of cell membrane components and structures and have different adsorption pathways for NPs . The susceptibility of bacteria to NPs depends on their biochemical composition since different NPs target different biomolecules (da Silva *et al.*, 2019).

Both organic and inorganic nanoparticles are reported to have antibacterial and anti-biofilm potencies (Mohid *et al.*, 2020).

These are also used as surface-coating and drug-delivery agents and thus offer a very promising alternative to conventional methods of biofilm control (Fam *et al.*, 2020).

However, this raise concerns on the specificity of nanoparticles to kill a particular pathogen. Unlike traditional antibiotics, inherited

resistance towards organic and inorganic nanoparticles has not been observed in bacteria. However, a recent study suggested that bacteria could evolve to acquire resistance through genetic mutations on continuous treatment with AgNPs for 225 generations. Hence, care should be taken to avoid unintentional and unnecessary exposure of microorganisms towards these nanoparticles (Graves Jr *et al.,* 2015).

Metal nano particles such as gold, silver and copper are of the bactericidal category. Silver reacts with thiol groups of microorganism enzymes and leads to the denaturation of enzymes and ultimately cell death. Packaging containing silver nanoparticles releases silver ions which reduce, prevent or delay microbial growth and thereby increase shelf life and maintain the quality of the food products. Therefore, advanced packaging techniques are based on the smart nano -materials which could respond to the environmental condition and contain an external and internal indicator to show any contamination, temperature and humidity changes and water penetration, as well as detect spoilage and pathogenic microorganisms(Anvar *et al.,* 2019).

It is well known that silver ions show good antibacterial properties without any toxic effects in comparison to other heavy metal ions. The biocidal activity of silver is related to the biologically active silver ion released from silver coatings. The silver ions (Ag^{+}), have the capacity to bind to the proteins and enzymes present on the bacterial cell wall and the cell membrane. This leads to the disruption of the cell membrane which causes permeability imbalance, cellular disintegration, ultimately cell death (Mantravadi *et al* .,2017).

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Equipment and Instruments

Equipment and instruments used in the study were listed in table 2-1.

Table 2-1 Equipment and Instruments Used in the study.

Equipment	Manufacturing Company/ Origin
Autoclave	Jeiotech/ South Korea
Centrifuge	Hettich / Germany
Cooling Centrifuge	Hettich /Germany
Deep-freezer	Hicool/ Denmark
Digital camera	Canon/ Japan
Electrical balance	KERN/ Germany
Eppendorf tubes	Sterellin Ltd /UK
Hot plate with magnetic stirrer	IKA/ Germany
Incubator	Memmert/ Germany
Light microscope	Olympus /Japan
Microcentrifuge	Hettich/ Germany
Microtiter plate (96) flat- shape	Sterilin Ltd/ UK
Millipore filter unit (0.45 µm)	Schleicher and Schuel / USA
Nanodroop	Avans/UK
PCR Thermocycler	Clever/ England
Refrigerator	Crafft/ Saudiaarabia
Safety cabinet (HOOD)	Gallenkamp/ USA
Spectrophotometer	Shimadzu/ Japan
Vitek 2 compact	Biomerieux/ France
Vortex	Fanem/ Brazil
Water bath	Memmert/ Germany
Water distiller	Paytekht Co.Ltd/ Iraq

2.1.2 Chemical and Biological Materials

Chemical and biological materials used in this study were summarized in the table 2-2.

Table 2-2 Chemical and Biological Materials Used in this Study.

Chemical and biological material	Manufacturing Company/ Origin
Agarose	Promega/USA
Ethanol 70%, absolute	BDH / UK
Ethidium bromide	Promega/USA
Glucose	Oxoid / UK
Glycerol	BDH / UK
Gram Stain kit	HiMedia/ india
Hydrogen peroxide (H_2O_2)	BDH / UK
Isopropanol 99%	BDH / UK
N-N-N-Ntetramethyl-P-phenylene diamine dihydrochloride	Hi-Media/ India
Normal Saline	PSI/ Saudi Arabia
Nuclease Free Water	Promega/USA
Phosphate buffer saline (PBS-pH=7. 2)	BDH/ England
Tris-Borate-EDTA (TBE) buffer	Promega/USA
Tris-EDTA buffer (TE)	Promega/USA
Kovacs reagent	BioMerieux (France)
Barritts reagent	Oxoid (UK)
Koh & alpha-naphthol	Oxoid (UK)

2.1.3 Culture Media

All the culture media were used in this study are listed in table 2-3.

Table 2-3 Culture Media Used in this Study.

Media	Manufacturing Company/ Origin
Blood agar	Himedia /India
Brain heart infusion broth	Himedia /India
Eosin methylene blue (EMB) agar	Himedia /India
MacConkey agar	Oxoid / England
Mueller Hinton agar	Oxoid / England
Nutrient broth & agar	Oxoid / England
Luria Bertani Medium	Oxoid / England
Simmon citrate media	Himedia /India
Kligler iron agar media	Himedia /India
MR-VP media	Himedia /India

2.1.4 Kits

The diagnostic kits used in the current study were presented in Table 2-4.

Table 2-4 Diagnostic Kit Used in this Study.

Kit	Manufacturing Company / Origin
DNA extraction kit	Geneaid／ USA
DNA ladder (100-1500) bp	Bioneer／ Korea
Green master mix 2X Kit	Promega/USA
Vitek $_2$ System kit	Biomerieux / France
RNA extraction kit	Sacace Biotechnologies Srl / Italy

2.1.5 Antibiotic Disks

The antibiotic used in the current study for each isolate was determined and recommended by CLSI, were presented in Table 2-5.

Table 2-5 Antibiotic Discs Used in this Study.

Groups	Antibiotic	Code	Disk potency (µg/disc)	Manufacturing Company /Origin
Cell Wall Synthesis (B-lactam)	Ampicllin	AM	10	Mastdiscs / UK
	Ceftriaxone	CRO	30	
	Cefotaxime	CTX	30	
	Imipenem	IPE	10	
Protein Synthesis (Aminoglycoside 30s)	Gentamicin	GEN	10	
Protein Synthesis (Tetracyclins 30s)	Doxycyline	DO	10	
	Tetracycline	TE	10	
DNA Synthesis (Fluorquinolones)	Ciprofloxacin	CIP	10	
	Norfloxacin	NOR	30	
DNA Damage	Trimethoprim-sulfamethoxazole	SXT	25 1.25/23.75	

2.2 Methods

2.2.1 Reagents and Buffer

2.2.1.1 Reagents

A- Catalase Reagent

It was prepared by adding 1ml of 30% Hydrogen peroxide to 9 ml of D.W. thefinal concentration of the solution was 3% and then it was kept in a dim bottle (Procop *et al.*, 2017). This reagent was used for detecting capability of bacteria isolates to produce catalase.

B- Oxidase Reagent

It was prepared by dissolving 1gm of N-N-N-N tetramethyl-P-phenylene diamine dihydrochloride in 100 ml of distilled water and kept in a dark bottle in refrigerator. This solution was used to detect the capability of bacterial isolates to produce oxidase (Shields & Cathcart, 2010).

C- Kovacs Reagent

It prepared by dissolving 5g of (p-dimethyl aminebenz aldehyde) in 75 ml of amyle alcohol and then 25 ml of concentrated HCL was added . It was used for the detection of indole production (MacFaddin, 2000).

D- Voges-Proskauer Reagents (Barritt's reagent)

It was used to detect the production of acetyl-methel carbinol. It is composed of two solutions as follows: A. 5 gm of anephthol dissolved in 100m! of 99% ethanol, then stored in a dark container away from light. B. 40 gm of KOH dissolved in 100m! of distilled water (Collee *et al.*, 1996).

2.2.1.3 Buffer

2.2.1.3.1 Working solution of Tris-Borate-EDTA (TBE buffer)

Tris-borate-EDTA buffer was used at dilution of 1X . The stock solution was diluted by D.W. and stored at room temperature (Sambrook and Rusell, 2005) .

2.2.2 Preparation of Culture Media

2.2.2.1 Ready-Culture Media

Ready-made culture media were prepared according to the manufacturing company instructions, while the other culture media were prepared in the laboratory according to the scientific references. All these media (with some exceptions) were autoclaved at 121°C for 15 minutes at 15 pound per square inch (Brown & Smith, 2017).

2.2.2.2 Laboratory Prepared Culture Media

A- Blood Agar (PH : 7.1)

According to the manufacturer company, Blood agar base was prepared. Then was autoclaved and cooled to (45-50)ºC, aseptically adding 5 ml of sterile defibrinated human blood for each 95 ml of the medium mixing well to homogeneity, distributed into sterile Petri dishes. Finally, the prepared medium was cooled to 37ºC and was left to solidify at room temperature (25ºC). (Harley & Prescott, 1996).

B- MacConkey Agar (PH: 7.3)

This media was prepared according to the instruction of the industrialized company. McConkey agar is used to isolate most Gram - negative bacteria and used to differentiate between lactose fermenter and non-fermenter bacteria (Govan and Deretic ,1996) .

C-Nutrient Agar Medium

This nutrient agar was prepared according to the instruction of supplier company by dissolving 28gm of nutrient agar in 1L of distilled water then sterilized in the autoclave at 121 Cº for 15 min. , this medium was used in general experiment such as cultivation and activation of bacterial isolates when it necessary (MacFaddin, 2000) .

D- Müller-Hinton Agar

This medium was prepared according to the instruction of supplier company by dissolving 38gm from the medium in 1L of distilled water and sterilization by autoclave at 121 Cº for 15 min., this medium used for an antibiotic sensitivity test (MacFaddin,2000).

E- Brain Heart Infusion (BHI) Broth -Glycerol Medium(Maintenance Medium) (PH : 7.4)

This medium used to preserve the bacterial isolated as the standard for a long time. This medium was prepared by adding 5ml of glycerol with 95 ml of BHI broth and sterilization by autoclave at 121 Cº for 15min. (Forbes *et al.*, 2007).

F- Luria Bertani Broth (PH : 7.2)

It was used for Enterobacteriaceae isolates and also for non- fastidious bacteria. It was prepared in the laboratory by dissolving (10 g of Trypton, 10 g of NaCl and 5 g of Yeast extract) in 1000 ml distilled water, to prepare solid media, 20 g of agar was added to the above broth medium .

G- Eosin methylene blue(EMB) agar

This medium was prepared according to the method recommended by the manufacturing company. The purple colour was due to the absorption of the eosin methylene blue complex formed in the presence of acid. Certain members of *E. coli* exhibit a greenish metallic sheen in reflected light (Murray *et al.*, 2003).

2.2.3Vitek $_2$ 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).

2.2.4 Ethical approval

The necessary ethical approval was taken from the ethical committee in Al-Hilla Teaching Hospital and Babylon Hospital,

Moreover, agreement from the family and patients for sampling and carrying out this work was obtained.

2.2.5 Study design

Cross-sectional study.

The design of study was illustrated in figure 2-1.

