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Occurrence and Molecular Characterization of Enteropathogenic *Escherichia coli* (EPEC) Serotypes Isolated from Children with Diarrhea in Najaf

A Thesis

Submitted to the Council of College of Medicine, University of Kufa in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology

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بسم الله الرحمن الرحيم

(وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللهِ عَلَيْكَ عَظِيمًا)

صدق الله العلي العظيم

سورة النساء ١١٣

Dedication

To....

The memory of My father

The kind heart My mother

My dear wife

My lovely daughters
Zahraa, Zainab and Fatema

All members of my family

Samer

I certify that this thesis was prepared under my supervision at the College of Medicine, University of Kufa, in partial Fulfillment for the requirement for the degree of Master of Science in Medical Microbiology /Bacteriology.

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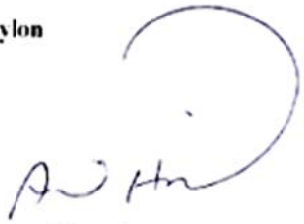

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الموسومة بـ (معدل الانتشار والنمط الجزيئي للأنماط المصلية لبكتريا إشيريشيا القولون المعوية الممرضة (EPEC) المعزولة من الأطفال المصابين بالإسهال في العراق).

وقومتها لغويا وأجد انها صالحة للمناقشة .

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Summery

Diarrhea continues to be one of the most common causes of morbidity and mortality among infants and children in developing countries. Enteropathogenic *Escherichia coli* (EPEC) are among the most important pathogens infecting children worldwide and are one of the main causes of diarrhea.

The aims of this study were to investigate the occurrence of EPEC as a cause of infectious diarrhea in children younger than 2 years of age, characterize their virulence genes, and determine the antibiotics susceptibility.

During the study period, *E. coli* isolates were serotypically identified with EPEC polyvalent and monovalent antisera. The isolated EPEC were examined for the presence of the attaching and effacing (*eaeA*), bundle-forming pilus (*bfpA*), Shiga like toxins (stx_1 and stx_2), enterohemolysin (EHEC *hlyA*), EAF plasmid, and β-lactams antibiotics resistance genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{PER}, bla_{VEB}, bla_{OXA}, bla_{GES}, bla_{AmpC}, bla_{VIM}, and bla_{IMP}) by PCR assay, in addition to determining the antibiotics susceptibility phenotypes.

A total of 656 fecal specimens from children with diarrhea and 54 from healthy children were analyzed. The study has shown that 22/656 children (3.35%) had diarrhea due to EPEC, while no EPEC isolates were detected in asymptomatic children. The highest number of the EPEC isolated belonging to polyvalent 2 were with following serotypes, O55:k59(B5) (22.7%), O111:k58(B4) (18.18%), O26:k60(B6) (13.63%), followed by polyvalent 3 with the following serotypes, O125:k70(B15) (9.09%), O128:k67(B12) (4. 54%), O127:k63(B8) (4.54%), O114:k90(B) (4. 54%) followed by polyvalent 4 with serotype O44:k74(L) (13.63%). The primers encoding virulence genes were tested against to all the 22 serotyped EPEC isolates. Only 2 (9.1%) isolates gave positive results

with an *eaeA* gene, 6 (27.27%) isolates gave positive results with a *bfpA* gene, and 2 (9%) isolates gave positive results with *EAF* gene. None of the EPEC isolates were positive for stx_1, stx_2 or enterohemorrhagic *E. coli* *hlyA* genes. EPEC isolates were classified as typical ($eaeA^+$, $bfpA^+$) or atypical ($eaeA^+$, $bfpA^-$). Typical EPEC was diagnosed in 2 (33.3%) isolates belonging to O125:k70(B15) serotype. While, atypical EPEC isolates (4, 66.7%) were belonging to O111:k58(B4) (2 isolates), O44:k74(L) (1 isolate) and O128:k67(B12) (1 isolate) serotypes.

Out of the 20 of antibiotics used, 20 (90.9%) isolates were multidrug resistance. None of the isolates were found to be resistant to imipenem, meropenem, amikacin, ciprofloxacin and levofloxacin.

All isolates have been found resistant to at least one β-lactams antibiotics, hence, the isolates were tested for detecting their ability to produce extended-spectrum β-lactamases using double-disk synergy test; the results revealed that 3 (13.6%) isolates were able to produce ESBLs. While PCR assay revealed that, 18 (81.8%), 22 (100%), 17 (77.3%), and 1 (4.54%) of the isolates carried bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{OXA} genes, respectively. AmpC β-lactamase production was detected (by modified three-dimensional and AmpC disk tests) in 4 (18.2%) isolates. The AmpC enzyme production was confirmed in only two (9%) isolates by PCR technique.

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List of Abbreviations

A/E	Attaching and Effacing
AA	Aggregative Adherence
AIDA	Adhesin Involved in Diffuse Adherence
AmpC	Molecular Class C β-lactamases
BFP	Bundle-Forming Pilus
bfpA	Bundle-Forming Pilus Gene
bla gene	β-lactamase Gene
bp	Base Pair
CDTs	Cytolethal Distending Toxins
CT	Cholera Toxin
CTX-M	Cefotaximase, B-Lactamase Active On Cefotaxime
DAEC	Diffusely Adherence *E. coli*
DDST	Double-Disk Synergy Test
eaeA	Intimin Gene
EAF	EPEC Adherence Factor Plasmid
EAggEC	Enteroaggregative *E. coli*
EAST-1	Heat-Stable Toxin-1
EDTA	Ethylene Diamine Tetra Acetic Acid
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
En	Isolates Codes
EPEC	Enteropathogenic *E. coli*
ESBL	Extended Spectrum β-lactamases

Esp	*E. coli*-Secreted Proteins
ETEC	Enterotoxigenic *E. coli*
F	Female
GES	Guiana Extended Spectrum β-lactamase
HC	Hemorrhagic Colitis
hlyA	Enterohemolysin Gene
HUS	Hemolytic–Uremic Syndrome
IEC	Intestinal Epithelial Cells
IMP	β-lactamase Active on Imipenem
LA	Local Adherence Pattern
LEE	Locus of Enterocyte Effacement
ler	LEE-Encoded Regulator
LT	Heat-Labile
M	Male
MTDT	Modified Three-Dimensional Test
NC	Needle Complex
OXA	Oxacillinases, β-lactamase Active on Oxacillin
PCR	Polymerase Chain Reaction
PER	Pseudomonas Extended Resistant and also The Initials of Its Discoverers: Patric, Esthel, and Roger
Per	Plasmid-Encoded Regulator
Pet	Plasmid-Encoded Toxin
RD	Resistance/Nodulation/Cell Division
SDS	Sodium Dodecyl Sulfate

SET buffer	Salt-EDTA-Tris Buffer
SHV	Sulfhydryl Variable β-lactamase
SLT, Stx	Shiga-Like Toxins
ST	Heat-Stable
STEC	Shiga toxin-producing *E. coli*
stx_1	Shiga-Like Toxins I Gene
stx_2	Shiga-Like Toxins II Gene
TBE buffer	Tris-Borate-EDTA Buffer
TE buffer	Tris EDTA Buffer
TEM	β-lactamase Named After First Patient Isolated From (Temarina)
Tir	Translocated Intimin Receptor
Tris	2-Amino-2-Hydroxymethylpropane-1,3-Diol
Tris-OH	Tris-(Hydroxymethyl) Methylamine
TSI	Triple Sugar Iron Test
TTSS	Type III Secretion System
UT	Untypeable
VEB	Vietnam Extended-Spectrum β-lactamase
VIM	Verona Integron-Encoded Metallo-β-lactamases

Chapter One

Introduction

Introduction

Diarrhea are abnormal fecal discharge characterized by frequent and/or fluid stool; usually resulting from disease of the small intestine and involving increase fluid and electrolyte loss (Mims *et al*., 2008). The causes of diarrhea include a wide range of viruses, bacteria, and parasites. Among the bacterial pathogens, *E. coli* plays an important role. Six different categories of *E. coli* may cause diarrhea worldwide: enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper 1998). EPEC is a major cause of infantile diarrhea among children in developing countries (Jong-Hyun *et al*., 2009). The central mechanism of EPEC pathogenesis is a lesion called 'attaching and effacing' (A/E), which is characterized by intimate adherence of bacteria to the intestinal epithelium (Khan *et al*., 2008). The *eaeA* gene, which is located in the 'locus of enterocyte effacement' pathogenicity island (LEE), and the *bfpA* gene, located on a plasmid called the EPEC adherence factor (EAF) have been used for the identification of EPEC and for subdivision of this group of bacteria into typical and atypical strains (Fujihara *et al*., 2009).

There has been an alarming increase in drug-resistant strains of EPEC in developing as well as developed countries. Several cases of antimicrobial resistance in EPEC have been observed in different parts of the world (Subramaniana *et al*., 2009). During the last 2 decades, extended-spectrum β-lactamases (ESBLs) found in Gram-negative bacilli has emerged as a significant mechanism of resistance to oxyimino-cephalosporin and monobactams antibiotics (Paterson *et al*., 2003).

In Iraq, till now, little information are available on the prevalence of EPEC infection. However, in Najaf, there is no molecular identification and categorization of EPEC associated with diarrhea in children younger than two years old, in addition to there is no information regarding the molecular studies of ESBLs or the occurrence of β-lactamases (AmpC and carbapenemases)-producing EPEC isolates. Therefore, there is an increase demand to investigate the role of EPEC in diarrhea in children and identify the prevalence of β-lactam resistance among these isolates. The present study is carried out to achieve the following objectives:

1. Determining the prevalence of EPEC serotypes in children with diarrhea in Najaf.
2. Detecting the virulence genes for EPEC including *bfpA*, *eaeA*, stx_1, stx_2, *EAF* and EHEC *hlyA* by using PCR technique.
3. Attempting to differentiation between typical and atypical EPEC using genotyping properties.
4. Evaluating antibiotic susceptibility for isolated EPEC and detection of extended-spectrum β-lactamase (TEM, SHV, OXA, CTX-M, PER, VEB and GES) and other β-lactamase enzymes (AmpC, VIM and IMP) in isolates using PCR technique.