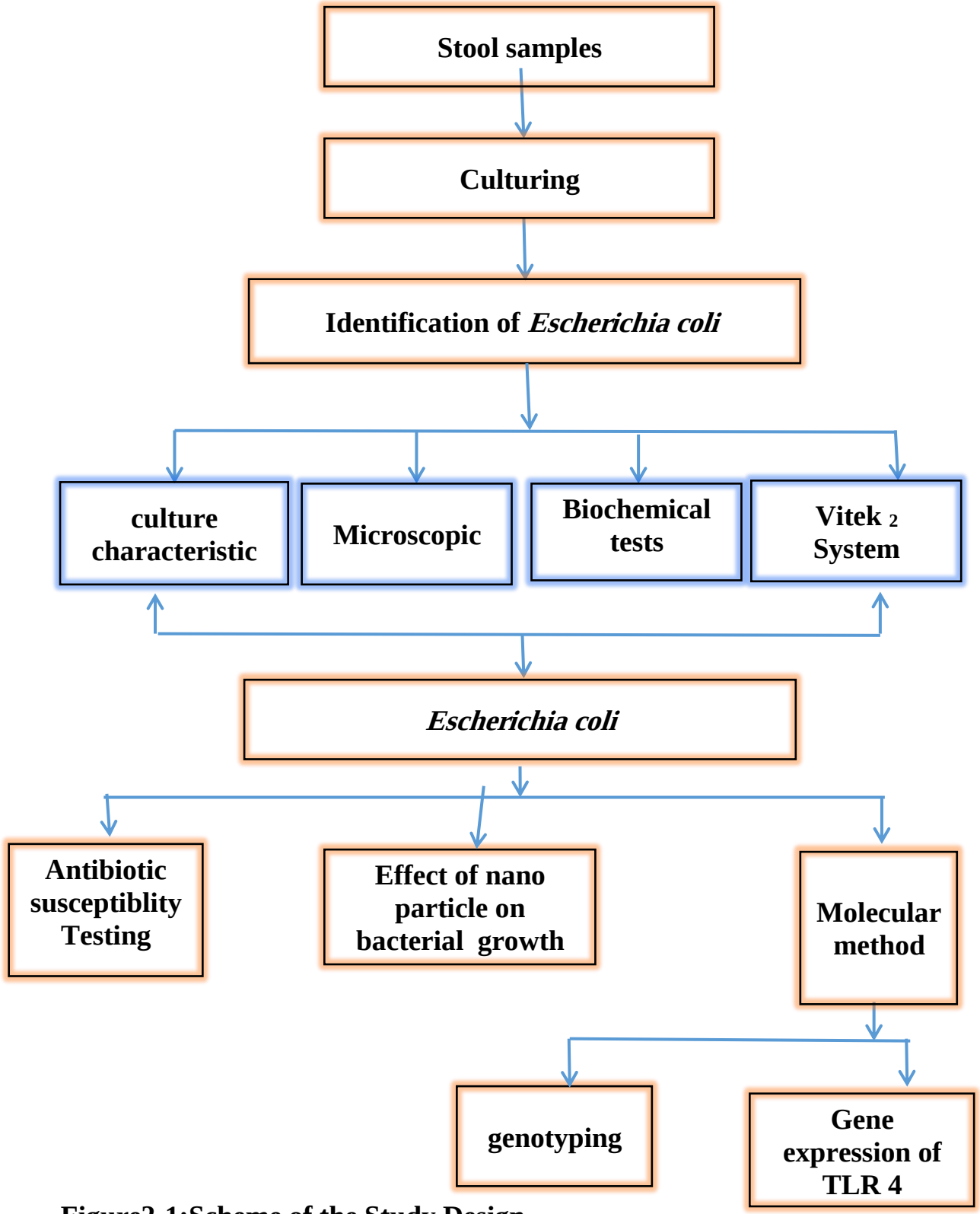


Figure2-1:Scheme of the Study Design.

2.2.6 Clinical specimens' collection

One hundred and fifty clinical specimens were collected during the present study period extended from October 2022 to January 2023, . from patients who were attended to the two main hospitals in Hilla city: Al-Hilla General Teaching Hospital and Babil Teaching Hospital for maternity and children.

Stool specimen were collected from patients who admitted the emergency room with specific symptom as diarrhea and vomiting that are suspected to get food poisoning according to physician by disposable sterile clean, leak-proof container proper way to avoid any _ possible contamination. these are taken and closed it then transported to the laboratory of College of Medicine.

fecal specimen were diluted serially 10-fold steps in pepton water and cultivated aerobically on *E.coli* media , the lowest level of detection was 300- 400 CFU w/g of feces . Colonies were isolated, subculture on other medium, gram stain were tested and culturing on Blood Agar , MacConkey Agar and EMB agar incubated at 37°C for 24hrs. for bacterial diagnosis.

2.2.7 Healthy contral group

A stool sample (16 sample) were collected from a healthy group who did not have any signs and symptoms of food poisoning and had a negative stool culture.

2.2.8 Bacterial Identification

2.2.8.1 Microscopic Examination (Gram Stain)

One isolated colony was transferred to a microscopic slide, fixed then stained with Gram stain, color, cell shape and arrangement were recorded. The results were compared with (Brooks *et al.*, 2013).

2.2.8.2 Biochemical Tests

All the biochemical tests were carried out according to (Brown & Smith, 2017).

A- Catalase Test

On a clean microscope slide this test was performed by using the end of a wooden swab, some cells from the bacterial culture were transferred to the surface of a clean microscope slide, two to three drops of 3% hydrogen peroxide were added to the cells, then were mix with the wooden stick, and vigorous bubbleswere observed (Brown & Smith, 2017).

B- Oxidase Test

A filter paper was saturated with little drops of 1% N-N-N-N-tetramethylpara- phenylenediamine dihydrochloride. With a woody applicator growth from an agar medium was smeared on the paper. A positive check was formed a purple color within 10 seconds (Brown & Smith, 2017).

C.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. (Forbes *et al.*, 2007).

D. 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 red ring after a few seconds indicates a positive result (Hemraj *et al.*, 2013).

E. 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).

F.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).

G. 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).

2.2.8.3 Vitek 2 system diagnosis

The Vitek 2 System was used to confirm the result of the manual biochemical test, this system used to identify microorganisms . This system was performed according to the manufacturer's instructions (Biomerieux-France).

This system consists of :

1- A personal computer.

2- Reader/incubator that consisting of multiple internal components including: card cassette, card filler mechanism, cassette loading processing mechanism, card sealer , bar code reader, cassette carousel and incubator.

3- The system also contains: transmittance optics , waste processing, instruments control electronics and firm ware. This system was performed according to the manufacturer's Instructions (Biomerieux-France):

1- Three ml of normal saline were placed in plane test tube and inoculated with a loopfull of single colony of overnight culture.

2- The test tube was inserted into a dens check machine for standardization of colony to McFarland's standard solution (1.5×10^8 CFU/ml).

3- The standardized inoculums were placed into the cassette.

4- Then a sample identification number was entered into the computer software via barcode . Thus the vitek 2 card was connected to the sample ID number. The cassette was placed in the filler module, when the cards were filled, transferred the cassette to the reader/incubator module.

2.2.9 Preservation of Isolates

2.2.9.1 Short Term Preservation

Bacterial isolates were maintained for few weeks on nutrient agar plates which were wrapped tightly with parafilm and then stored at 4ºC(Harley & Prescott, 1996) .

2.2.9.2 Long Term Preservation

Brain heart infusion broth with the addition of 5% glycerol was distributed into plain tube, autoclave and inoculated with 24 hr. ancient culture of bacteria isolates as wellas incubated at 37°C for 24 hr. then stored at -20°C (Vandepitte *et al.*, 2003).

2.2.10 Antibiotic Susceptibility Test

Kirby -Bauer method used to perform carry out the antibiotic susceptibility test for 10 different antimicrobial agent Bacterial suspension was prepared by picked 4-5 colonies of each bacterial isolate from original culture and was suspended into a test tube containing 5 ml of normal saline, then turbidity was adjusted to obtain approximately 1.5 $x10^8$ CFU/ml (MacFarland tube 0.5). By a sterile cotton swab a portion of bacterial suspension was transferred carefully and evenly spread on Mueller - Hinton agar medium, and then it was left for 10 min. Then after the antimicrobial discs were placed on the agar with sterile forceps pressed firmly to ensure contact with the agar.

Later the plates were inverted and incubated at 37 °C for 24 hr. Inhibition zones that developed around the discs were measured by millimeter (mm) unit by using a metric ruler. The isolate was interpreted as susceptible, intermediate, or resistant to particular antibiotic according to CLSI 2022.

2.2.11 Genotyping assays of *Escherichia coli*

2.2.11.1 DNA Extraction

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid (UK). Chromosomal DNAs obtained were used as templates for all PCR experiments,and according to the following steps:

Step1: Cell Harvesting/pre-lysis

- One ml of bacterial suspension containing approximately up to1.2×10^9 cell/ml (equal to 4.0 McFarland standard) was transferred to a 1.5ml microcentrifuge tube, centrifugation for 1 minute at 14-16,000×g and discard the supernatant.
- A volume of 200 µl of GT buffer was added to the tube and resuspend the cell pellet by shaking vigorously or pipetting, then left at room temperature for 5 minutes.

Step 2: Lysis

- A volume of 200 µl of GB buffer was added to the sample and mix by shaking vigorously for 5 seconds, then incubated at 70ºC for 10minute
- or until the sample lysate is clear. During incubation, the tube was inverted every 3 minutes. At this time, the required Elution Buffer (200µl per sample) incubated at 70ºC (for step 5 DNA elution).

Step 3: DNA Binding

- A volume of 200 µl of absolute ethanol was added to the sample lysateand immediately mixed by shaking vigorously. If precipitate appears, broke it up by pipetting.
- GD column was placed in a 2ml Collection Tube.
- All of the mixture (including any precipitate) was transferred to the

GDColumn and centrifuged at 14-16,000×g for 2 minutes.

- The 2 ml Collection Tube flow-through was discarded and placed theGD Column in s new 2 ml Collection Tube.

Step 4: Washing

- A volume of 400 µl of W1 Buffer was added to the GD Column.
- Then, centrifugation at 14-16000×g for 30 second
- The flow-through was discarded and placed the GD Column back inthe 2 ml Collection Tube.
- A volume of 600 µl of Wash Buffer (ethanol added) was added to theGD Column.
- Centrifugation at 14-16,000×g for 30 seconds.
- The flow-through was discarded and placed the GD Column back inthe 2 ml Collection Tube.
- Step 5: DNA Elution
- ❖ The dried GD Column was transferred to a clean 1.5 micro centrifuge
- tube And 100 µl of preheated Elution Buffer was added to the center of
- the column matrix and let stand for 3-5 minutes or until the Elution
- Buffer is absorbed by the matrix.
- ❖ Centrifugation at14-16,000×g for 30 seconds to elute the purified DNA.

2.2.11.2 Detection of DNA concentration and purity by Nanodroop

The extracted DNA was checked by using nanodrop spectrophotometer, wich measured DNA concentration (ng/µL) and chek the DNA purity by reading the absorbance at (260/280nm) as following steps:

1. After opening up Nanodrop software, chosen the appropriate application

(Nucleic Acid, DNA).

2. A dry wipe was taken to clean instrument pedestals several times. then carefully pipette 2 µl of ddH2O on to the surface of the lower measurement pedestals for blank system.

3. The sampling arm was lowered and clicked OK to initialized the nanodrop, then cleaning off the pedestals and 1 µl of extracted DNA carefully pipette onto the surface of the lowered measurement pedestals,then concentration and purity of extracted DNA was checked (Wilfinger *et al.*, 1997).

2. 2.11.3 PCR amplification

2.2.11.3.1 Oligonucleotides primer pairs

All primer pairs used in this study were purchased from macrogen (Korea) in lyophilized form. Primarily, the stock solution was prepared by dissolving the lyophilized powder in nuclease free water buffer according to manufacturer's protocol.

2.2.11.3.2 PCR Master Mix

A. Master Mix kit:

GoTaq Green Master Mix kit is a premixed ready-to-use solution for amplification of DNA templates by conventional PCR. This kit was purchased from Promega/USA. This master mix contains the following:

- *Taq* DNA polymerase: This enzyme responsible for the synthesis of new DNA strand during PCR reaction.
- Reaction buffer (2X) important for maintenance, pH about 8.5 also contains a compound that increases sample density.
- Deoxyribose Triphosphate (dNTPs) 400 µM of the following: dATP, dGTP, dCTP and dTTP.

- Magnesium ions $MgCl_2$ (3mM), which is a co-factor for enzyme action.
- Loading dye: containing a mixture of yellow dye (xylene cyanol) and blue dye (bromophenol blue), important for direct PCR product loading for analyzed by agarose gel electrophoresis.

B. GoTaq Green Master Mix kit protocol:

The multiplex and uniplex PCR amplification mixture which used for detection of MLVA genes were listed in tables below, table (2-6).

Table (2-6): Contents of the Reaction Mixture uniplex PCR

Contents of the reaction mixture uniplex	**Volume**
Green master mix	12.5µl
Upstream primer	2.5µl
Downstream primer	2.5µl
Nuclease free water	5µl
DNA template	2.5µl
Total volume	**25µl**

2.2.11.3.3 Primer Sequences and PCR conditions

The primer sequences and PCR conditions thatused are listed in tables 2-7

Table 2-7 The primer sequences and PCR conditions

No	MLVA locus	Sequence 5'-3'	PCR condition	product	Reference
1	*Ms 06 F*	AAACGGGAGAGCCGGTTATT	94°C for 5 min 94°C for 1min 55°C for1 min 72°C for1 min *35 cycles 72°C for10 min	-Cell division protein	Farajzadeh-Sheikh 2020
	Ms06 R	TGTTGGTACAACGGCTCCTG		-Cell division protein	
2	*Ms 07 F*	GTCAGTTCGCCCAGACACAG		-electron transport	
	Ms 07 R	CGGTGTCAGCAAATCCAGAG			
3	*Ms 09F*	GTGCCATCGGGCAAAATTAG		-complex protein RNFC	
	Ms 09 R	CCGATAAGGGAGCAGGCTAGT		-complex protein RNFC	
4	*Ms 11F*	GAAACAGGCCCAGGCTACAC		-dependent protein	
	Ms 11 R	CTGGCGCTGGTTATGGGTAT			
5	*Ms 21 F*	GCTGATGGCGAAGGAGAAGA		-hypothetical protein	
	Ms 21 R	GGGAGTATGCGGTCAAAAGC			
6	*Ms 23 F*	GCTCCGCTGATTGACTCCTT			
	Ms 23 R	CGGTTGCTCGACCACTAACA			
7	*Ms 32F*	TGAGATTGCCGAAGTGTTGC			
	Ms 32 R	AACTGGCGGCGTTTATCAAG			

2.2.11.4 Detection of Amplified Products by Agarose Gel Electrophoresis

PCR products were visualized on 1.5%. Agarose gel electrophoresis stained with Ethidium bromide according to(Lee *et al.*, 2012).

A- Preparation of gel:

- The gel was prepared at a concentration of 1.5% by dissolving 1.5gram of agarose in 100ml of TBE working buffer (1X).
- Heating mixture until agarose was completely dissolved.
- The mixture was left to cool to about 50-60°C then 1 µl of ethidium bromide was added to the gel.

B-preparation of casting horizontal agarose gel:

- The casting platform was placed with well former sideways in gel stand where the gel was poured.
- The gel was poured on an electrophoresis plate fixed on an even surface.
- The comb was placed and the gel was left to cool and solidify for 30 min at room temperature.
- After the gel was set, the combs were removed carefully and the tank was placed into electrophoresis system. Then added 1X TBE buffer until covered gel (approximately 1 - 2 mm above the gel surface).

C-Running of products

- When the thermocycling was finished, the PCR tube was handled outside the thermocycler. 5 µl of each PCR product along with 100bp DNA ladder was loaded into the gel wells.
- The system cover put into place and then turned on. The gel was run for 60min at 75 volts.

2.2.11.5 Visualization

Following electrophoresis, visualization was conducted with a UV transilluminator and image was captured by the digital camera.

2.2.12 Gene expression of TLR4

2.2.12.1 RNA Extraction

After collection of Stool samples from patients and healthy individuals, the total RNA was extracted by the protocol of Sacace DNA/RNA prep Kit (Sacace Biotechnologies Srl, Italy). Briefly,

1- Prepare 10-20% feces suspension, (by adding 4ml of Saline Solution and 1,0 gr (approx. 1,0 ml) of feces in 5 ml tube).
2- Vortex to get an homogeneous suspension and centrifuge for 5 min to 7000-12000g.
3- Mix 300 µl of Lysis Sol with 100 µl of samples (supernatant) to the 1.5 ml disposable polypropylene micro centrifuge tubes using pipette tips with aerosol barriers.
4- Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 7-10 sec.
5- Add 400 µl of Prec Sol and mix by vortex. Centrifuge all tubes at 13,000 r/min for 5 min and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
6- Add 500 µl of Wash Sol 3 into each tube. Vortex vigorously to ensure pellet washing. Centrifuge all tubes at 13,000 r/min for 60 sec and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
7- Add 200 µl of Wash Sol 4 into each tube. Vortex vigorously to ensure pellet washing. Centrifuge all tubes at 13,000 r/min for 60 sec and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.

8- Incubate all tubes with open caps at 65 °C for 5 min.

9- Resuspend the pellet in 50 µl of RE-buffer. Incubate for 5 min at 65°C and vortex periodically.

10- Centrifuge the tubes at 13000g for 60 sec. The supernatant contains RNA ready for amplification.

2.2.12.2 Real-time qPCR

The real-time qPCR reactions were performed by using specific primers targeting reference gene GAPDH and the target gene of TLR4 in table 2-9 . Conversion the total RNA to cDNA and amplification of DNA was done according to instructions provided by GoTaq® 1-Step RT-qPCR System (Promega) using BRYT Green® dye, where RT-qPCR Mixture and conditions were summarized in tables 2-10 and 2-11, where the final volume of RT-qPCR reaction was 20 µl. Relative expression fold was calculated by delta delta method (2^-(ΔΔCt) according to (Livak and Schmittgen, 2001).

Table 2-8 The primer sequences of Gene expression of TLR4 by Real-time qPCR

No	MLVA locus	Sequence 5'-3'	Reference
1	TLR4-F	ATATTGACAGGAAACCCCATCCA	(Livak KJ, Schmittgen ,2001)
	TLR4-R	AGAGAGATTGAGTAGGGGCATTT	
2	GAPDH-F	GTCTCCTCTGACTTCAACAGCG	
	GAPDH-R	ACCACCCTGTTGCTGTAGCCAA	

Table (2-9) RT-qPCR Mixture

Component	Volume
GoTaq® qPCR Master Mix, 2X	10 µl
GoScript™ RT Mix for 1-Step RT-qPCR (50X)	0.5 µl
Forward primer (20X)	1µl
Reverse primer (20X)	1µl
RNA Template	5 µl
Nuclease-Free Water	2.5 µl
Total volume	20 µl

Table (2-10) RT-qPCR conditions

STEP	CYCLES	TEMPERATURE	TIME
Reverse transcription	1	37 c˚	15 minutes
Reverse transcription inactivation and GoTaq DNA polymerase activation	1	95 C˚	10 minutes
Denaturation	40	95 C˚	10 seconds
Annealing and data collection		60 C˚	30 seconds
Extension		72 C˚	30 seconds

2.2.13 Preparation of $AgTio_2$ nanoparticles suspension:

Ten mg of $AgTio_2$ nanoparticles ,the size of $AgTio_2$ is 20nm, dissolves in 10 ml D. W to prepare a suspension of 1000µg/ml , continuous ultra- sonication(over-night) for the suspension was achieved at the time of preparation and each time prior to use so as to re-disperse the particles in the solution (Kharisov *et al.,* 2013).

2.2.14 The effect of $AgTio_2$NPs on Bacterial Growth at 24 and 48-hours Incubation time

In brief, first well contain only 300 µl of BHI broth to adopt it as negative control while the remaining wells were filled with 150 µl of bacterial suspension except positive controls well which is filled with double volume. Subsequently, the $AgTio_2$ (150 µl) was added to the third wells. Then, double fold serial dilution was carried out across the plate beginning with 1/2,1/4,1/8,1/16 and finally 1/32 dilution all dilution wells with duplicated ,then the plates were incubated for 24 hours and 48 hours at 37°C. the effect of $AgTio_2$ Nanoparticles was read by ELISA reader at 405 nm. For 48 h the experiment was applied with the same conditions used in 24 h experiment but the incubation period was 48 h

2.2.15 Statistical analysis

All frequency data was analyzed by Pearson's chi-squared test and Fisher's exact test . Data were processed and analyzed by using statistical program social science (SPSS22)and the result were expressed percentages (McDonald, 2014).

Chapter Three

Results & Discussion

3.1 Collection and Isolation of *E. coli* Isolates

During the present study period extended from October 2022 to January 2023, a total of (150) stool specimens were collected from patients suggested to have diarrhea infection .All clinical specimens were cultured on different media. All isolates were identified by culturing on Blood agar and MacConkey agar and Eosin Methylene Blue agar plates and confirm by biochemical test and vitek 2 compact system, The results indicated that 22 (14.7%) of the isolates belonged to *E.coli* ,other bacterial isolates (50%) while no growth detect in (35.3%) as shown in Table and Figure (3-1).

Table (3-1) Numbers and Percentages of *E. coli* Isolates From patients with diarrhea

Total No. Of samples	Bacterial growth		
	Positive *E. coli*	No growth	Other growth
150	22 (14.7%)	53 (35.3%)	75 (50%)

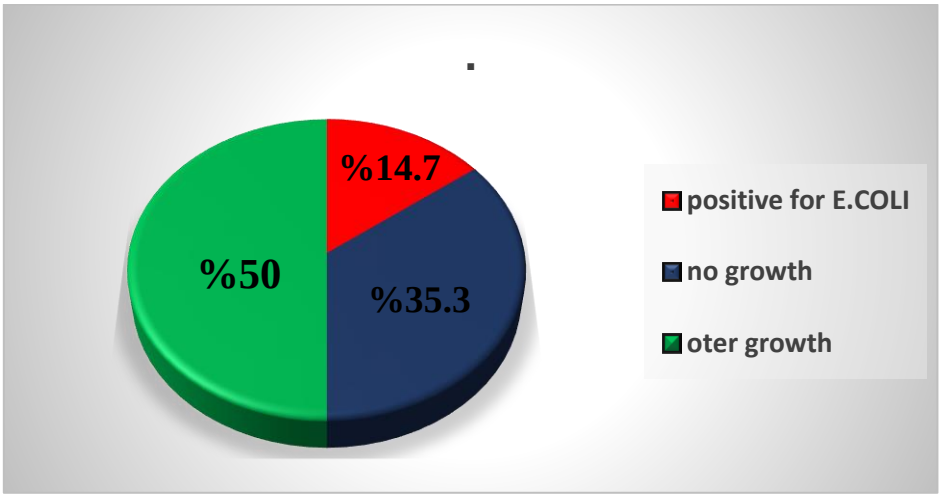


Figure (3-1) percentage of *Escherichia coli* isolates from patients with diarrhea

The result in this study agree with study by (Kibret and Abera,2011) who found that 14.2% of sampling were positive for *E. coli* by using culture techniques.