Chapter Two

Literature Review

2-1 Diarrhea

Diarrheal disease continues to be a health problem worldwide (Alrifai *et al*., 2009). Diarrhea is abnormal fecal discharge characterized by frequent and /or fluid stool, usually resulting from disease of the small intestine and involving increase fluid and electrolyte loss, leading to the production of unformed or liquid feces and can be thought of as the method by which the loss forcibly expels the pathogen (and in doing so, aids its dissemination) (Mims *et al*., 2008). For many individuals, this consists of daily stool production in excess of 250 g, containing 70% to 95% water. Over 14 L/day of fluid may be lost in severe cases of diarrhea, equivalent to circulating blood volume (Robbins *et al*., 2003). Thus, during diarrhea, there is a loss of water and electrolytes (sodium chloride, potassium, and bicarbonates) from the body through the diarrheal stools. Fluid and electrolytes are also lost through sweet, urine, breathing, and by vomiting.

Dehydration occurs when these are not replaced effectively and the body develops a deficit of water and electrolytes (Mohmand, 1996). Incidence of diarrhea may be acute and may last for hours or days, while chronic diarrhea lasts for weeks or months. In case of acute diarrhea, death is most often due to dehydration caused by excessive passage of fluids, water and salts in stools. Severely dehydrated infants are associated with insufficiency of salts like potassium, sodium, beside water. Sodium loss of the body has been estimated to be 80-120 mmol/liter of water loss (Mansourian and Sayers, 1979). Infective diarrhea is a common childhood disease, the etiology of which varies according to geography, climatic conditions, nutritional status, and other circumstances (Mohmand, 1996). The incidence of diarrhea is most common in children, especially those between 6 months and 2 years old

(Hassan 1990). Diarrheal diseases in children cause much more harm than just loss of fluids and electrolytes, as is the general concept. Diarrhea has severe impact on the growth and development of a young child. The effect of causative organism determines whether an episode of diarrhea is dehydration or malabsorption (Molla, 1992).

2-2 *Escherichia coli*

Escherichia coli was first identified by the German pediatrician Theodore Escherich in 1885 during his studies of the intestinal flora of infants. It is a component of the normal intestinal flora of both human and warm-blooded animals. The organism is excreted with the feces and may survive in the environment. However, it appears that there is no independent existence outside the body. Accordingly, *E. coli* is considered an indicator organism for fecal contamination and is an important parameter in food and water hygiene (Brenner *et al*., 2004).

Escherichia spp. form rod-shaped cells of between 2.0 and 6.0 μm in length and 1.1 and 1.5 μm in width with rounded ends (Gross and Holmes, 1990). *E. coli* are usually motile by a set of peritrichous flagella, and also have fimbriae (pili) or fibrillar proteins often extending in great numbers from the bacterial surface and far out into the surrounding medium (Brenner *et al*., 2004). In some strains, the outer membrane of *E. coli* is covered by a polysaccharide capsule composed of K antigens. In other polysaccharides, the M antigens are synthesized under conditions of high osmolarity, low temperature, and low humidity (Schaechter, 2009).

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 antigens are associated with flagella; therefore, only flagellated (motile) bacteria have H antigens. The K antigens are most often associated with the capsule or, less commonly, with the fimbriae (Harvey *et al*., 2007).

Escherichia coli typically produces positive tests for indole, methyl red, and produce acid and gas from glucose, mannitol, maltose, lactose, sucrose, while it gives negative test for Voges-Proskauer, urease test, H_2S production, phenylalanine deaminase test, and gelatin liquefaction (Arora and Arora, 2008).

Escherichia coli are facultatively anaerobic organisms. They are chemo-organotrophic, having both a respiratory and a fermentative type of metabolism, but growth is less plentiful under anaerobic conditions. The optimal growth temperature is 37°C at which they grow well on ordinary media containing 1% peptone as carbon and nitrogen source (Holt and Krieg, 1994).

Escherichia coli strains can cause an impressive variety of different types of disease, including septicemia, pneumonia, meningitis, bladder, and kidney infections, hemolytic–uremic syndrome (HUS), diarrhea, and dysentery. However, different strains that have acquired distinct sets of virulence genes cause different clinical syndromes. This extremely broad spectrum of pathogenicity, the majority of *E. coli* strains have to be considered a virulent pathogen in the intestine (Holt and Krieg, 1994).

Phylogenetic analyses have shown that most *E. coli* strains belong to four main phylogenetic groups, A, B1, B2, and D. Whereas, most commensal and diarrheagenic strains belong to groups A and B1, extraintestinal *E. coli* strains belong mainly to group B2 and group D.

Recent phylogenetic studies indicated that Shiga toxin-producing *E. coli* and enterohemorrhagic *E. coli* strains fell into phylogenetic groups A, B1, and D (Girardeau *et al*., 2005).

2-3 Diarrheagenic *E. coli*

Escherichia coli may cause several different gastrointestinal infection syndromes, based on definitive virulence factors, clinical manifestation, epidemiology, and different O and H serotypes. There are six distinct groups have been defined within gastrointestinal pathogenic *E. coli* commonly associated with intestinal disease: [enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), verotoxigenic *E. coli* (VTEC), enteroaggregative *E. coli* (EAggEC) and the diffusely adherent *E. coli* (DAEC)] (Gillespie and Hawkey, 2006; Mahon *et al*., 2007).

2-3-1 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* is an important category of diarrheagenic *E. coli* which has been linked to infant diarrhea in the developing world. As with other diarrheagenic *E. coli* strains, transmission of EPEC is faecal-oral, with contaminated hands, contaminated foods, or contaminated fomites serving as vehicles. EPEC adhere to the mucosal cells of the small bowel. The result of EPEC infection is watery diarrhea, which is usually self-limited, but can be chronic (Dedeićljubović *et al*., 2009). EPEC strains are noninvasive, infecting their hosts by attaching to intestinal epithelial cells (IEC), effacing the epithelial microvilli, and producing pedestal-like structures. The formation of attaching and effacing (A/E) lesions is required for these microbes to cause diarrheal disease (Khan *et al*., 2008). EPEC possessing both the bundle-forming pilus gene (*bfpA*) and intimin gene (*eae*) for *E. coli*-attaching and effacing. It is a well-recognized pathogen in developing countries as class

I EPEC or typical EPEC. However, atypical EPEC organisms possessing *eae* alone have been reported to be more prevalent in both developing and developed countries, and animals can be reservoirs of atypical EPEC, in contrast to typical EPEC, in which humans are the sole reservoir (Fujihara *et al*., 2009).

2-3-1-1 Pathogenicity of EPEC

2-3-1-1-1 Pathogenicity-associated Genes and Factors

2-3-1-1-1-1 Chromosomal PAI (LEE)

Several genes implicated in attaching and effacing lesion formation are located on large (35.6 kb) PAI termed the locus of enterocyte effacement (LEE) (McDaniel *et al*., 1995). LEE is located on a chromosome and contains more than 40 genes responsible for the production of components of the type III secretory system, a series of effector proteins called *E. coli*-secreted proteins (Esp), the 94 kDa outer membrane adhesin intimin, and the 90 kDa protein translocated intimin receptor (Tir**)** (Schaechter, 2009). LEE is organized into five polycistronic operons (LEE1 to LEE5). The LEE1, LEE2 and LEE3 operons encode the type III secretion system (TTSS) and a global regulator Ler (LEE-encoded regulator). LEE4 encodes TTSS-secreted proteins EspA, EspB and EspD (EPEC-secreted proteins) that are also components of the translocation apparatus by which other effector proteins are translocated into the cell. LEE5 encodes intimin, Tir and the Tir-chaperone CesT (Elliott *et al*., 1998).

a.Type III Secretion System (TTSS)

Type III secretion system is a multiprotein needle-like complex evolutionarily related to the flagella apparatus that comprises more than 20 proteins spanning both the inner and outer membranes of the bacterial

envelope (Badea *et al*, 2009). TTSS is capable of transporting proteins across the cytoplasmic and outer membrane of the bacterial pathogen and the membrane of the host cell where they interfere with host-cell signalling cascades (Gillespie and Hawkey, 2006).

b. *E. coli*-Secreted Proteins (Esp)

Proteins reported to be essential for A/E lesion formation are themselves components of the extracellular translocation apparatus. These include EspA (25 kDa), EspB (38 kDa) and EspD (40 kDa) **(**Buttner and Bonas, 2002). EspA protein is a major component of a filamentous sheath-like structure, sometimes called a needle complex (NC) or EspA filament which is a characteristic feature of TTSS. This NC connects the bacteria to the host cells to form a pore or translocon through which other LEE-encoded effector proteins are secreted. These translocated effector proteins which include Tir, EspF, EspG and MAP (mitochondria-associated protein) perform specific functions or attack specific targets after introduction into and sub-localisation in the host cell (Gillespie and Hawkey, 2006).

c. Intimin

Intimin, outer membrane protein 94-kDa, encoded by the *E. coli* attaching and effacing (*eaeA*) gene in the LEE (Gillespie and Hawkey, 2006), is responsible for the intimate adherence to the enterocyte membranes that triggers the A/E lesion (Caron *et al*., 2006). This protein plays an important role in virulence and host-cell invasion, including activation of signal transduction pathways induced by products of the TTSS and subsequent pedestal formation (Gillespie and Hawkey, 2006). Intimins belong to a growing family of proteins. In human EPEC strains, the *eae* genes of several strains have been cloned, sequenced and described as presenting a highly conserved 5′-terminal region and a

variable 3′-terminal region. This variable region determines the intimin types and subtypes, namely, α, α2, β1–3, γ1, γ2, δ, ε, ε2–4, ζ, η, η2, θ, ι, ι2, κ, λ, μB, νB, ξ, ο, π, ρ and σ (Menezes *et al*., 2009).

d. Translocated Intimin Receptor (Tir)

The attachment of bacteria is by means of intimin binding to a 90 kDa tyrosine phoshorylated protein in the host membrane. This receptor is known as translocated intimin receptor (Tir) and is of bacterial origin; it is translocated on to the host membrane where its tyrosine residues become phosphorylated and binds to intimin. Subsequently, signal transduction events that occur within the host cells are the activation of protein kinase C, inositol triphosphate and calcium release. This leads to the formation of an actin-rich pedestal that forms a dome-like anchoring site for the bacteria which is an essential feature of EPEC pathogenesis (Ouwehand and Vaughan, 2006).