Also (Al-Sorchee *et al.*,2013) found that Bacterial pathogens were found in 24.8% of the stool samples from patients with diarrhea, 82 (16.4%) of which were *E.coli* of different pathogenic serotypes.

The results in this study disagree with(Wolde *et al.*,2021)who found that from a total of 110 stool samples from diarrheic children, 68 (61.8%) were positive for *Escherichia coli*. The occurrence of *E. coli* in child diarrhea showed a significant difference by age .

Also (Dela *et al.*, 2022) isolates *E. coli* in (62.3%; 76/122) it was the predominant among the pathogens recovered.

High isolation rate detect in (Poyil *et al* .,2022) show A total of 123 diarrheal stool samples were collected 37 of them (30.08% of the total samples) were found to have the presence of *E. coli*.

Also study found that from the total of diarrheic children, 37.8% of them were positive for *E. coli* by using culture techniques (Belete *et al.* ,2022)

The observed contrasts between the present study and previous reports could be due to variations in sample size, culturing techniques, culture media, and specific incubation conditions and the difference in isolation rate might be attributed to different socio demographic characteristics, and the season when the studies were carried out.

Diarrheagenic *E.coli* strains transmission is through the fecal-oral route, with contaminated hands, contaminated weaning foods or formula etc. The reservoir of diarrheagenic *E.coli* infection is thought to be

symptomatic or asymptomatic children and asymptomatic adult carries including mothers and persons who handle infants.

In this study, the distribution of isolates according to age were studed and it was found that (50%) of the samples belonged to infant patients (from 1 day to 11 month) , (22.7%) belong to patients with an average age between (1-10) years, (9%) of the samples belong to patients with an average age between (11-20) years, (13.8%) of the samples belong to patients with an average age of (21-30) years. While (1%) of the samples were recorded for patients over 30 years old as shown in table (3-2) and figure (3-2).

Table 3-2 : Frequency Distributions of culture positivity to *E.coli* isolates according to age group:

Age	NO.		present
Infant (1 day to 11 month)	**11**		**50 %**
	***male* 5**	***Female* 6**	
1-10	**5**		**22.7%**
	***Male* 2**	***Female* 3**	
11-20	***2***		**9 %**
	***Male* -**	***Female* 2**	
21-30	***3***		**13.8 %**
	***Male* 1**	***Female* 2**	
More then 31	**1**		**4.5 %**
	***Male* -**	***Female* 1**	

Found the highest percentage of diarrhea infection with *E. coli* in males and female was within the age group (1-12) months by (48%), followed by the age group of (12-24) months by (28%), and then the age group (36-48) by 14%, while the lowest rate of *E.coli* infection in males and females found in the age group (24-36) months by (6%) followed by the age group (48-60) months by(4%)(Al-Azzawi *et al* .,2023) .

Other study done in Kurdistan Region, Iraq refer that males of the age group 6 months to 2 years showed the highest rate (62.16%,) whereas females of the age group 9-12 years had the highest rate (51.16%) Statistically the relationships between the rate of infection, age and gender were non-significant (Abdulqader *et al* .,2022)

According to the age, patients with diarrhea between 1 month and 10 years were grouped into 7 age groups, which demonstrates that diarrhea is common in the age group less than 3 years ($P \leq 0.05$) (Al-Sorchee *et al.* ,2013).

Found Overall, 66 (20.6%) of isolates were identified as DEC among children with acute diarrhea. Of these, 16/66 (24.2%) was aged< 1 year 22/66 (33.4%) of them were between 1 and 2 years old and 28/66 (42.4%) of them aged between 2 and 5 years the incidence of different DEC pathotypes was not uniform in all age groups of the current study. EPEC was more common in children under 2 years old, while EAEC was more in children aged from 2 to 5 years old. This is consistent with previous studies reported that, EPEC is among the most important pathogens infecting children under 2 years old in the developing country (Khairy *et al.*,2020).

The epidemiological significance of enteric pathotypes of *E. coli* in children varies with geographical region. Studies have also shown that factors such as the health status of the host as well as the environmental,

geographic and social conditions could influence the distribution of *E. coli* phylogroups in humans (Alfinete *et al.*, 2022).

3.1.1 Identification of *E.coli*

Overall, all the *E.coli* isolates were phenotypically identified on both Blood and MacConkey agar with some different characteristics observed. The morphology of colonies has been seen on the blood agar within 24 h at 37°C. While, the colonies properties which were appeared on the MacConkey agar at the same lab conditions can be discriminated with, pink, fermented lactose sugar

The isolates taken from MacConkey agar plate and stained by Gram stain appear under light microscope as a red rod which referred Gram –ve bacteria. In biochemical test, all isolates showed negative results for oxidase test and motility test, whereas a positive results for catalase.as shown in table (3-3)

Table 3-3 biochemical tests used for Identification of *E. coli*.

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

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 peptone, and the fermentation of these sugars caused a reduction in the pH of the medium. **(**Brown and Smith, 2017) and (Sharmin *et al.,* 2010).

3.1.2 Identification of *E.coli* by Vitek - 2 compact system

The clinical isolates identified as *E.coli* using VITEK 2 system used the identification card for Gram negative strains (ID-GNB). The obtained result shows an interesting percentage of accuracy (99%). The accurate and rapid identification of *E.coli* was critical for the appropriate infection control in hospital settings. Up to date, the most common and widespread detection methods include characterization via a phenotypic system and commercial phenotypic methods (e.g.,the Vitek-2 compact system, Biomerieux).

3.2 Antibiotic Susceptibility profile of *E.coli* by disc diffusion method

All the identified *E. coli* isolates from patient with diarrhea were subjected to *in vitro* susceptibility test by modified Kirby - Bauer disc diffusion method , selective antibiotics are used to show their effect on *E. coli* isolates which are 10 commonly used antibacterial agents, the results were interpreted according to the diameter of inhibition zone and compared with stander zones of inhibition determined by CLSI (2022) .

In this study , the results revealed that (22) isolates of *E. coli* resistance to most commonly antibiotics used in treatment of diarrhea , Susceptibility testing showed that all 100% (22) isolates were resistant to Ampicllin & Cefotaxime , Ceftriaxone , 40.9% of all isolates resist to

Doxycyline and Trimethoprim-sulfamethoxazole ,also the results show that all *E. coli* isolates exhibited maximum susceptibility to Norfloxacin 86.3%, Gentamicin 91%, Imipenem 77.3% as shown in table (3-4) .

Table 3-4 Antimicrobial sensitivity pattern of the Diarrhea by *E.coli* isolates against 10 antibiotics.

Antibiotics	Symbol	Number of bacterial isolates and their percentage	
		R	S
Ampicllin	AM	100 %	--
Cefotaxime	CTX	100 %	--
Ceftriaxone	CRO	100 %	--
Imipenem	IPM	22.7 %	77.3 %
Gentamicin	GEN	9 %	91 %
Doxycyline	DO	40.9 %	59.1 %
Tetracycline	TE	31.8 %	68.2 %
Ciprofloxacin	CIP	36.3 %	63.7 %
Norfloxacin	NOR	13.6 %	86.3 %
Trimethoprim-sulfamethoxazole	SXT	40.9 %	59.1 %

R=resistance S=sensitive

Antibiotic resistance is a major clinical problem in treating infections , *E. coli* is one of the most important multidrug-resistant (MDR) opportunistic Gram-negative bacteria cause different illness with high mortality and morbidity due to hospital-acquired infections and non-hospital-acquired infections , susceptibility patterns against different antibiotics vary in different geographical regions , antibiotics resistance among *E.coli* isolates has also reported with an increased frequency all over the world due to aberrant use of antibiotics (Maraki , *et al.*,2013) ; (Soltani *et al.*,2018).

The use of antibiotics in the management of acute gastroenteritis is quite challenging. This is because the pattern of pathogens causing diarrhea does not only vary from one region to another but also according to age, season, immunization status and symptoms. Also complicating treatment is that diarrhea infections could be caused by multiple pathogens , the prevalence of antibiotic resistance in any given locality is also a huge factor in considering the choice of empiric antibiotic for treatment in order to minimize the rates of treatment failure and further resistance development **(**Mkuhlu *et al* ., 2020**).**

Many factors have contributed to high antibiotics resistance rates in hospitals and community, such as excessive antibiotics prescription, antibiotics can be purchased without a prescription and inadequate surveillance also account for the spread of resistance bacteria ,

The results obtained in this study showed that the antibiotic Trimethoprim-sulfamethoxazole was resistant to (40.9%), and these results agree with the results obtained by (Wafa,2022), as well as with the results obtained by (Zenebde *et al.,* 2022).

Also the results showed, the resistant rate of isolates to doxycyline (40.9%), and these results agreed largely with the study (Zenebde *et al.*, 2022), in which the resistant rate was 38% .

The results also showed very high resistance (Cefotaxime, Ampicllin), where all 22 isolates were resistant by (100%), as these results agreed with the study (Zenebde *et al.*, 2022), who found that (87.5) of the isolates were resistant, also agree with (Hussein *et al.*, 2018) who found the resistant rate was 95.5% .

Also showed that 88% were resistant to ampicillin, trimethoprim (84%), tetracycline (83%), doxycycline (82%), High susceptibility of the isolates was observed for gentamicin (95%), amikacin (91%), nitrofurantoin (91%), meropenem (90%), norfloxacin (84%) and imipenem (83%). (Mkuhlu *et al.*, 2020)

These findings suggest that these antibiotics should not be considered as empiric treatment of DEC infections as it could further fuel the spread of antibiotic resistance in the community. The most important factors that contribute to the spread of antibiotic resistance could be abuse and poor stewardship in the prescription and usage of the drug in developing countries for infections that may not require antibiotics as well as administration of inadequate regimen of the drug .

The results showed that Ceftriaxone resistance was also high, reaching (100%) this result agree with , (Webale *et al.*, 2020) who refer that diarrheagenic *Escherichia coli* isolates identified as phenotypic resistant to ampicillin, ceftriaxone, streptomycin, gentamycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline, respectively, 70(98.6%), 15(88.2%), 83(93.3), 60(65.9%), 38(95.0%), 85(87.6%), 11(91.7%) and 102(91.9%). and this resistance due to inappropriate antibiotic use selects antibiotic resistance, it was not

surprising that this study found high phenotypic antibiotic resistance rates to ampicillin, streptomycin, gentamycin, ciprofloxacin, chloramphenicol and tetracycline suggesting that these six drugs should not be used as a first-line therapeutic drug for diarrheagenic *Escherichia coli* (Webale *et al* ., 2020) .

Possible explanation for the persistence of resistance to these antibiotics includes the frequent co-existence of resistant genes on large transferable plasmids (Aslam *et al.*,2018)

The results showed that the resistance of (Ciprofloxacin) was (36.3%), which was significantly associated with (Zenebde *et al.,* 2022), who found that (33.9%) and showed a difference from (Hussein *et al.,* 2018).

While the results of (Norfloxacin) resistance were (13.6%) as they were associated with the results of (Zenebde *et al.,* 2022).

The results also showed the resistance of the isolates to the antibiotic Tetracycline by (31.8%), as it agreed with the results obtained by (Wafa, 2022) and contradicted the results of (Hussein *et al.,* 2018).

The results showed that the resistance of the isolates to the Gentamicin antibiotic was (9%), and these results indicated a correlation with the results of (Wafa ,2022) and showed a contradiction with (Zenebde *et al.,* 2022).

The results indicated that the resistance to Imipenem reached (22.7%) this agree with (Khairy *et al.,* 2020) found that the resistance to meropenem was only (9.1%).

3.3 Effect of $AgTIO_2$ Nanoparticles on *E.coli* growth

In this study the effect of $AgTIO_2$ was examined against *E.coli* isolates growth by making five double serial dilutions at (1/2, 1/4, 1/8, 1/16 and 1/32) from stock solutions (concentration 400 ug/ml); then after incubation for 24 hours the bacterial growth had been monitored and checked with spectrophotometer. Results showed that there was a significant ($p \leq 0.05$) decrease in growth of bacteria after 24hr of incubation with different concentrations of $AgTIO_2$ nanoparticles especially with the first and second dilution, as shown in figure (3-2).

The highest concentration of Ag Tio_2 after diluted for (1/2) made the highest inhibition effect for bacterial growth and this inhibition minimized the bacterial growth from (1.36) to (0.5) after 24hours of incubation While the lowest concentration of Ag Tio_2 that made by diluted the nanoparticles for (1/32) showed the lowest inhibition effect on *E.coli* growth after 24 hours of incubation. As an antibacterial agent, silver-titanium dioxide nanocomposite materials are widely known for their anti-pathogenic ability; studies have reported efficacy against various strains of Gram-positive bacteria (*B. subtilis, S. aureus, MRSA*), Gram-negative bacteria (*E. coli, K. pneumonia, P. aeruginosa*) and fungi (C. albicans) (Muflikhun *et al.*, 2019).

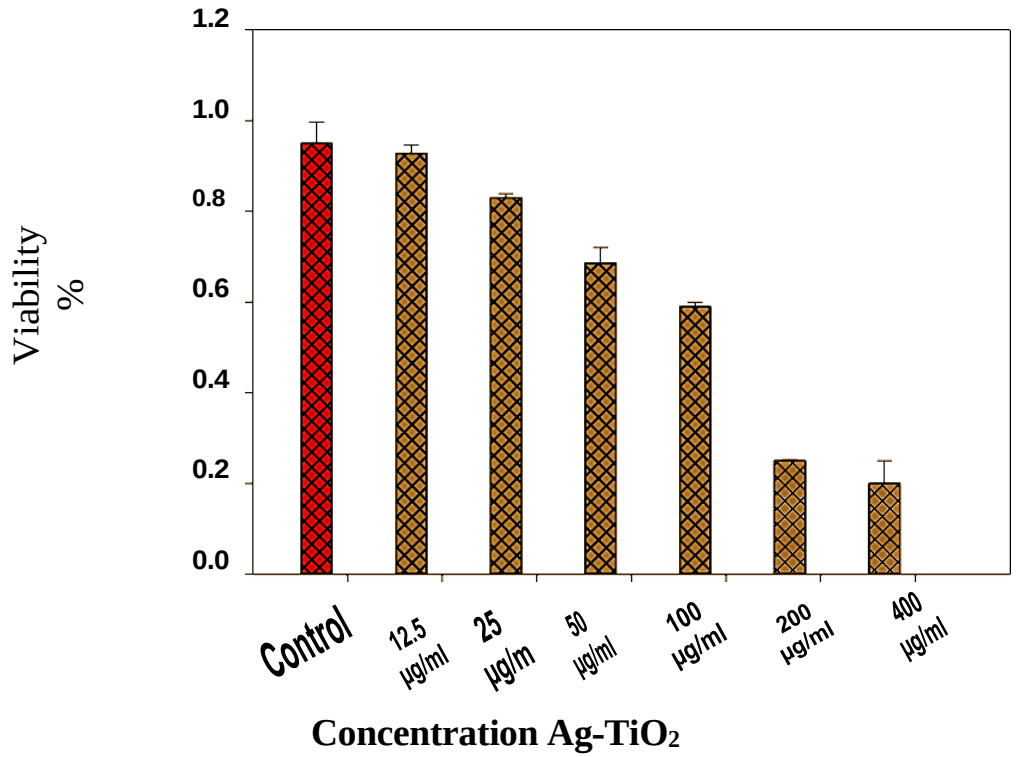


Figure (3-2) effect of AgTIO$_2$ on bacterial growth after 24h incubation

Different modes of action of the nanoparticles on bacteria have been described. One of them is said to be by oxidative damage on the cell membrane of the organism. Another mode of action described is an alteration of the Coenzyme A dependent enzymes and their activity , while damage to the DNA by means of hydroxyl radicals have also been reported to be one of the causes(Mantravadi *et al.*,2017)

Ag-TiO$_2$ nanoparticles has been discussed to be a solution to antibiotics resistance problem. It also can destroy microbes and bioaerosols (dust mites, mold spores) by disintegrating their DNA. Silver nanoparticles may directly attach to and penetrate the cell membrane to kill spores, although penetration of silver nanoparticles into microbial cell membranes is not completely understood. The use of the low-cost photocatalyst and the possibility of its activating with solar light offer economically reasonable and environmentally friendly solutions to the disinfection

process, development of self-cleaning materials and the protection of technical materials from biodeteriorating microorganism (Ahmad Barudin *et al.,* 2013).

Several mechanisms have been reported for the antimicrobial property of silver doped TiO_2 nanoparticles ,The first step is that the adhesion of nanoparticles to the cell surface rated by the TiO_2 photocatalyst attacks the outer membrane. The second step is the effective uptake of the silver ions into the cytoplasmic membrane, degrade lipoolysaccharide molecules, accumulate inside the membrane and cause membrane permeability ,Silver particles penetrating inside bacterial cell, thus damage the DNA Dissolution of silver releases antimicrobial Ag ions .In this case, the TiO_2 photocatalytic reaction assists the intrusion of the silver ions into the cell (Ahmad Barudin *et al.,* 2013).

The main mechanism of TiO_2 NPs toxicity is potentially associated with metal oxides carries the positive charge even though the microorganisms bear negative charges; this results in electromagnetic interaction between microorganisms and metal oxides leading to oxidation and finally death of microorganisms. Bactericidal action of TiO_2 nanoparticles on bacteria is of extreme importance due to the ability of pathogenic bacteria to join the food chain of the ecosystem . The antibacterial activity of TiO_2NPs was due to the capability of TiO_2 particles to cause free hydroxyl radicals (OH) (Hamza and Yaaqoob, 2020)

(Ahmad Barudin et al., 2013) found that the antibacterial activity of TiO_2 nanoparticle solution was tested against *E. coli* under visiblelight irradiation. Bare TiO_2 significantly has a weak antibacterial activity while Ag loading to TiO_2 matrix lead increase in inhibition area where it diffuses radially outward through the agar, producing zone of inhibition. However

increasing the silver concentration in TiO_2 showed not much effect towards zone of inhibition. Optimum amount of silver was needed to rapidly trap electron.

In a study by (Sangchay W *et al.*, 2013) silver doped TiO_2 thin films eliminated *E. coli* much faster than TiO_2 alone even under UV radiation. These results were in accordance to our study, where TiO_2 doped with Ag were much more effective than only pure TiO_2. With increasing concentrations of silver, the antibacterial effect was faster.

3.4 Multiple-Locus Variable-number tandem-repeat Analysis (MLVA) for *Escherichia coli* Isolate Genotyping

Every single *E. coli* isolate underwent MLVA. A total of seven VNTR loci were used to genetically type *E. coli* isolates. The results of the PCR were electrophoresed on a 1.5% gel. The MLVR was able to detect allele Profiles.

The (22) *E. coli* strains' amplicon size distributions for each VNTR were as follows: 300-400 bp for **Ms06**, 380-800 bp for **Ms07**, 400-1400 bp for **Ms09**, 700-950 bp for **Ms11**, 200-1500 bp for **Ms21**, 550-1000 bp for **Ms23**, and 350-800 bp for **Ms32** as shown in figure (3-3).

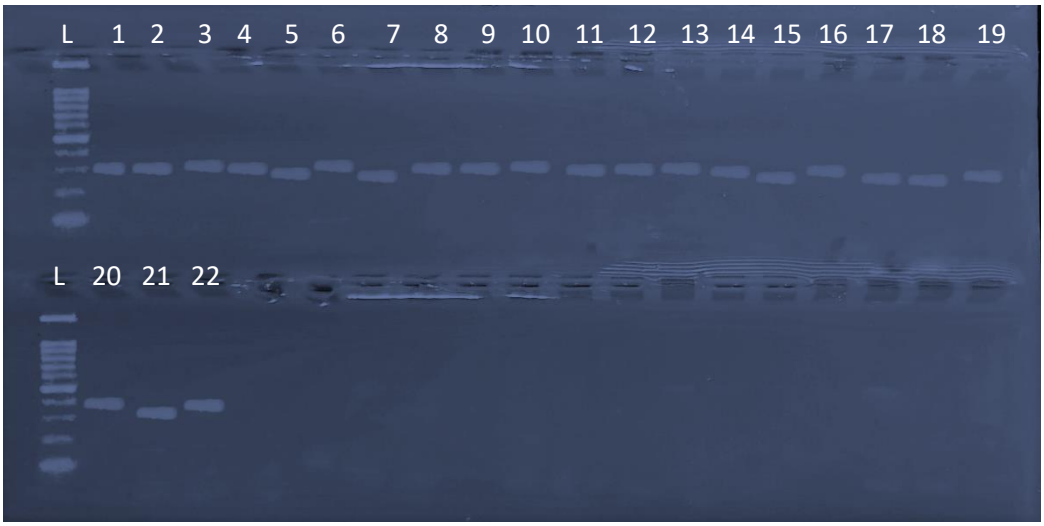


figure 3-3 A: MS6 Electrophoresis of genomic *E. coli* isolates DNA. Running conditions: Agarose gel 1.5%, 75 volt for 1 hrs, stained with ethidium bromide dye and visualized under UV transilluminator documentation ladder (100-1500) bp.

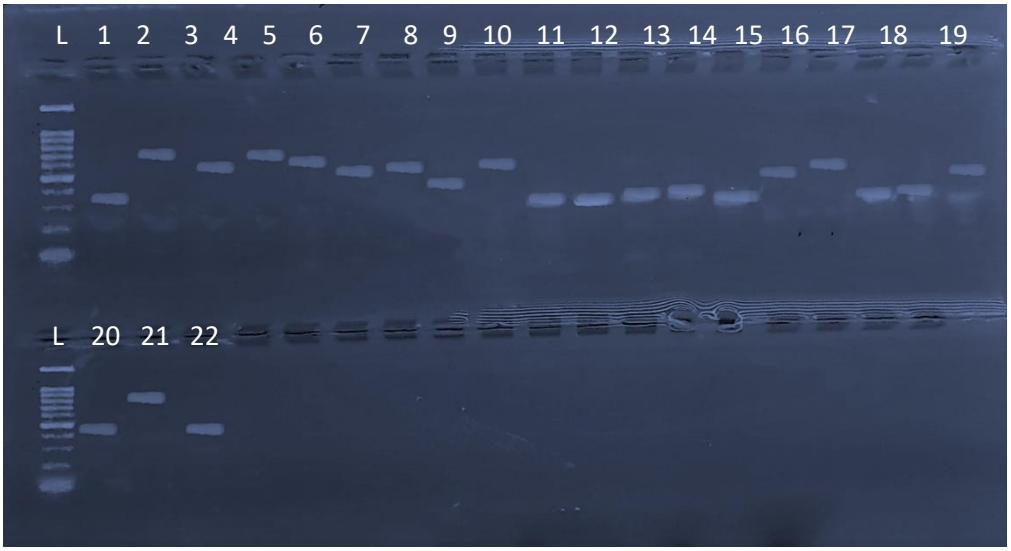


figure 3-3 B: MS7 Electrophoresis of genomic *E. coli* isolates DNA. Running conditions: Agarose gel 1.5%, 75 volt for 1 hrs, stained with ethidium bromide dye and visualized under UV transilluminator documentation.

ladder (100-1500) bp.