2-3-1-1-1-2 EPEC Adherence Factor (EAF) Plasmid

Enteropathogenic *E. coli* contains a large plasmid, referred to as the EPEC adherence factor (EAF) plasmid. The EAF plasmid encode three pathogenicity-associated genes which are bundle-forming pilus (BFP), plasmid-encoded regulator (Per) and EAF probe (Nataro and Kaper., 1998).

a. Bundle Forming Pilus (BFP)

Enteropathogenic *E. coli* is known to produce a type IV fimbria called the bundle forming pilus (BFP) (Brenner *et al*., 2004). The first stage in EPEC pathogenesis is the localized non-intimate attachment of the organism to the intestinal epithelium via the inducible bundle-forming pilus (BFP) (Gillespie and Hawkey, 2006), which promote autoaggregation and microcolony formation in EPEC strains. After initial

attachment, an energy-dependent conformational change in the quaternary structure of BFP appears to be needed for the further dispersal of EPEC over human intestinal cells and for the full virulence of this pathogen (Schaechter, 2009). This stage, encoded by the *bfp* operon on a 50–70-MDa plasmid, designated the EPEC adherence factor (EAF) plasmid. The BFP facilitates interbacterial aggregation, leading to a characteristic local adherence (LA) pattern (Gillespie and Hawkey, 2006). Genes at two loci are necessary for expression of the LA phenotype: a cluster of 14 genes on the EAF plasmid involved in biogenesis of the BFP, a type-IV pilus, which includes genes encoding bundlin (*bfpA*), the major structural subunit of the type-IV pilus, a prepilin peptidase, which processes pre-bundlin to its mature form, and 12 other proteins, and *dsbA* on the chromosome (Donnenberg *et al*., 1997).

b. Plasmid Encodes a Regulator (Per)

The EAF plasmid encodes a regulator of virulence genes called Per (plasmid- encoded regulator) consisting of three open reading frames: *perA, perB,* and *perC* (Nataro and Kaper., 1998). These genes encode proteins that form a regulatory complex, which activates the transcription of several genes in the chromosome and on the EAF plasmid (Nataro and Kaper., 1998). The gene is involved in the regulation of the LEE, which activates transcription of the chromosomal regulator protein LEE-encoded regulator (ler). This in turn upregulates the transcription of other LEE genes including *EspA, EspB* and *EspD* and increases the expression of chromosomal *eae* (Gillespie and Hawkey, 2006). Per also involve in activates the expression of the *bfp* operon (Moreira *et al*., 2006).

c. EAF Probe

The DNA sequence of this fragment has been determined, but the contribution to EPEC pathogenesis of the genes encoded in the EAF

probe region is unknown, as is the contribution of genes contained in the major portion of the EAF plasmid. EAF probe is a 1-kb restriction fragment that has been extensively used as a diagnostic DNA probe (Nataro and Kaper., 1998). Recently, EPEC has been classified into 2 subcategories on the basis of hybridization results with the EAF probe: EPEC strains that hybridize with the EAF probe have been designated "typical EPEC," and EPEC strains that do not hybridize with the EAF probe designated "atypical EPEC" (Dulguer *et al*., 2003).

2-3-1-1-2 Other Virulence Factors

a. Cytolethal Distending Toxins (CDTs)

Cytolethal distending toxins, first detected in 1987 in *E. coli* O128 isolated from the stool of a child who was less than 2 years old and suffering from gastroenteritis, are an emerging family of toxins and produced by several pathogenic bacteria (Jong-Hyun *et al*., 2009). The CDTs are characterized by a unique structure of three overlapping genes, cdtA, cdtB, and cdtC, which have molecular masses of approximately 30, 32, and 20 kDa highly homogeneous in all bacteria, yet in widely varying degrees (Jong-Hyun *et al*., 2009). There is a growing CDT family as a result of reports that this toxin, or close homologs, is produced by *Campylobacter* spp., *S. dysenteriae*, and *Haemophilus ducreyi*, an agent of genital ulcers. The mechanism of CDT action in diarrhea is not known (Nataro and Kaper, 1998).

b. *E. coli* Heat-Stable-Like Toxin-1 (EAST-1)

Toxins that have also been associated with strains of EAggEC include an *E. coli* heat-stable-like enterotoxin termed EAST-1, although the role of these toxins in diarrheal disease remains unclear and not all EPECs encode these toxins (Gillespie and Hawkey, 2006).

c. Shiga Like Toxins (Stx)

Shiga like toxins (Stx1 and Stx2) are the most critical virulence factors responsible for the principal manifestations of hemorrhagic colitis and hemolytic-uremic syndrome (Girardeau *et al*., 2005). Bacteriophage - mediated cytotoxins referred to as Stx_1 or VT_1 (first described as Shiga-like toxin I, SLTI) and Stx_2 or VT_2 (first described as Shiga-like toxin II, SLTII) (Brenner *et al*., 2004). Strains may carry genes vtx/stx for the production of either VT1 or VT2 alone or both together and both toxins consist of an enzymatically active A subunit (32 kDa) and a pentameric B subunit (7.7 kDa monomers). The B subunits form a ring which mediates binding to a neutral glycolipid receptor (Lingwood, 1996). Stxs/VTs inhibit protein synthesis (Brenner *et al*., 2004). Stx exerts its lethal effect by inhibiting the host-cell 60S ribosomal subunit via cleaving a specific adenosine residue of the 23S rRNA (Gillespie and Hawkey, 2006). In Vero cells the result is cell death by apoptosis (Inward *et al*., 1995).

2-3-1-1-3 Mechanism of Pathogenesis

The hallmark of infections due to EPEC is the attaching-and-effacing (A/E) histopathology, which can be observed in intestinal biopsy specimens from patients or infected animals and can be reproduced in cell culture (Nataro and Kaper, 1998).

Multiple steps are involved in producing the characteristic A/E histopathology. Donnenberg and Kaper, (1992) proposed a three-stage model and Knutton *et al*. (1998) proposed a four-stage model.

In the first stage and under the correct environmental conditions, EPEC cells express bundle-forming pili (Bfp), the intimate adhesin intimin, and short, surface-associated filaments (EspA filaments); the expression of these determinants is dependent on both plasmid and chromosomal genes. In the second stage, EPEC cells adhere to the

epithelial cell via Bfp and EspA filaments, and a type III secretion system injects the translocated intimin receptor (Tir) and an as yet undetermined number of effector molecules directly into the host cell. Effector molecules activate cell-signaling pathways, causing alterations in the host cell cytoskeleton and resulting in the depolymerization of actin and the loss of microvilli. Tir is modified by the action of both protein kinase A and tyrosine protein kinase and inserts into the host membrane. Finally, in the fourth stage, further actin polymerization and accumulation of cytoskeletal elements at the site of bacterial attachment result in the production of the mature A/E lesion with the characteristic pedestal structure. Host cell processes are disrupted by the translocated effector molecules allowing a loss of tight-junction integrity and mitochondrial function, resulting in electrolyte loss and cell death (Knutton *et al*., 1998).

2-3-1-1-4 Typical and Atypical EPEC

Enteropathogenic *E. coli* is defined as *E. coli* strains that produce A/E lesions and carry the *eaeA* gene but not *stx* genes (Wani *et al*., 2006). The EPEC pathotype was subdivided into typical (tEPEC) and atypical (aEPEC) with the basic difference being the respective presence and absence of EAF plasmid. *E. coli* strains with the A/E genotype ($eaeA^+$) that harbor the EAF plasmid ($bfpA^+$) are classified as 'typical EPEC' and strains with the A/E genotype that do not possess the EAF plasmid ($bfpA^-$) are classified as 'atypical EPEC' (Kaper, 1996). Typical EPEC strains are further subdivided into EPEC 1 (expressing flagellar antigen H6 and intimin a) and EPEC 2 (expressing flagellar antigen H2 or H- and mainly intimin b) lineages. Typical EPEC O119:H6 (which express flagellar antigen H6 and intimin b) is unusual and classified as non-EPEC 1 non-EPEC 2 (Frankel and Phillips, 2008). Typical EPEC have rarely been isolated from animals, as humans are the major natural reservoir for

these pathogens, whereas atypical EPEC strains have been isolated from both animals and humans (Jong-Hyun *et al*., 2009).

2-3-1-2 Typing Methods

There are numerous methods available for subtyping *Escherichia* spp. Each method has advantages and disadvantages when applied to a specific situation. 'Conventional' typing methods such as biotyping, phage and colicin typing, resistotyping, and serotyping have been replaced by or used in combination with molecular techniques that are applicable to a wide variety of organisms with only minimal adaptation in the procedures (Borriello *et al.*, 2005).

2-3-1-2-1 Conventional Typing Methods-Serotyping

The variations in chemical arrangement of the bacterial cell wall, capsule and flagellate within strains of the same species act as distinct antigens. These variations can be used to produce antibodies that can discriminate between the antigenic types, in other words, serotype the organism (Hardy, 2002). Consequently, *E. coli* strains can be defined mainly by their antigenic composition. Taxonomic relevance are over 180 different serological types of lipopolysaccharide antigens (O antigens) and 80 types of capsular (K antigens). Other properties that are used to define individual strains are H antigens (flagellar proteins), and F antigens (fimbrial proteins) (Borriello *et al.*, 2005). A serotype is recorded in the following way: O18ac:K1:H7 or O111:H2 (the latter antigenic formula indicates that K antigens are not present in the strain). Fimbriae, which are present only in some, often pathogenic, serotypes, can also be used for the serological characterization in which case the complete serotype is recorded as O4:K3:H5; F13 or O147:H19; F4ac. Although complete serotyping involving the many known O, K, H, and F antigens has been carried out in only a very few laboratories, it is well

known that the existing number of serotypes is very high (Brenner *et al*, 2004).

2-3-1-2-2 Molecular Typing Methods

Recent advances in molecular methods are having a direct effect upon strain typing and can be seen to complement and enhance conventional culture-based methods for the detection, identification, typing, and epidemiological analysis of the wide variety of *E. coli* strains associated with causing disease. Genomic molecular methods can offer more discrimination by 'fingerprinting' than phenotypic methods. Such methods include detection of specific genes by oligonucleotide probes and PCR and the use of PCR for amplification of chromosomal sequences (Borriello *et al.*, 2005).

2-3-1-3 Epidemiology

Endemic diarrhea is a major public health concern around the world, especially in developing countries. It is one of the world's leading causes of morbidity and mortality, it is estimated to account for approximately 1.9 million or 18% of deaths among children under 5 years of age annually, of which approximately 40% occur in the African region (Eijk *et al*., 2010). *E. coli* has been described as an important bacterial etiologic agent of this pathology (Kaper *et al*., 2004). Diarrheagenic *E. coli* pathotypes represent a leading bacterial cause of pediatric diarrhea in developing regions (Nguyen *et al*., 2005), and are also an emerging cause of diarrhea in industrialized countries (Donnenberg *et al*., 1995; Fei *et al*., 2003). EPEC strain are diarrheagenic *E. coli* belonging to specific serotypes, historically associated with outbreaks of infantile diarrhea, which are one of the main causes of severe diarrhea in many developing countries (Gomes *et al*., 1991; Cravioto *et al*., 1996; Levine *et al*., 1996 ;Nara *et al*., 2009). EPEC strains were first identified as the cause of

explosive outbreaks of diarrhea in hospital nurseries in the United States and Great Britain during the 1950s. The disease seems to have disappeared in industrialized nations, although it may be underestimated due to the difficulty of diagnosis (Ryan and Ray, 2004). In contrast to the situation in Britain and the US, EPEC strains are among the most frequent causes of diarrhea in infants in developing countries (Trabulsi *et al*., 2002). EPEC infection is primarily a disease of infants younger than 2 years and infections show a marked seasonality and are associated with warm season peaks. (Nataro and Kaper, 1998). Since 1996, EPEC has been divided into two categories: typical EPEC (tEPEC) and atypical EPEC (aEPEC). Atypical EPEC strains have been found in association with endemic diarrhea in children and diarrhea outbreaks in adults in developing and industrialized countries. Apparently, this pathotypes emerged recently and it is among the leading causes of childhood diarrhea in many countries (Bando *et al*., 2009).

2-3-1-4 Treatment

As with other diarrheal pathogens, the primary goal of treatment of EPEC diarrhea is to prevent dehydration by correcting fluid and electrolyte imbalances. Oral rehydration may be sufficient for milder cases, but more severe cases require parenteral rehydration. Correction of nutritional imbalance with lactose-free formula or breast milk may be insufficient for some severely ill patients, and total parenteral nutrition may be required (Fagundes-Neto, 1996). Varieties of antibiotics have been used to treat EPEC and have proved useful in many cases, but multiple antibiotic resistances are common for EPEC (Donnenberg *et al*., 1995).

2-3-1-4 Antibiotic Resistance

The seemingly perfect nature of antibiotics, originally hailed as "wonder drugs," has been steadily eroded by the appearance of strains resistant to their action. This resistance may be inherent to the organism or appear in a previously susceptible species by mutation or the acquisition of new genes (Ryan and Ray, 2004).

There has been an alarming increase in drug-resistant strains of EPEC in developing as well as developed countries. Several cases of antimicrobial resistance in EPEC have been observed in different parts of the world (Subramaniana *et al*., 2009).

E. coli like other Gram-negative bacteria is intrinsically resistant to hydrophobic antibiotics, such as macrolides, novobiocins, rifamycins, actinomycin D, and fusidic acid. Resistance to these compounds is attributed, in part, to the low permeability of the outer membrane bilayer to lipophilic solutes; however, active efflux mechanisms may have a synergistic effect on resistance in certain cases (Brenner *et al*., 2004).

Enzymatic inactivation of the invading antibiotic is the most powerful and robust of the resistance mechanisms. Literally, hundreds of distinct enzymes produced by resistant bacteria may inactivate the antibiotics in the cell, in the periplasmic space, or outside the cell. They may act on the antibiotics molecule by disrupting its structure or by catalyzing a reaction that chemically modifies it (Ryan and Ray, 2004).

2-3-1-4-1 Natural Resistance

a. Membranes and cell surfaces initially, intrinsic differences in resistance to antibiotics and other chemotherapeutic agents were attributed to structures such as the Gram-negative outer membrane. The mechanism depending on lipophilic molecules are slowed by the

low fluidity of the lipid bilayer surrounding the cell wall and that hydrophobic molecules enter the mycobacterial cell slowly because the porins are inefficient and few in number (Schaechter, 2009).

b. Multidrug efflux pumps; a cell has different ways to export material across its membrane. All of them involve the expenditure of energy. The most well-characterized multiple drug resistance pumps in mammalian cells are the ABC (ATP binding cassette) transporters. In bacteria, active export system has been characterized in which the energy to drive the pump comes from the proton motive force (PMF) of the transmembrane electrochemical proton gradient. The multidrug efflux pump found in *E. coli* is one of the most well characterized of the resistance/nodulation/cell division (RD)-type pumps (Schaechter, 2009).

c. Topoisomerase mutants, involving changes in the chromosomal genes encoding drug targets (such as gyrAB/parCE, which causes fluoroquinolone resistance) are evolving and emerging at a rapidly increasing rate, and are also being recognized more consistently in epidemiological surveys (Hawkey and Munday, 2004).

2-3-1-4-2 Resistant to β-lactam (Production of β-lactamases)

β-lactamases is a general term referring to any one of hundreds of bacterial enzymes able to break open the β-lactam ring and inactivate various members of the β-lactam group (Ryan and Ray, 2004). β-lactam antibiotics are the most common treatment for bacterial infections and consist of four major groups: penicillins, cephalosporins, monobactams, and carbapenems. All four groups have β-lactam structures and are susceptible to hydrolytic activity of β-lactamases (Mahon *et al*., 2007).

The β-lactamases of Gram-negative rods are responsible for the most rapidly evolving mechanisms of resistance in pathogenic bacteria under the selection pressure of antibiotic use. One of the major aspects of this evolution is the accumulation of mutations, which result, for example, in modifications of the enzymes' catalytic efficiencies, substrate spectra, and susceptibilities to inhibitors (Baraniak *et al*., 2005).

The enzymes can be classified in a number of ways, such as by their amino acid sequences or by their enzymatic activity spectrum. In the latter classification, four groups have been defined: group 1, cephalosporinases on which the β-lactamase inhibitor clavulanic acid has a weak activity; group 2, penicillinases sensitive to clavulanic acid and extended spectrum β-lactamases; group 3, metallo-β-lactamases; and group 4, other β-lactamases weakly sensitive to clavulanic acid (Schaechter, 2009).

Genes encoding resistance to many clinically valuable, widely prescribed β-lactam antibiotics include the 'mobile' *ampC* genes; the extended-spectrum β-lactamases (ESBL), such as bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M}$; and carbapenem resistance genes, bla_{IMP} and bla_{VIM}, which also convey resistance to all β-lactams currently used (Hawkey and Munday, 2004).

a. Extended-Spectrum β-lactamases (ESBLs).

Extended-spectrum β-lactamases confer resistance to expanded-spectrum cephalosporins and monobactams due to their ability to hydrolyze these compounds. Many ESBLs are derived from non-ESBL precursors by point mutation of *bla* genes, and the prevailing assumption is that these variants are selected by exposure to expanded-spectrum

cephalosporins and/or monobactams in health care facilities (Hammond *et al*., 2008).

New generations of 'extended spectrum' β-lactam antibiotics were designed to be effective in the presence of the (then) existing β-lactamases, but, following the use of these newer drugs, new variant (mutant) forms of β-lactamase have evolved; these can hydrolyze at least some of the newer antibiotics and are becoming widely disseminated (Singleton and Sainsbury, 2006).

The first plasmid- mediated β-lactamase in Gram-negative, TEM-1, was first reported in the early 1960s, and originally found in a single strain of *E. coli* isolated from blood culture from a patient named Temoniera in Greece, hence the designation TEM (Medeiros and Crellin, 1997). The most common extended-spectrum phenotypes arise from point mutations in the bla_{TEM}, bla_{SHV} or $bla_{CTX\text{-}M}$ gene resulting in alterations of the primary amino acid sequence of the enzyme (Mulvey *et al*., 2004). However, the first ESBLs observed at the teaching hospital of Clermont-Ferrand, France, in 1984, the TEM-3 was produced by *K. pneumoniae* (Sirot *et al*., 1987).

The ESBLs are classified into two subgroups. The first group (type I) consists of variants of the penicillinases TEM-1, TEM-2, and SHV-1 differing by one to four point mutations leading to either an extended hydrolytic capacity to third generation cephalosporins (Tomanicek *et al*., 2010), and do not hydrolyze oxyiminocephalosporins or aztreonam (Kiratisin *et al*., 2008). The origins of the TEM types remain uncertain, but SHV-1 was later realized to be derived from the chromosomal β-lactamase of *Klebsiella pneumoniae*. The spread of plasmid-mediated TEM and SHV enzymes provided a major impetus for the development

of 'β-lactamase-stable β-lactams' from the mid-1970s onwards (Livermore *et al*., 2008). The second and the most widespread group of ESBLs (type II) includes the CTX-M-type enzymes that are distantly related to TEM-1 or SHV-1.4 These enzymes share less than 40% sequence identity with other type I ESBLs, whereas they share a 70% or higher identity within the subgroup (Tomanicek *et al*., 2010). The CTX-M-type ß-lactamases constitute a novel group of enzymes that have a typical ESBL resistance phenotype: are capable of hydrolyzing broad-spectrum cephalosporins and are inhibited by clavulanic acid, sulbactam, and tazobactam. They also confer a high level of resistance to cefotaxime but have a low level of activity against ceftazidime (Fam and El-Damarawy, 2008). The CTX-M-type β-lactamases, along with a host of smaller groups such as the VEB, PER, GES and some OXA enzymes to form a collection of enzymes referred to as the ESBLs (Evans *et al*., 2007). These enzymes are mostly plasmid-coded and thus more easily horizontally transmissible. They hydrolyze β-lactam antibiotics resulting in resistance to penicillins, cephalosporins, and aztreonam (Reinthaler *et al*., 2010).

All the β-lactamase variants so far discussed belong to molecular class A, except, OXA-type β-lactamases, belonging to molecular class D and functional group 2d, are characterized by their high hydrolytic activity against oxacillin and cloxacillin and are poorly inhibited by clavulanic acid (Bhattacharjee *et al*., 2007).

b. AmpC Cephalosporinases

The first major mechanism of resistance to extended spectrum cephalosporins was the derepression and over expression of chromosomal cephalosporinases in a number of different Gram-negative species. These chromosomally located genes were thought to remain within the species

they characterized. It was hypothesized that, if they could be mobilized, they could become a significant cause of resistance (Hawkey and Munday, 2004). AmpC β-lactamases confer resistance to a wide variety of β-lactam drugs except for cefepime and carbapenems. They are known to be responsible for nosocomial outbreaks, therapeutic failure, and multidrug resistance (Oteo *et al*., 2010). This AmpC phenotype may result from overexpression of the chromosomally encoded AmpC enzyme or from the acquisition of a transferable plasmid encoded AmpC gene (Naseer *et al*., 2009).

c. Carbapenemases

Carbapenemases are ESBL enzymes that hydrolyze or partially hydrolyze imipenem and/or meropenem. Because they often confer only partial resistance and are hard to detect, their presence may be underestimated (Siegel, 2008). Carbapenems are the most potent antibiotics for the treatment of Gram-negative infections because of their stability against hydrolysis by the majority of β-lactamases and their high rate of permeation through the bacterial outer membrane (Hawkey and Munday, 2004). Most of carbapenemases confer resistance not only to carbapenems, but also to other β-lactams. These enzymes have been classified into the class A group (clavulanic acid-inhibited carbapenemases) which is a class of serine β-lactamases (most of these β-lactamases have a serine residue at the active center of the enzymes), the class B (metallo-β-lactamase) requiring zinc ion as cofactor for the maximal activity, and the class D (oxacillinases) (Jamklang, 2004).