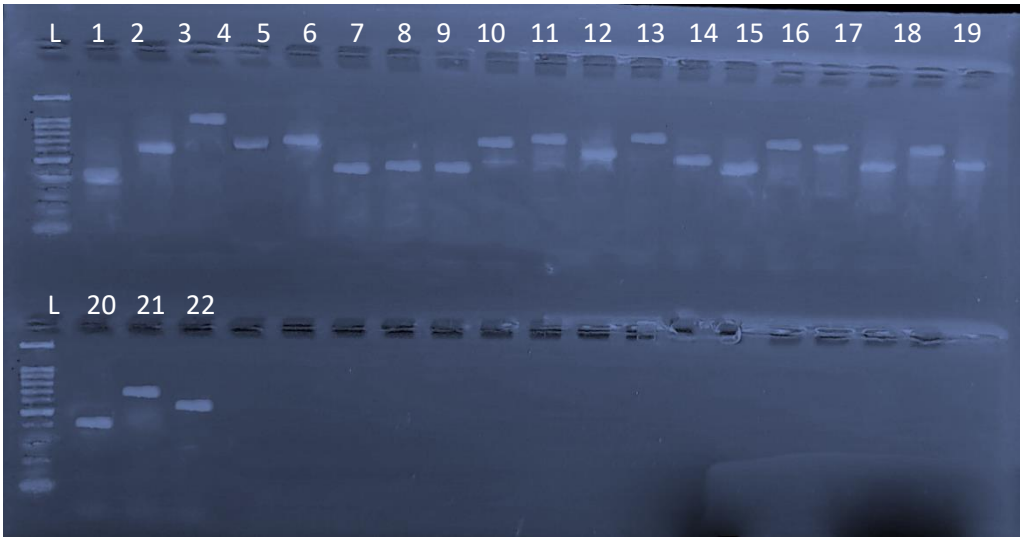


figure 3-3 C: MS9 Electrophoresis of genomic *E. coli* isolates DNA. Running conditions: Agarose gel 1.5%, 75 volt for 1 hrs, stained with ethidium bromide dye and visualized under UV transilluminator documentation.

ladder (100-1500) bp.

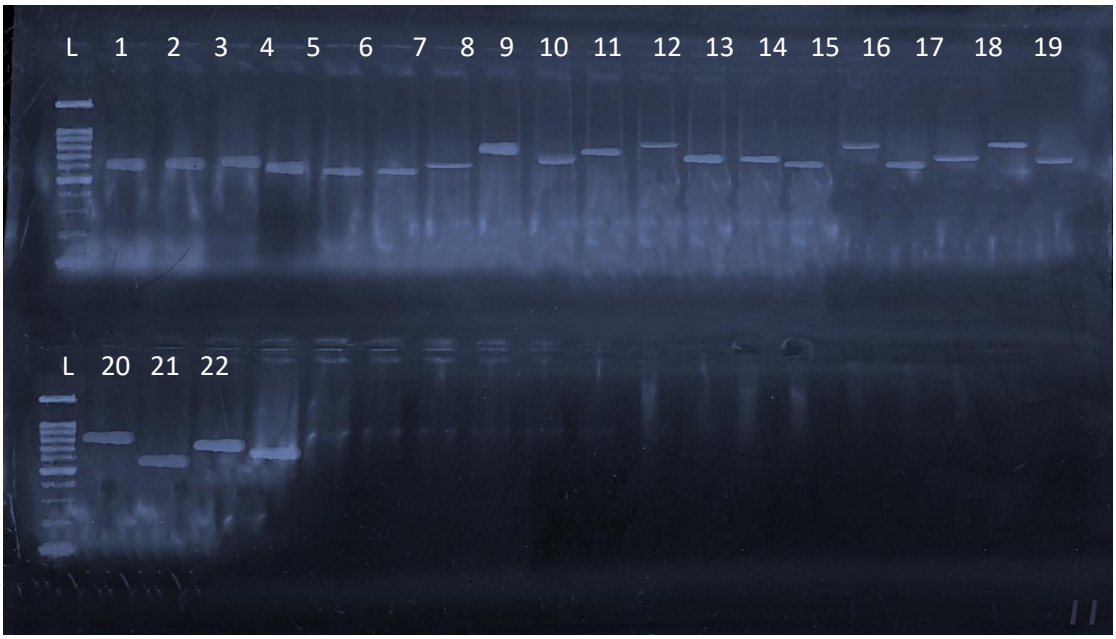


figure 3-3 D:MS11 Electrophoresis of genomic *E. coli* isolates DNA. Running conditions: Agarose gel 1.5%, 75 volt for 1 hrs, stained with ethidium bromide dye and visualized under UV transilluminator documentation.

ladder (100-1500) bp.

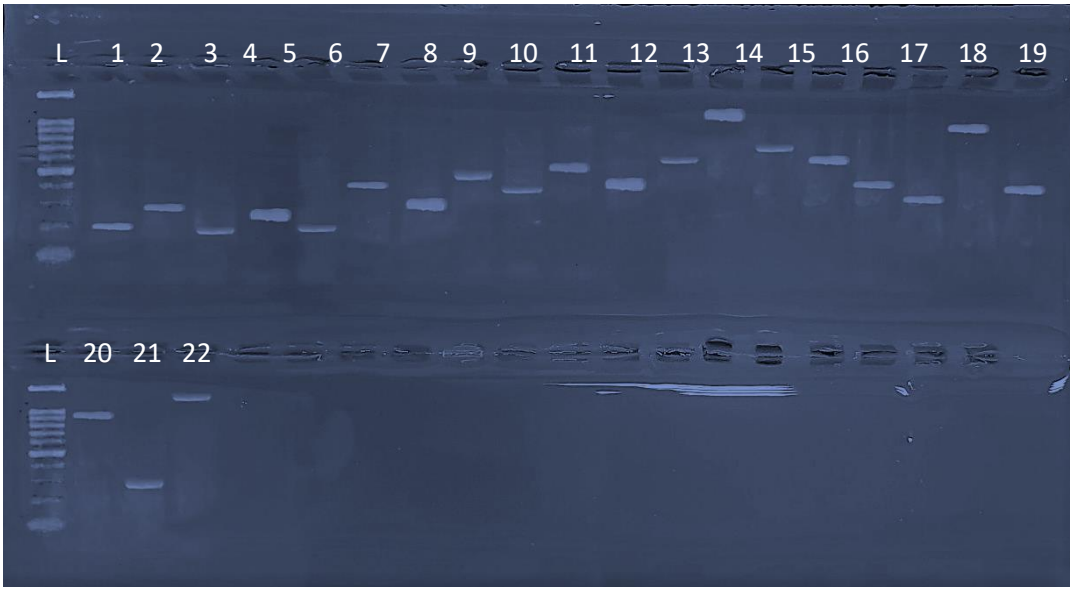


figure 3-3 E: MS21 Electrophoresis of genomic *E. coli* isolates DNA. Running conditions: Agarose gel 1.5%, 75 volt for 1 hrs, stained with ethidium bromide dye and visualized under UV transilluminator documentation. ladder (100-1500) bp.

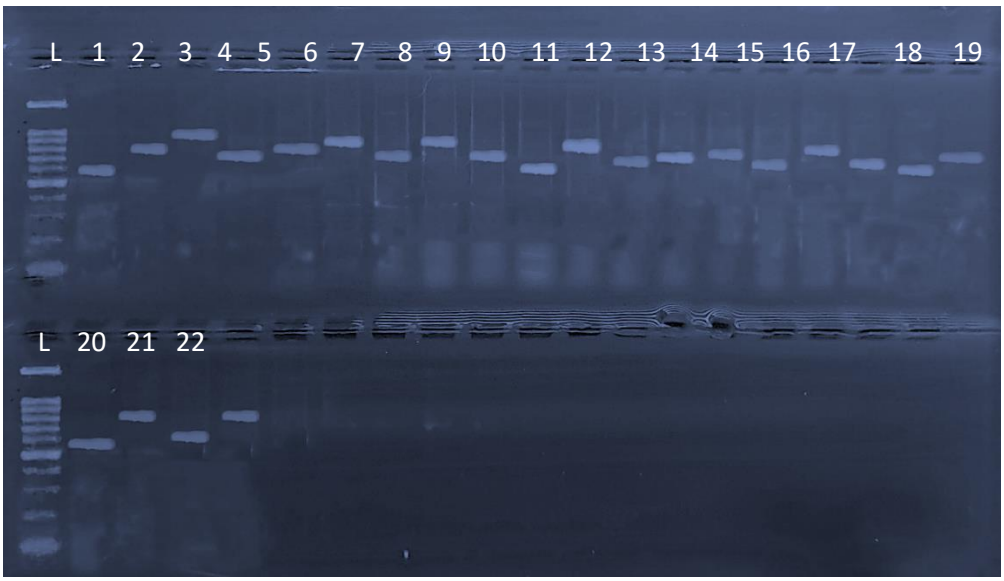


figure 3-3 F:MS23 Electrophoresis of genomic *E. coli* isolates DNA. Running conditions: Agarose gel 1.5%, 75 volt for 1 hrs, stained with ethidium bromide dye and visualized under UV transilluminator documentation. ladder (100-1500) bp.