Chapter Three
Materials and Methods

3-1 Materials

3-1-1 Equipment and Instrument

Type of equipment	Model name (Origin)
Sensitive balance	AND
Gel documentation system	Biometra (Germany)
Calipers	China
Petridish 15 cm	China
Petridish 9 cm	China
Refrigerator	Concord (Lebanon)
Laminar flow	Cruma (Spain)
Millipore filter (0.22µm)	Difco (USA)
Micropipette 1-10 µl	Eppendorf(Germany)
Micropipette 2-20 µl	Eppendorf
Micropipette 10-100 µl	Eppendorf
Micropipette 10-1000 µl	Eppendorf
PCR system	GeneAmp (Singapore)
Distilator	GFL
Deep freezer	GFL (Germany)
Bench centrifuge	Hettich (Germany)
High speed centrifuge	Hettich
Autoclave	Hiclave- HIRAYAMA (Japan)
Calibrated loop 0.01	Himedia (India)
Water bath	Kottermann
Electrophoresis	Labner (Taiwan)
pH-meter	LKB (Sweden)
Incubator	Memmert(Germany)
Centrifuge	Memmert
Electric oven	Memmert
Digital camera	Sony (Japan)
UV- transilluminator	Taiwan
Vortex mixer	Thermolyne

3-1-2 Biological and Chemical Materials

Biological and Chemical type	Manufacturer and Origin
Sodium dodecyl sulfate (SDS)	AppliChem (Germany)
Ethanol (96%)	BDH (England)
Ethyenediamine tetra-acetic acid (EDTA)	BDH
Hydrochloric acid (HCl)	BDH
Isoamyl alcohol	BDH
Methyl red	BDH
Potassium hydroxide (KOH)	BDH
Sodium chloride (NaCl)	BDH
Sodium hydroxide (NaOH)	BDH
Sulfuric acid (H_2SO_4)	BDH
Tris-(hydroxymethyl)methylamine ($NH_2.(CH_2OH)_3$ (Tris-OH)	BDH
α-naphthol (C10H8O)	BDH
Cloroform	BDH
Barium chloride dihydrate ($BaCl_2$). $2H_2O$	Fluka (Switzerland)
Glycerol ($C_3H_8O_3$)	Fluka
Gram stain	Himedia (India)
Kovac's reagent	Himedia
Urea solution	Mast Diagnostic (UK)
Isopropanol	Mast Diagnostic
Iodine	Mast Diagnostic
Tris- EDTA (TE) buffer molecular grad	Promega (USA)
Tris-Borate-EDTA Buffer (TBE buffer)	Promega
Agarose	Promega
Phenol	Scharlau
Ethidium bromide	Sigma (USA)

3-1-3 Culture Media

Medium	Manufacturer Origin
Urea agar base	Biolife(Italy)
Tryptic soy broth	Biolife
Tryptic soy agar	Biolife
Triple sugar iron agar	Biolife
Peptone water	Biolife
Brain heart infusion agar	Himedia (India)
MacConkey agar	Himedia
Muller-Hinton agar	Himedia
Nutrient broth	Himedia
Nutrient agar	Himedia
Blood base agar	Himedia
Brain heart infusion broth	Himedia
Simmons citrate agar	Mast Diagnostic (UK)
MR-VP broth	Oxoid (UK)

3-1-4 Antibiotics

Antibiotic class	Antibiotic subclass	Antibiotic Name	Symbol	Content	origin
penicillins	aminopenicillin	**amoxicillin**	**Am**	25 µg	Bioanalyse (Turkey)
	Ureidopencillin	**piperacillin**	**Pc**	100 µg	Himedia (India)
	carboxypenicillin	**carbenicillin**	**PY**	100 µg	Bioanalyse
		ticarcillin	**TIC**	75 µg	Bioanalyse
β-lactam/β-lactamase inhibitor combinations		**amoxicillin-clavulanic acid**	**Ac**	30 µg (20µg / 10µg)	Himedia
cephems (parenteral)	cephalosporin III	**cefotaxime**	**CTX**	30 µg	Bioanalyse
		ceftazidime	**CAZ**	30 µg	Bioanalyse
		ceftriaxone	**CRO**	30 µg	Bioanalyse
	cephalosporin IV	**cefepime**	**FEP**	30 µg	Bioanalyse
	cephamycin	**cefoxitin**	**FOX**	30 µg	Bioanalyse
monobactams		**aztreonam**	**ATM**	30 µg	Bioanalyse
penems	carbapenem	**imipenem**	**IPM**	10 µg	Bioanalyse
		meropenem	**MEM**	10 µg	Bioanalyse
aminoglycosides		**amikacin**	**Ak**	30 µg	Himedia
		tobramycin	**Tb**	10 µg	Himedia
		gentamicin	**CN**	10 µg	Bioanalyse
quinolones	fluoroquinolone	**ciprofloxacin**	**CIP**	5 µg	Bioanalyse
		levofloxacin	**LEV**	5 µg	Bioanalyse
folate pathway inhibitors		**trimethoprim**	**TMP**	5 µg	Bioanalyse
tetracyclines		**tetracyclin**	**TE**	30 µg	Bioanalyse

3-1-5 Agglutinating Sera (Oxoid and Remel, UK)

Polyvalent	Monovalent
Anti-coli Polyvalent 2	*E. coli* type O26:K60 (B6)
	E. coli type O55:K59 (B5)
	E. coli type O111:K58 (B4)
	E. coli type O119:K69 (B14)
	E. coli type O126:K71 (B16)
Anti-coli Polyvalent 3	*E. coli* type O86:K61 (B7)
	E. coli type O114:K90 (B)
	E. coli type O125:K70 (B15)
	E. coli type O127:K63 (B8)
	E. coli type O128:K67 (B12)
Anti-coli Polyvalent 4	*E. coli* type O44:K74 (L)
	E. coli type O112:K66 (B11)
	E. coli type O124:K72 (B17)
	E. coli type O142:K86 (B)
	E. coli type O18c:K77 (B21)

3-1-6 Rapid Multitest System

Test	Manufacture, Origin
Api 20 E test kit	(BioMerieux, France)

3-1-7 Standard Strain Bacteria

Strain name	Laboratory identifier	Key characteristic	Source
E. coli	American Type Culture Collection (ATCC 25922)	Susceptible to ampicillin, cephalosporins, and gentamicin	University of Kufa College of Medicine Department of Microbiology

3-1-8 PCR Materials

3-1-8-1 Master Mix

Go Tag Green Master mix	Source
Go Tag DNA polymerase is supplied in 2x Green Taq Reaction buffer pH 8.5, 400µm dATP, 400µm dGTP, 400µm dCTP, 400µm dTTP, and 3mM $MgCl_2$.	Promega (USA)

3-1-8-2 Molecular Weight Marker

DNA marker	Description	Source
100 bp Ladder	100-1500 base pairs. The ladder consists of 11 double strand DNA fragment with size of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500bp. The 500bp present at triple the intensity of other fragments and serve as a reference. All other fragments appear with equal intensity on gel.	Promega (USA)

3-1-8-3 Primers (Alpha DNA, Montreal)

a. Virulence Properties Primers

Primer Name	DNA Sequences (5'-3')		Product size bp	Reference
eae	F	ACGTTGCAGCATGGGTAACTC	815	Brian *et al.*, 1992 Kobayashi *et al.*, 2001 Hazarika *et al.*, 2007
	R	GATCGGCAACAGTTTCACCTG		
bfpA	F	AATGGTGCTTGCGCTTGCTGC	326	Afset *et al.*, 2003 Leomil *et al.*, 2005 Alikhani *et al.*, 2006
	R	GCCGCTTTATCCAACCTGGTA		
bfpA	F	ATTGGTGCTTGCGCTTGCTGC	326	Obi *et al.*, 2004
	R	GCCGCTTTATCCAACCTGGTA		
EAF	F	CAGGGTAAAAGAAAGATGATAA	397	Nataro and Kaper, 1998 Woodford and Johnson, 1998 Kobayashi *et al.*, 2001
	R	TATGGGGACCATGTATTATCA		
stx1	F	AAATCGCCATTCGTTGACTACTTCT	370	Brian *et al*, 1992 Brandal *et al*, 2007 Altalhi and Hassan, 2009
	R	TGCCATTCTGGCAACTCGCGATGCA		
stx2	F	CGATCGTCACTCACTGGTTTCATCA	282	Brian *et al*, 1992 Altalhi and Hassan, 2009 Herrera-Luna *et al*, 2009
	R	GGATATTCTCCCCACTCTGACACC		
hlyA	F	ACGATGTGGTTTATTCTGGA	165	Nataro and Kaper, 1998 Fagan *et al*, 1999 Posse *et al*, 2007
	R	CTTCACGTCACCATACATAT		

B. β-lactam Resistance Primers

Primer name		DNA Sequence(5'-3')	Product size	Reference
***bla*$_{TEM}$**	F	AAACGCTGGTGAAAGTA	822	Paterson *et al.*, 2003 Hujer *et al.*, 2006
	R	AGCGATCTGTCTAT		
***bla*$_{SHV}$**	F	ATGCGTTATATTCGCCTGTG	753	Paterson *et al.*, 2003 Hujer *et al.*, 2006
	R	TGCTTTGTTATTCGGGCCAA		
***bla*$_{CTX-M}$**	F	CGCTTTGCGATGTGCAG	550	Paterson *et al.*, 2003 Bhattacharjee *et al.*, 2007
	R	ACCGCGATATCGTTGGT		
***bla*$_{AmpC}$**	F	ATCAAAACTGGCAGCCG	550	Paterson *et al.*, 2003 Kaczmarek *et al.*, 2006
	R	GAGCCCGTTTTATGCACCCA		
***bla*$_{GES}$**	F	ATGCGCTTCATTCACGCAC	846	Kiratisin *et al.*, 2008
	R	CTATTTGTCCGTGCTCAGG		
***bla*$_{VEB}$**	F	GCGGTAATTTAACCAGA	961	Wang *et al.*, 2006
	R	GCCTATGAGCCAGTGTT		
***bla*$_{PER}$**	F	AGTCAGCGGCTTAGATA	978	Wang *et al.*, 2006
	R	CGTATGAAAAGGACAATC		
***bla*$_{IMP}$**	F	CGGCCKCAGGAGMGKCTTT	587	Yin *et al.*, 2008
	R	AACCAGTTTTGCYTTACYAT		
***bla*$_{VIM}$**	F	ATTCCGGTCGGRGAGGTCCG	633	Yin *et al.*, 2008
	R	GAGCAAGTCTAGACCGCCCG		
***bla*$_{OXA}$**	F	ATATCTCTACTGTTGCATCTCC	619	Karami and Hannoun, (2008)
	R	AAACCCTTCAAACCATCC		

3-2 Methods

3-2-1 Preparation of Buffers and Solutions

The following solutions and reagents were used in the present study. Those, which require sterilization, were autoclaved at 121˚C for 15-20 minutes. Millipore filters (0.22 µm) were used for sterilization of heat-sensitive solutions like urea. The pH of the solution was adjusted using 1M NaOH and 1M HCl.