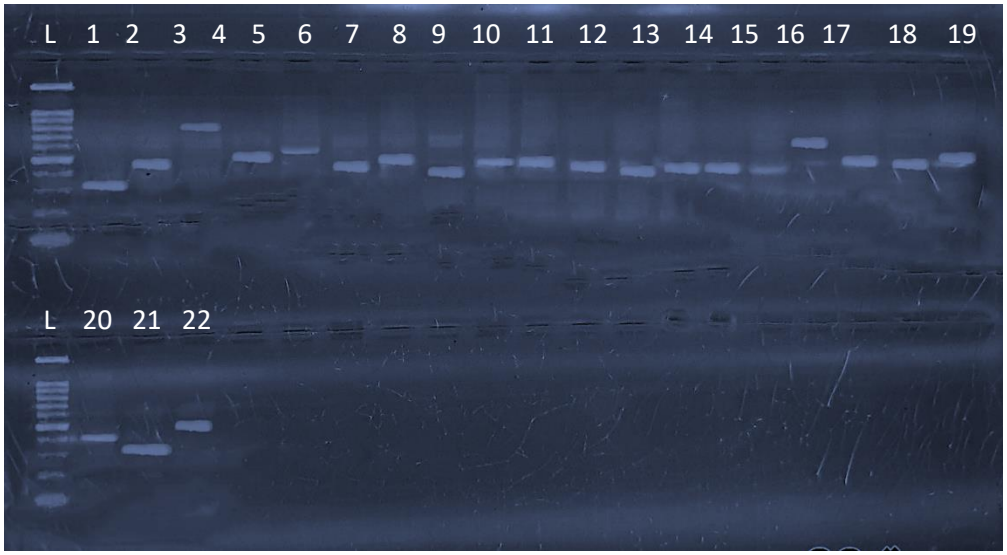
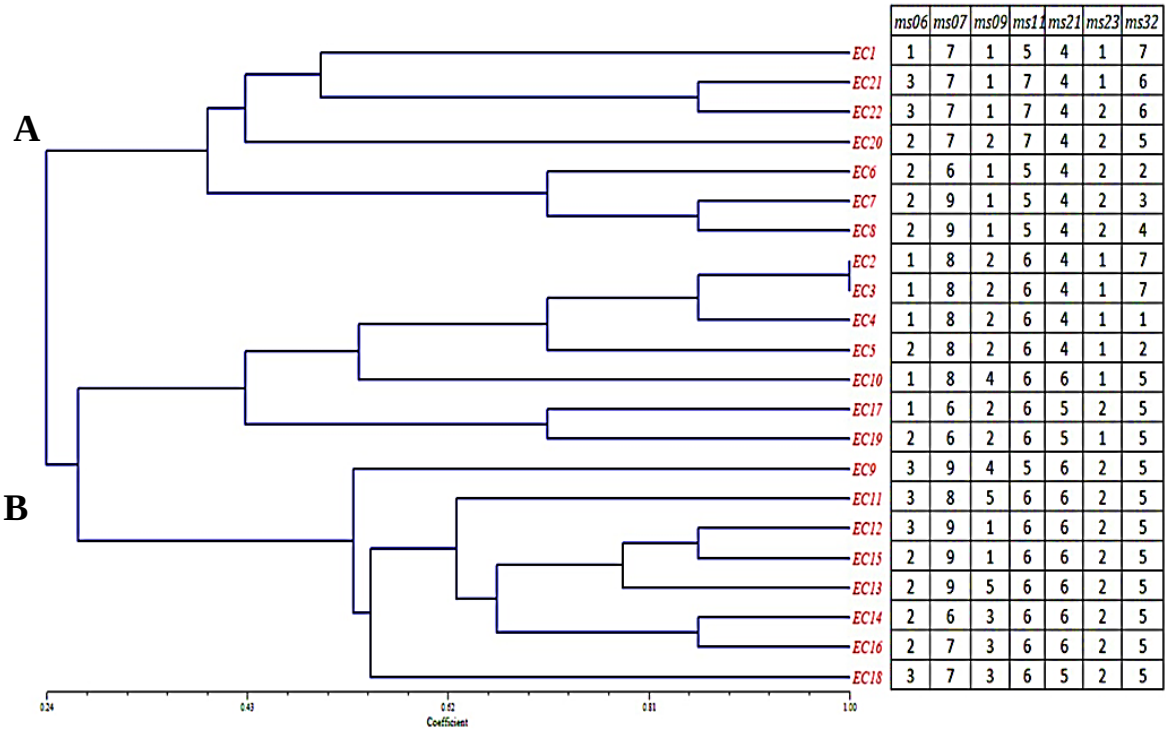


figure 3-3 G: MS32 Electrophoresis of genomic *E. coli* isolates DNA. Running conditions: Agarose gel 1.5%, 75 volt for 1 hrs, stained with ethidium bromide dye and visualized under UV transilluminator documentation. ladder (100-1500) bp.

All *E. coli* strains were categorized into 21 different MLVR types with 2 clusters, according on an analysis of MLVR profiles as shown in figure (3-6).



	ms06	ms07	ms09	ms11	ms21	ms23	ms32
EC1	1	7	1	5	4	1	7
EC21	3	7	1	7	4	1	6
EC22	3	7	1	7	4	2	6
EC20	2	7	2	7	4	2	5
EC6	2	6	1	5	4	2	2
EC7	2	9	1	5	4	2	3
EC8	2	9	1	5	4	2	4
EC2	1	8	2	6	4	1	7
EC3	1	8	2	6	4	1	7
EC4	1	8	2	6	4	1	1
EC5	2	8	2	6	4	1	2
EC10	1	8	4	6	6	1	5
EC17	1	6	2	6	5	2	5
EC19	2	6	2	6	5	1	5
EC9	3	9	4	5	6	2	5
EC11	3	8	5	6	6	2	5
EC12	3	9	1	6	6	2	5
EC15	2	9	1	6	6	2	5
EC13	2	9	5	6	6	2	5
EC14	2	6	3	6	6	2	5
EC16	2	7	3	6	6	2	5
EC18	3	7	3	6	5	2	5

Figure (3-4): UPGMA dendrogram according to the similarity coefficient values obtained from MLVA different patterns.

The study data also demonstrate that cluster A was split into two subclusters. Subcluster 1 has four isolates, while Subcluster 2 has three isolates. While cluster B was split into two subclusters, the first of which contains seven isolates, while second Subcluster has eight isolates.

It was found that isolates No. 2 and No. 3 were 100% identical within this subcluster. indicated both share the same MLVA type (M2) as shown in figure (3-4) .

The results of this study provided data to accurately measure the variety of seven TR loci and identify the various alleles. MS 09 and MS 32 had the highest levels of diversity because they were the most polymorphic loci, whereas MS 23 had the lowest levels.

The MLVA approach offers data in the form of code that can be stored in a database and shared between several isolates. Additionally,

these methods made it possible to establish a correlation between genotypes and variables like virulence factors (Sheikh *et al.*,2020).

The disparity of the *Escherichia coli* isolates found in the current investigation may be caused by the horizontal transfer of "mobile genetic elements".

Farahani (*et al.*,2020) demonstrate that the MLVA analysis utilized for bacterial stream identification has a substantially greater discrimination power than MLST (Farahani *et al.*, 2020).

Through PCR amplification and consequent fragment analysis of targeted regions, MLVA is utilized to create a DNA fingerprint of abacterial isolates (Helldal *et al.*, 2017).

According to Farahani (*et al.*,2020), the MLVA method could identify 56 distinct genotypes among 70 *E. coli* bacterial .

It can be difficult to determine the associations between isolates because of the large allele diversity in isolate genotypes, which also revealed that the MS 32 locus has the most alleles. Whereas the MS 06 and MS 23 loci have the fewest alleles.

When attempting to predict the associations between isolates, the VNTR loci can present challenges. Detecting the frequency of VNTR mutations and its contributing factors is also important, and can present the MLVA for study on legal epidemiology (Naseer *et al.*, 2012).

3.5 Gene expression of TLR4 by using real-time PCR

A total from 22 stool patients samples , positive to *E.coli* . RNA was extracted to study gene expression of TLR4 by using the real time - PCR Relative gene expression (2 ddct) methods . and 16 stool samples Were collected from healthy as control groups. in this method the level of expression of TLR4 gene in test samples as well in Control Samples were

normalized with house - keeping for test samples as shown in figure (3-5)and table (3-5) , (3-6)

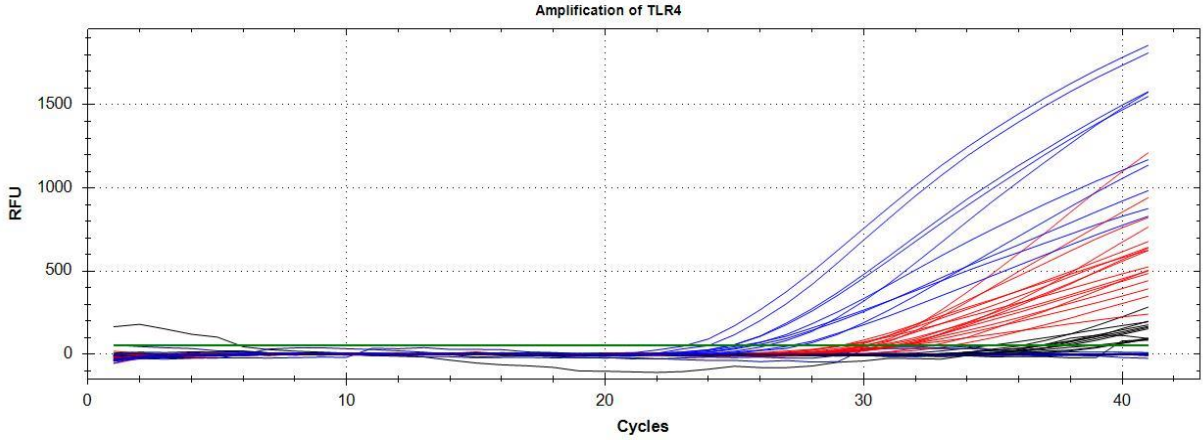


Figure (3-5): Gene expression level of TLR4. This is the first run for 15 samples, Blue lines represent amplification of Reference gene (GAPDH), Red lines represent amplification of samples for Patients, Black lines represent amplification of control samples.

RFU=relative fluorescence units.

Table (3-5): TLR4 Fold Gene Expression among Control and Patients versus the reference gene (GAPDH).

Groups	N	Expression levels (2^-(ΔΔCt))		
		Mean	SD	SE
Control	16	1.09	0.47	0.12
Patient	22	7.89	1.46	0.31
P value		<0.0001 *		

* Represent a significant difference at $p \leq 0.05$.

Table (3-6) : Distribution of Ct values between Control and Patients versus the reference gene (GAPDH).

Groups	N	Ct		
		Mean	SD	SE
Control	16	36.74	0.88	0.22
Patient	22	33.22	0.51	0.11
P value		<0.0001 *		

* Represent a significant difference at $p \leq 0.05$.

In the present study, the results showed that the expression of the TLR4 gene was very high in the samples of the patients compared with the control group. so the expression of gene is increase in Comparison with control set more than 7 Fold in compared with control as shown in figure (3-6)

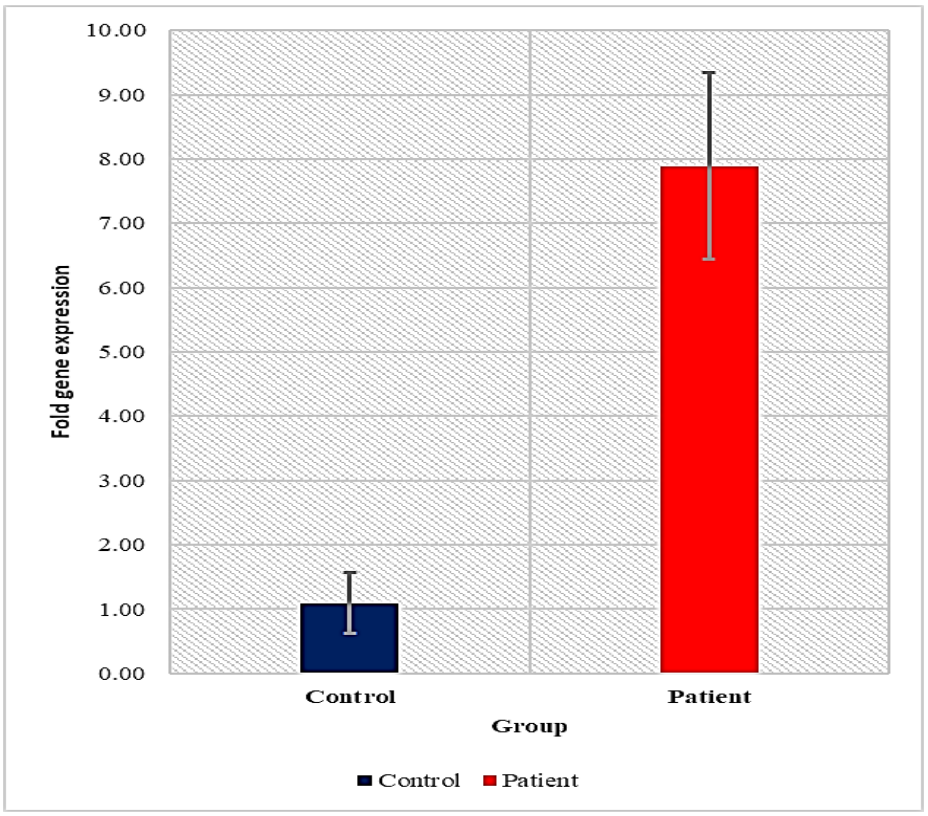


Figure (3-6): TLR4 fold Gene Expression among Control and Patients versus the reference gene (GAPDH)

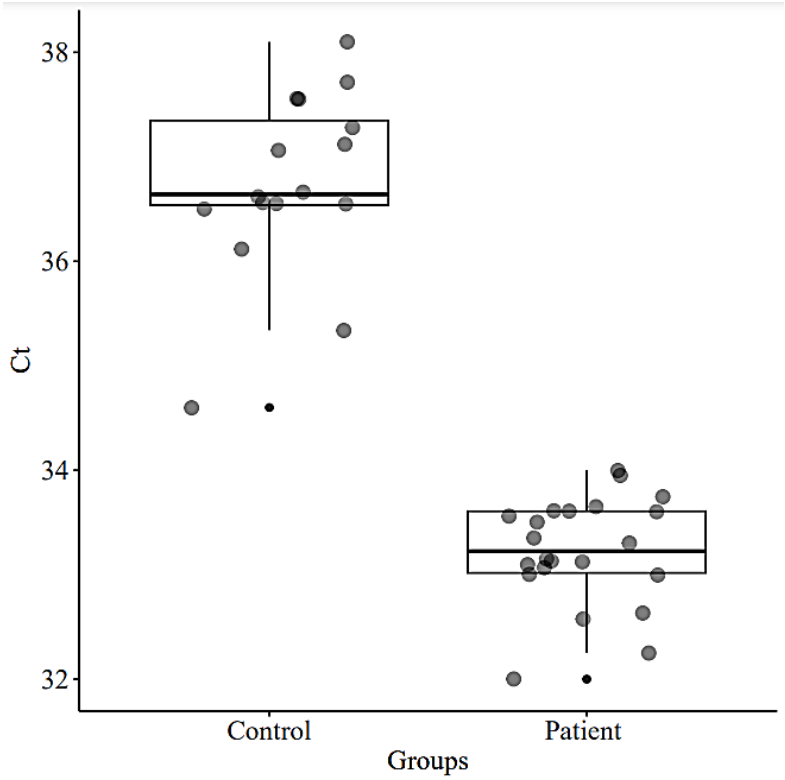


Figure (3-7): Boxplot represent the distribution of Ct values between Control and Patients versus the reference gene (GAPDH).

TLR-4 recognizes lipopolysaccharide in the cell wall of gram negative bacteria, So the expression of TLR4 Varies by region of the gut and is determined primarily by the bacterial composition of the region (Dheer *et al.*, 2016).

Also refer that the expression of TLR**-** 4 increased in epithelial and lamina propria in inflammatory bowel disease patients. (Abreu *et al.*, 2010)

The TLR signaling pathway plays an important regulatory role in autoimmune diseases ,viral infection and inflammatory bowel disease , . The TLR4 signaling cascade is a complex network of protein interactions, and the potential variances of reaction across distinct developmental stages of swine have yet to be fully elucidated. The alterations in the gene

expression levels within the TLR4 signaling pathway during immune system responses have been previously documented (Jalanka *et al.,* 2021).

Found that the TLR4 were significantly increased in diarrhea – predominant irritable bowel Compared to healthy controls group, and increased mucosal expression of TLR4 (4-5 fold). (Jalanka *et al.*,2021)

Study found the increased TLR4 expression was associated with increased days with diarrhea, and also expression of TLR4 correlated with Stool frequency (McKernan *et al.*,2009) .

Reported that a significant increase in TLR4 mRNA and protein expression compared with the healthy group, this may be due to the increased innate immune cell line expressing TLR 4. (Tam *et al.*,2021)

Other studies have observed an increase in the expression of TLR4 in pathogenic *E. coli* and a decrease in the beneficial gut microbiota (*bifidobacterium* spp and *lactobacillus* spp.) (Dong *et al.*, 2012).

the expression of TLR4 was significantly up regulated in inflammatory bowel disease and the expression fold increase than the control group, also it is refer that TLR4 considered to be a type of substance of intestinal immune response which role in maintaining intestinal membrane homeostasis (Wang *et al.*, 2022).

Conclusion

Conclusions

1- *E.coli* are considered as one of the major causative agent of diarrheal infection

2- The highest percent of *E.coli* was found between the ages one day to one year.

3- The isolate of *E.coli* were multidrug resistance to most of commonly used antibiotics.

4- It was found that there was a highly significant inhibition in growth of bacteria after exposure to the different dilution of nanoparticles $AgTIO_{2.}$

5- Analysis of MLVA profile showed that all *E.coli* isolates categorized into 21 different MLVR types with 2 clusters.

6- It was found that the expression of TLR4 was significantly increased in patients with diarrheal infection associated with *E.coli* when compared with control group. as risk factors for diarrheal infections.

Recommendations

Recommendations

1- future studies should carefully compare MLVA with older genotyping techniques such as PFGE that is the gold standard genotyping method

2- study the combination of nanoparticales and antibiotics against resistant strains.

3- Study the role of others, specific pro and anti-inflammatory cytokines levels and their gene polymorphism, which may have impact on the disease management

4- study provided new data on the complexity and diversity of genetic features associated with the antibiotics resistance genes, which are of great importance for further analyses of the dissemination routes and mechanisms

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Appendixes

Appendixes.....

Patient Name: 5 — Patient ID: 3320221
Location: — Physician:
Lab ID: 3320221 — Isolate Number: 1

Organism Quantity:
Selected Organism : Escherichia coli

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:** 4.35 hours	**Status:** Final
Selected Organism	99% Probability **Escherichia coli**	
	Bionumber: 0405610550526610	
ID Analysis Messages		

Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	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	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Appendixes.....

Patient Name: 20 — Patient ID: 3320222
Location: — Physician:
Lab ID: 3320222 — Isolate Number: 1

Organism Quantity:
Selected Organism : Escherichia coli

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	4.85 hours	**Status:** Final
Selected Organism	97% Probability	**Escherichia coli**	
	Bionumber:	0405610540560610	
ID Analysis Messages			

Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	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	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Patient Name: 26
Location:
Lab ID: 3320223

Patient ID: 3320223
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Escherichia coli

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	4.37 hours	**Status:**	Final
Selected Organism	98% Probability **Bionumber:**	**Escherichia coli** 0405610570526600		
ID Analysis Messages				

Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	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	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	-	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Patient Name: 54
Location:
Lab ID: 3320224

Patient ID: 3320224
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Escherichia coli

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	4.87 hours	**Status:**	Final
Selected Organism	96% Probability	**Escherichia coli**		
	Bionumber:	0405610570526600		
ID Analysis Messages				

Biochemical Details																
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	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	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	-	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

الخلاصة :ـ

تم جمع ما مجموعه (150) عينة براز من مرضى مصابين بعدوى الإسهال ، أشارت النتائج إلى أن 22 (14.7٪) من العزلات تنتمي إلى الإشريكية القولونية ، وكشفت عن عزلات بكتيرية أخرى بنسبة (50٪) بينما لم يتم اكتشاف نمو في (35.3٪). تم تحديد هذه العزلات وفقا لخصائص الاستزراع والأنظمة المورفولوجية والبيوكيميائية والفسيولوجية و.2- VITEK

تم دراسة توزيع العزلات حسب العمر ووجد أن (50٪) من العينات تعود لمرضى تتراوح أعمارهم بين يوم واحد إلى سنة واحدة ، و (22.7٪) تنتمي لمرضى يتراوح متوسط أعمارهم بين (1-10) سنوات ، و (9٪) من العينات تعود لمرضى تتراوح أعمارهم بين (11-20) سنة ، و (13.8٪) من العينات تعود لمرضى بمتوسط عمر (21-30) سنة. بينما تم تسجيل (1٪) من العينات لمرضى تزيد أعمارهم عن 30 سنة.

أظهر اختبار الحساسية للمضادات الحيوية لعزلات الإشريكية القولونية أن جميع العزلات ال 22 كانت مقاومة بنسبة 100٪ للمضادات الحيوية الثلاثة (أمبيسلين ، سيفوتاكسيم ، سيفترياكسون) بينما أظهرت النتائج أقل مقاومة للمضادات الحيوية جنتاميسين 9٪ ونورفلوكساسين 13.6٪.

تم إجراء MLVA لجميع عزلات الإشريكية القولونية عندما تم اختيار سبعة مواقع VNTR (سبعة مواقع عناصر متكررة) للتنميط الجيني للإشريكية القولونية.

تم إجراء تفاعل البوليميراز المتسلسل و تم إجراء الترحيل الكهربائي على (1.5) من هلام الأغاروز المحتوي على بروميد الإيثيديوم وتم الكشف عن ملف الأليل بواسطة MLVA أظهر تحليل ملف MLVA أن جميع عزلات الإشريكية القولونية تم تجميعها في (21) نوع MLVA متميز وتنقسم الى المجموعتين (A) و.(B)

كما تمت دراسة التعبير الجيني TLR4 mRNA بواسطة تفاعل البوليميراز المتسلسل الكمي في الوقت الحقيقي (التعبير الجيني النسبي) في المرضى الذين يعانون من الإسهال وأظهرت النتائج أن TLR4 كان يزداد في مرضى الإسهال الذين يعانون من الإشريكية القولونية عند مقارنته بالمجموعات السيطرة. يتم زيادة تعبير TLR4 أكثر من (٪ 30) أضعاف عند مقارنتها مع مجموعة السيطرة.

كما تم دراسة أن أعلى تركيز ل $Ag\ Tio_2$ بعد تخفيفه لمدة (2/1) حقق أعلى تأثير تثبيطي لنمو البكتيريا وهذا التثبيط قلل من نمو البكتيريا من (1.36) إلى (0.5) بعد 24 ساعة من الحضانة بينما بعد 48 ساعة من الحضانة انخفض نمو البكتيريا من 1.29 إلى 0.22. في حين أن أقل تركيز ل $Ag\ Tio_2$ الذي صنعته الجسيمات النانوية المخففة ل (32/1) أظهر أقل تأثير تثبيطي على نمو الإشريكية القولونية بعد 24 و 48 ساعة من الحضانة.

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وزارة التعليم العالي
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كلية الطب

دراسة النشاة التطورية باستخدام مواقع متغايرة متعددة التكرارات لبكتريا الإشريكية القولونية المعزولة من حالات الاسهال

رسالة

مقدمة الى مجلس كلية الطب / جامعة بابل

كجزء من متطلبات نيل درجة الماجستير في العلوم / الأحياء المجهرية الطبية

من قبل

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بأشراف

الاستاذ

د. ميساء صالح مهدي الشكري

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