3-2-1-1 McFarland (0.5) Turbidity Standard

The 0.5 McFarland was prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2.2H_2O$) solution to 99.5 ml of 1% (vol/vol) H_2SO_4. The turbidity standard was liquated into test tubes identical to those used to prepare the inoculum suspension. The McFarland standard tubes were sealed with parafilm to prevent evaporation and stored for up to 6 months in the dark at room temperature (22-25 C°). The accuracy of the density of a prepared 0.5 McFarland standard was chacked by using a spectrophotometer. The absorbance of the wavelength of 625 nm should be 0.08 to 0.1 (NCCLS, 2003a).

3-2-1-2Normal Saline Solution

This solution was prepared by dissolving 0.85 gm of NaCl in 90 ml distilled water and further completed to 100 ml with D.W (Collee *et al*., 1996)

3-2-1-3 Solutions Used in DNA Extraction

The following solutions were prepared as described by Pospiech and Neumann (1995) with some modifications:

3-2-1-3-1 Tris-EDTA Buffer (TE) Buffer

This buffer was prepared by adding 0.05 M Tris-OH and 0.001 M EDTA to 800 ml D.W, the pH was adjusted to 8 and completed to one litter by D.W, then autoclaved at 121˚C for 15 minutes, and stored at 4˚C until used.

3-2-1-3-2 Salt-EDTA-Tris (SET) Buffer

This buffer was prepared by dissolving 20 mM Tris-OH, 25 mM of EDTA and 75mM NaCl in 750 ml D.W, the pH was adjusted to 8 and the volume completed to 1000 ml by D.W, then autoclaved at 121°C for 15 minute

3-2-1-3-3 Sodium Dodecyl Sulfate (SDS) Solution (25%)

Sodium dodecyl sulfate (25 mg) was dissolved in 100 ml of D.W, then sterilized in autoclave, and stored at 4˚C.

3-2-1-3-4 Sodium Chloride (NaCl) Solution (5 M)

Sodium chloride (14.625 gm) was dissolved in 50 ml D.W, sterilized in autoclave, and stored at 4˚C.

3-2-1-3-5 Phenol: Chloroform: Isoamyl Alcohol (25:24:1) Mixture

The solvent was composed from 25 ml phenol, 24 ml chloroform, and 1 ml isoamyl alcohol.

3-2-1-4 Solution Used for Agarose Gel Electrophoresis

3-2-1-4-1 Working Solution of Tris-Borate-EDTA (TBE) Buffer

Tris-borate-EDTA buffer was used at concentration of 1X (1:10 dilution of the concentration stock). The stock solution was diluted by D.W. and stored at room temperature.

3-2-1-4-2 Ethidium Bromide Solution

A stock solution (5 mg/ml) was prepared by dissolving 0.05 gm of ethidium bromide in 10 ml of D.W and stored in dark reagent bottle.

3-2-2 Preparation of Reagents

3-2-2-1 Methyl red Reagent

It was prepared by dissolving 0.1 gm of methyl red in 300 ml of 96% ethanol, and then completed to 500 ml with D.W. This reagent was used as indicator in methyl red test (MacFaddin, 2000).

3-2-2-2 Voges-Proskauer reagents

The reagents were prepared as follows:

Reagent A: 5% α-naphthol in 96% ethanol.

Reagent B: 40% KOH in D.W.

These reagents were used as indicators in Voges-Proskauer test (MacFaddin, 2000).

3-2-3 Preparation of Culture and Diagnostic Media

Media used in this study listed in (3-1-3) were prepared in accordance with the manufacturer's instructions fixed on their containers. All the above media were sterilized in the autoclave at 121˚C for 15

minutes. After sterilization urea agar base was supplemented with 20% sterile urea solution.

3-2-4 Biochemical Test

3-2-4-1 Indole Production Test

Peptone water was inoculated with a young bacterial culture and incubated at 37 ˚C for 24-48 hours. A few drops of Kovacs reagent were added to each tube. Formation of pink ring indicates a positive test (MacFaddin, 2000).

3-2-4-2 Methyl red Test

Methyl red-voges proskauer broth was inoculated with a young bacterial culture and incubated at 37˚C for 24 hours. Five drops of methyl red solution were added, mixed, and the result was read immediately. A positive test was bright red (MacFaddin, 2000).

3-2-4-3 Voges-Proskauer Test

Methyl red-Voges-Proskauer broth was inoculated with a young bacterial culture and incubated at 37˚C for 48 hours. One ml of 40% KOH solution and 3 ml of 5% solution of α-naphthol were added to each tube. A positive reaction was indicated by the development of a pink color in 15-20 minutes (MacFaddin, 2000).

3-2-4-4 Simmons citrate Test

Simmons citrate slant was inoculated with a young bacterial culture and incubated at 37˚C for 48-72 hours. A blue color and streak of growth appearance indicate as positive test (MacFaddin, 2000).

3-2-4-5 Urease Test

Urea agar slant was streaked with bacterial culture and incubated at 37˚C. the result was read after 6 hours, 24 hours, and every day for 6 days. Urease test is positive if the indicator was changing the color of medium to purple-pink (MacFaddin, 2000).

3-2-4-6 Triple Sugar Iron Test (TSI)

A heavy growth was streaked over the surface of the slope and stabbed into the butt, and incubated at 37˚C for 24 hours. Result were configured as follows:

Slant/butt	Colour
Alkaline/Acid	Red/Yellow
Acid/ Acid	Yellow/ Yellow
Alkaline/ Alkaline	Red/ Red
H_2S	Black precipitate

(MacFaddin, 2000).

3-2-5 Patients and Stool collection

This study were conducted in Najaf, in order to establish EPEC occurrence and determine their virulence properties and antimicrobial susceptibility. Two hospitals (Alzahraa teaching hospital, and Alhakeem teaching hospital) are included in this study. A total of 656 stool specimens were collected from children younger than two years old suffering from watery diarrhea characterized in Nguyen *et al* (2005); Hien *et al.* (2008); Albert *et al* (2009), and 54 apparently healthy control. The specimens collected during the period from 7 September to 15 November 2009. Specimen were collected with their medical records reviewed for each patient which including: name, gender, age, address, and date of collection. Depending on children medical records the patients were divided into two categories, 109 hospitalized patients lies in Alzahraa hospital, and outpatient (547). Stool specimens were collected in sterile containers. Rectal swab specimen were taken with sterile cotton-tapped swabs from a patients whom stool specimens could not be obtained at the time of collection. Of 656 patients, 356 male, and 300 female were involved in this study.

3-2-6 Isolation and Identification of Bacterial Isolates

All stool specimens were cultured on MacConkey agar plates. They were incubated overnight at 37°C in bacteriological incubators under aerobic conditions. Depending on morphological features of the colonies and microscopically examination with Gram's stain, pure cultures on MacConkey agar plates were made from each single group of colonies. The pure cultures were prepared for biochemical tests to differentiate *E. coli* from other *Enterobacteriaceae* depending on six biochemical tests (positive for methyl red and indole tests, negative in the

Voges–Proskauer, Simmon citrate, and urease tests), acid/acid with gas production in the triple sugar iron agar test (MacFaddin, 2000).

3-2-7 *Escherichia coli* Serotyping and Confirmatory Tests

The *E. coli* positive cultures were set for further serological test. Isolates biochemically identified as *E. coli* were serologically examined by slide agglutination test, according to Oxoid and Remel procedures, using polyvalent O antisera for EPEC, (2, 3, and 4), separately. From the colony which showed a positive reaction with polyvalent antisera a subculture was prepared and the isolates were retested by using EPEC monovalent O:K antisera mentioned in (3-1-5). A slide agglutination test was performed on a small amount of bacterial mass from 5 colonies of a pure culture transferred onto a glass slide containing two separate drops (40 µl each) of saline. The bacterial mass under test was emulsified with a loop in each drop of saline to give a smooth, fairly dense bacterial suspension. One drop (40 µl) of saline was added to one bacterial suspension and mixed as a control. To the other bacterial suspension a drop (40 µl) undiluted antiserum was added and mixed. The slide was rocked for one minute. The results were read with the naked eye by holding the slide against a dark background using indirect light source while swaying the slide (tilting it back and forth). Positive result performed by seeing the aggregation which should be strong and clearly visible within one minute and there was no visible aggregation in control suspension. A positive polyvalent result should be checked in confirmatory test using Api 20 E test.

3-2-8 Storage of Isolates

a. Short Term Storage:

The *E. coli* positive cultures were stored on nutrient agar slant at 4°C until further testing (Thomas, 2007).

b. Long Term Storage:

Fresh 24 hours, blood agar cultures of isolates were frozen in 20% glycerol nutrient broth and stored at -70°C until required (Thomas, 2007).

c. Subculture of Frozen Stocks

Frozen stock cultures stored at -70°C were sub-cultured on fresh blood agar plates, and then incubated in aerobic condition at 37°C for 24 hours (Thomas, 2007).

3-2-9 Polymerase Chain Reaction Assay

3-2-9-1 Preparing the Primers

The Alpha DNA primers were prepared depending on manufacturer instruction by dissolving the lyophilized product with TE buffer molecular grad after spinning down briefly. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

3-2-9-2 Enteropathogenic *E. coli* DNA Extraction (salting out method)

According to Pospiech and Neumann (1995), the DNA extraction was done as follow: a loopfull of EPEC growth were inoculated in 5 ml nutrient broth at 37°C for 24 hours. The bacterial growth was centrifuged at 6000 rpm for 5 minutes. The precipite was washed in TE buffer by adding 2 ml of TE buffer and vibrated by vortex then centrifuged at 6000

rpm for 10 minutes, and the washing repeated twice. The pellet re-suspended in 2.5 ml SET buffer. 300 µl of freshly made 25% SDS and 1ml of 5M NaCl solution were added to the lysate. The lysate was mixed by inversion, incubated at 55°C for 5 minutes, then mixed again thoroughly by inversion, and cooled to 37°C. A mixture of equal volume (3.8 ml) of [phenol: chloroform: isoamylacohol (25:24:1)] was added to the lysate and mixed by inversion for 30 minutes at room temperature. It was spin by centrifuging at 6000 rpm for 15 minutes. The aqueous phase was transferred to a fresh tube, then 0.6 volume of isopropanol was added to the extract and mixed by inversion for 3 minutes; the DNA spooled on to a pasture pipette. DNA rinsed in 5 ml of 70% ethanol, air dried for 15 minutes, and dissolved in 1 ml TE buffer, heated to 55°C in water-bath, and the DNA extract was stored in freezer at -20°C until used.

3-2-9-3 PCR Supplies Assembling and Thermocycling Conditions

Enteropathogenic *E. coli* DNA templates were subjected to PCR using 17 sets (F and R) of primers targeting two groups of genes: the first group listed in (3-1-8-3) to determine the virulence properties and the second group listed in (3-1-8-4) to determine β-lactam antibiotics resistance genes. The reaction mixture moreover contain Go Taq® Green Master Mix, X2 which is premixed ready-to-use solution containing bacteriology derived *Taq*DNA polymerase dNTP, $MgCl_2$, and reaction buffers at optimal concentrations and its recommended for any amplification reaction that to visualized by agarose gel electrophoreses and ethidium bromide staining.

Assembling PCR materials were done according to the procedure of Promega corporation (USA), using PCR reaction mixtures prepared in 0.2 ml eppendorf tube with 25 µl reaction volumes, which contain: 12.5

µl Go Taq® Green Master Mix X2, 2.5 µl upstream primer, 2.5 µl downstream primer, 5 µl DNA template, 2.5 µl nuclease-free water. All the appending was done in laminar flow on ice.

3-2-9-4 PCR cycling profiles

Polymerase chain reaction assays were carried out in a 25 µl reaction volume, and the PCR amplification conditions performed with a thermal cycler were specific to each single primer set depending on their reference procedure, as follows:

a. The first group:

primer name	Thermocycling condition		
	Denaturation	Anneling	Extension
Intimine (*eae*)	1 Hld 94.0 2:00	3 Tmp 30 Cycles 94.0 1:00 / 55.0 1:00 / 72.0 1:00	2 Holds * 72.0 ** 7:00 *** / 4.0 ∞
Bundle Forming Pilus (*bfpA*)	1 Hld 94.0 2:00	3 Tmp 30 Cycles 94.0 1:00 / 60.0 1:00 / 72.0 2:00	2 Holds 72.0 7:00 / 4.0 ∞
Bundle Forming Pilus (*bfp*)	1 Hld 95.0 5:00	3 Tmp 30 Cycles 94.0 0:30 / 56.0 1:00 / 72.0 2:00	2 Holds 72.0 10:00 / 4.0 ∞
EAF	1 Hld 94.0 5:00	3 Tmp 30 Cycles 94.0 1:00 / 57.0 0:45 / 72.0 1:00	2 Holds 72.0 7:00 / 4.0 ∞
Shiga toxin I (stx_1)	1 Hld 94.0 5:00	3 Tmp 35 Cycles 95.0 0:40 / 65.0 0:20 / 72.0 0:45	2 Holds 72.0 5:00 / 4.0 ∞
Shiga toxin II (stx_2)	1 Hld 94.0 5:00	3 Tmp 35 Cycles 95.0 0:40 / 65.0 0:20 / 72.0 0:45	2 Holds 72.0 5:00 / 4.0 ∞
EHEC (*hlyA*)	1 Hld 95.0 5:00	3 Tmp 35 Cycles 95.0 1:00 / 58.0 1:30 / 72.0 1:30	2 Holds 72.0 5:00 / 4.0 ∞

*The thermocycling condition diagram is a picture of PCR monitor for each primer
**The number above the line of thermocycling condition to each primer refer to tempretures
***The number above the line of thermocycling condition to each primer refer to time in minutes

b. The second group:

primer name	Thermocycling condition			
	Denaturation	Anneling	Extension ***	
bla_{TIM}	1 Hld 94.0 0:30	3 Tmp 35 Cycles 94.0 0:30 / 45.0 1:00 / 72.0 1:00	2 Holds * 72.0 10:00 **	4.0 ∞
bla_{SHV}	1 Hld 94.0 0:30	3 Tmp 35 Cycles 94.0 0:30 / 60.0 1:00 / 72.0 1:00	2 Holds 72.0 10:00	4.0 ∞
bla_{CTX-M}	1 Hld 94.0 0:30	3 Tmp 35 Cycles 94.0 0:30 / 60.0 1:00 / 72.0 1:00	2 Holds 72.0 10:00	4.0 ∞
bla_{AmpC}	1 Hld 94.0 0:30	3 Tmp 35 Cycles 94.0 0:30 / 60.0 1:00 / 72.0 1:00	2 Holds 72.0 10:00	4.0 ∞
bla_{IMP}	1 Hld 93.0 3:00	3 Tmp 40 Cycles 93.0 1:00 / 55.0 1:00 / 72.0 1:00	2 Holds 72.0 7:00	4.0 ∞
bla_{VIM}	1 Hld 93.0 3:00	3 Tmp 40 Cycles 93.0 1:00 / 55.0 1:00 / 72.0 1:00	2 Holds 72.0 7:00	4.0 ∞
bla_{GES}	1 Hld 93.0 3:00	3 Tmp 40 Cycles 93.0 1:00 / 55.0 1:00 / 72.0 1:00	2 Holds 72.0 7:00	4.0 ∞
bla_{PER}	1 Hld 93.0 3:00	3 Tmp 40 Cycles 93.0 1:00 / 55.0 1:00 / 72.0 1:00	2 Holds 72.0 7:00	4.0 ∞
bla_{VEB}	1 Hld 93.0 3:00	3 Tmp 40 Cycles 93.0 1:00 / 55.0 1:00 / 72.0 1:00	2 Holds 72.0 7:00	4.0 ∞
bla_{OXA}	1 Hld 94.0 5:00	3 Tmp 30 Cycles 94.0 0:50 / 55.0 0:50 / 72.0 1:00	2 Holds 72.0 5:00	4.0 ∞

*The thermocycling condition diagram is a picture of PCR monitor for each primer
**The number above the line of thermocycling condition to each primer refer to tempretures
***The number above the line of thermocycling condition to each primer refer to time in minutes

3-2-9-5 Preparation of Agarose Gel

Agarose gel was prepared by adding 1 gm of agarose powder to 100 ml of TBE buffer previously prepared (90 ml D.W. were added to 10 ml TBE buffer 10X, the final concentration was 1X and pH 8). The muddle was placed in boiling water bath until it become clear, then allowed to cool to 50˚C, and 1.5 µl ethidium bromide at concentration of 0.5 mg/ml was added. The agarose poured kindly in equilibrated gel tray earlier set with two combs fixed in the end and in the middle, and the two ends of gel tray were sealed. The agarose allowed solidifying at room temperature for 30 minutes. The comb was removed gently from the tray and the seal was removed from the ends of the tray. The comb made wells used for loading DNA samples.

3-2-10 PCR Product Analysis

3-2-10-1 Agarose Gel Electrophoresis

The amplified PCR products were detected by agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR products were loaded to the agarose gel wells: 5µl from single product to single well in known sequence, followed by 100 bp ladder to one of the wells in each row. The gel tray was fixed in electrophoresis chamber. 1X TBE buffer was added to the chamber until covered the surface of the gel. The electric current was performed at 60 volt for 1.5 hour.

3-2-10-2 Electrophoresis Results

The electrophoresis result was detected by using gel documentation. The base pair of DNA bands were measured according to the ladder. The positive results were distinguished when there was DNA band equal to the target product size. Finally, the gel was photographed using gel documentation saving picture.

3-2-11 Antibiotic Susceptibility Testing

All enteropathogenic *E. coli* isolates performed identification to susceptibility testing by modified disc-diffusion method (Kirby–Bauer) (Bauer *et al*., 1966). The selection of antibiotic disc was performed according to the guidelines recommended by the Clinical and Laboratory Standard (CLSI, 2007). Inoculums from the pure culture plate was prepared; a loopful of the growth is similarly suspended in a tube of tryptic soy broth and incubated for one hour at 37°C. The density of the suspension was adjusted depending on 0.5 McFarland standard by adding sterile normal saline. Mueller-Hinton agar plates were inoculated by sterile swab dipped into the inoculums. The excess inoculums were removed by pressing and rotating the swab firmly against the side of the tube above the level of the liquid. The swab was streaked all over the surface of the medium several times; the plate was rotated through an angle of 60° after each application finally, and the swab was pressed around the edge of the agar surface. Antibiotic disk list in (3-1-4) was applied to each plate by a pair of sterile forceps. Twenty discs were placed on a 15 cm plate, approximately 15 mm from the edge of the plate. Each disc gently pressed down to ensure even contact with the medium. The plates were placed in an incubator at 37°C. After 18 hours incubation, the diameter of each zone (including the diameter of the disc) were measured with a pair of calipers, and recorded in mm. The results then interpreted according to (CLSI, 2007), the critical diameters and to the leaflet of antibiotics manufactures. *E. coli* ATCC 25922 was used as negative control.

3-2-11-1 ESBL Screening Test

3-2-11-1-1 Initial Screening Test

Extended spectrum β-lactamase-producing EPEC isolates were detected by using an initial screening test and phenotypic confirmatory test (CLSI, 2007). Incubation conditions and procedure according to modified disc-diffusion method (Kirby–Bauer) (Bauer *et al*., 2003). In brief, 4 antimicrobial disks containing ceftazidime, ceftriaxone, cefotaxime, and aztreonam were applied to Mueller-Hinton agar plates inoculated with tested EPEC. The distances between antibiotic discs were 20 mm from each other. The plates were overnight incubated at 37°C. The diameter of each zone was measured. *E. coli* ATCC 25922 was used as negative control.

3-2-11-1-2 Double-Disk Synergy Test (DDST)

Confirmatory test was done by antimicrobial disks containing ceftazidime, ceftriaxone, cefotaxime, and aztreonam plus Amoxyclav in the center of the plate. This antibiotics were placed on Mueller-Hinton agar plates inoculated with tested EPEC. Comparing with initial screening test, enhancement of the zone of inhibition of ceftazidime, cefotaxime, and/or ceftriaxone, and aztreonam towards the clavulanic acid disc was reported as a positive result (Bedenić *et al*., 2010). *E. coli* ATCC 25922 was used as negative control.

3-2-11-2 AmpC Screening Test

3-2-11-2-1 Modified Three-Dimensional Test (MTDT)

Enteropathogenic *E. coli* isolates were overnight inoculated on Mueller-Hinton agar plates. 15 mg of bacterial growth were transferred into pre-weighted micro-centrifuge tube and then the tube re-weighted

again to make sure of the desired weight of e bacterial mass. The bacterial mass was suspended in peptone water and centrifuged at 3000 rpm for 15 minutes, than the supernatant were decanted. Crude enzyme were prepared by repeating freezing-thawing of the bacterial pellet 10 times. Lawn culture of *E. coli* ATCC 25922 was prepared on Mueller-Hinton agar plates and cefoxitin (30 µg) disk were placed on the center of the plates. Linear slits (3 cm) were cut by a sterile surgical blade, 3 mm away from cefoxitin disk. A small circular well at the other end of the slits was made and loaded with 25 µl of the bacterial extract. Overnight incubation at 37°C and the results were recorded. Any enhanced growth of the indicator strain, decreasing the radius of the cefoxitin inhibition zone at the end point of the slit, was considered as positive for Amp C (Coudron *et al*., 2000).

3-2-11-2-2 AmpC Disk

AmpC disk test for detection of plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* was done by the following procedure: sterilized circular filter papers (6mm Whitman No.1) were prepared. The circular filter each paper was moistened with (20 µl) of normal saline. Lawn cultures of *E. coli* ATCC 25922 were prepared on Mueller-Hinton agar plates. Several colonies of each test organism were applied to moistened filter paper disk. The inoculated disk was then placed beside a cefoxitin disk (almost touching) on the inoculated plate. The plates were incubated at 37°C for 24 hours. After incubation, plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result), or the absence of a distortion, indicating no inactivation of cefoxitin (negative result). Coudron *et al*., 2000; Black *et al*., 2005; Singhal *et al*., 2005).

3-2-11-2-3 Disc Antagonism Test

Screening for the inducible AmpC β-lactamase were done by placing cefoxitin disc at a distance of 20 mm from ceftazidime on the surface of Mueller-Hinton agar plates inoculated with EPEC isolates and incubated for 24 hours at 37°C. β-lactamase inducibility was recognized by blunting of the ceftazidime zone adjacent to cefoxitin disc (Taneja *et al*., 2008).

3-2-12 Statistical Analysis

The Chi-square test was applied to determine the statistical significance of the data. P value of <0.05 was considered significant (Paulson, 2008).

Chapter Four
Results
and
Discussion

4-1 Study population

The children age categorized into four age groups (Figure 4.1). Higher incidence of diarrhea was recorded at age group 7 to 12 months, with the number of recorded patients was 248 (37.8%), followed by high incidence at age group since birth up to 6 months, where the number of recorded patients was 218 (33.3%), followed by the lowest incidence in

Figure 4.1: The occurrence of diarrhea according to the age groups.

age group 13 to 18 months, where the number of recorded patient was 91 (13.9%), and the final group was in age 19 to 24 months, were the number of patients was 99 (15.1%). The high incidence of diarrhea was record in age groups from birth months to 12 months as they are most groups prone to this disease. The reason of higher infection in these two groups of infants may be due to low immunity, as the amount of tranceplacental antibodies of the child starts dwindling after 6 months of age. Also those children/infants may not have been breast-fed but bottle-fed instead, which is a source of infection and contamination. This becomes a matter of public health importance because such children may serve as a source of infection in the community. With the time, the child is exposed to pathogens and gets gradual resistance against infection.

Similar observation regarding the relative occurrence of the two age groups have been documented in other developing countries as well as the developed countries by several workers (Jafari *et al*., 2008; Dedeić-Ljubović *et al*., 2009; Usein *et al*., 2009).

The children with diarrhea were divided into two categories, 109 hospitalized patients lying in Alzahraa teaching hospital, and 655 outpatients. From this investigation, it was observed that the number of patients was higher in male (356) compared to females (300) (54.3% vs. 45.7%) (Figure 4.2).

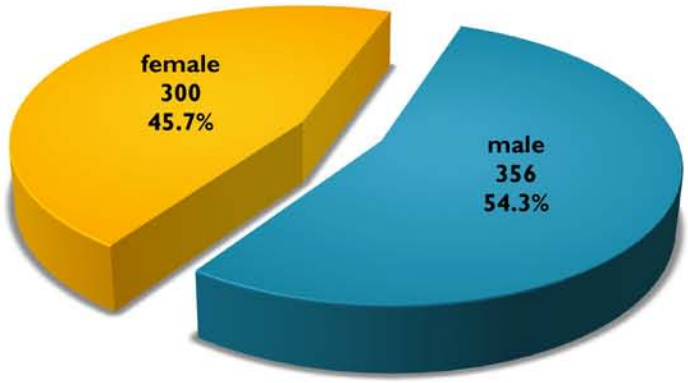


Figure 4.2: Number of infant patients with diarrhea depending on gender.

From these examinations, it was observed that the number of *E. coli* isolates from 656 stool specimens was 535 (81.6%) (basically, one *E. coli* isolate from each patient), while *E. coli* was not found in 121 (18.4%) specimens (Figure 4.3). A total of 52 (96.3%) *E. coli* isolates were obtained from 54 healthy individuals. The absence of *E. coli* in some specimens may be due to oral or parenteral antibiotic therapy that suppresses the bacterial normal flora. The antibiotic therapy may be given to the children in primary medical cares before they came to the hospital.

Another reason may be due to competitive bacteria that the fecal flora of breastfed infants differs from that of adults, with anaerobic Gram-positive rods of the genus *Bifidobacterium* constituting as much as 99% of the total, because of acidic environment (pH 5–5.5) due to breast milk (Ryan and Ray, 2004). However, pathogenic organisms other than *E. coli* were disregarded in this study.

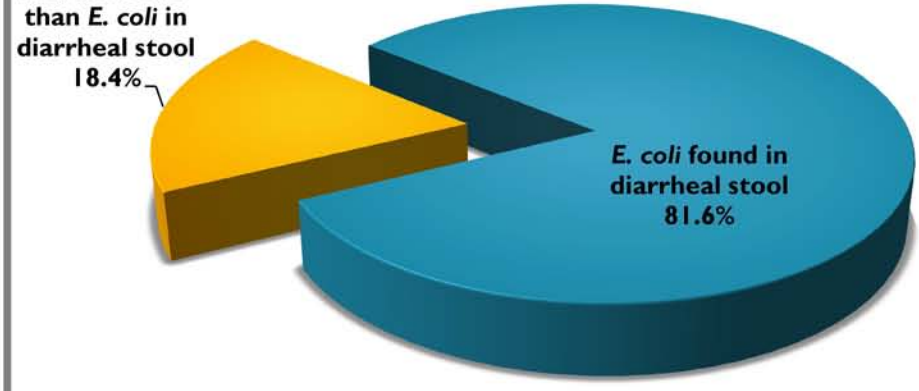


Figure 4.3: The occurrence of *E. coli* isolated from 656 children with diarrhea.

4-2 Detection of EPEC Serotypes

In the present study, biochemical characteristics of the *E. coli* isolates showed that they behaved as typical *E. coli* when they were screened serologically. Out of 535 *E. coli* isolates recovered from 656 children with diarrhea, only 22 (3.4%) were agglutinated with EPEC polyvalent antisera (Figure 4.4 and 4.5). Moreover, these 22 isolates were confirmed as *E. coli* by biochemical tests with the API 20 E miniaturized diagnosis test. By contrast, no EPEC isolate belonging to classical EPEC polyvalent serotypes was detected in 54 stool of healthy individual in this study.

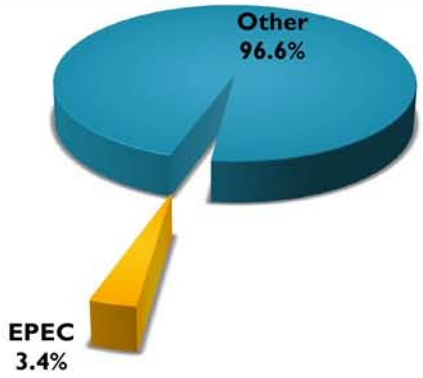


Figure 4.4: The frequency of EPEC isolates in 656 children with diarrhea.

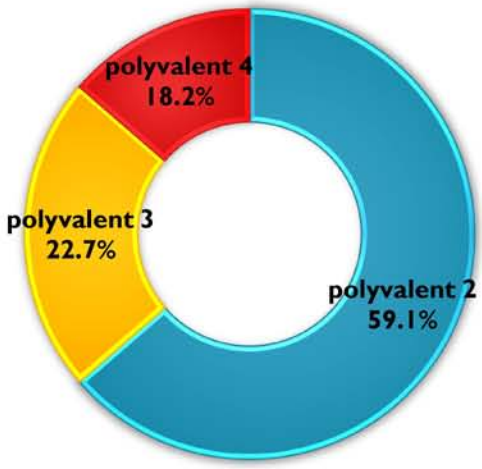


Figure 4.5: The spread of polyvalent serotypes in 22 EPEC recovered from children with diarrhea.

The relative frequency has also been demonstrated in the past by Almohana(2004) who reported that 37 (2.1%) *E. coli* isolates recovered from 1798 patients suffering from diarrhea were assigned as EPEC. Alternatively, this increase in distribution of EPEC in the present study might be attributed to decrease hygiene level in Najaf due to tragedic situations in Iraq due to terrorism, which lead to large numbers of

emigrates to this safer area. EPEC has been identified as important cause of infantile diarrhea in all the developing countries where it has been looked for, but the incidence has varied greatly in different studies. Present study revealed that EPEC was relatively infrequency isolated (3.4%), as was also found in study conducted in Tehran, Iran (8.4%) (Jafari *et al*., 2008), as was also found in studies conducted in Somalia (4.0%) (Casalino et al., 1988) and Thailand (5.5%) (Echeverria *et al*., 1991); however, a high frequency of EPEC was observed in Chile (38.3%) (Vine *et al*., 1988), Korea (56%) and Brazil (34.0%) (Gomes *et al*., 1991). In another study, EPEC serogroups were isolated as the sole pathogen from 44.9% of the Iranian children with diarrhea (Alikhani *et al*., 2006). However, the role of these pathogens in most probably underestimated in Najaf due to inappropriate diagnostic methods in clinical practice.

The study also demonstrated that high occurrence of EPEC were detected in hospitalized children (8.3%) as compare with outpatients (2.4%) (Figure 4.6). This may be due to nosocomial infections. In

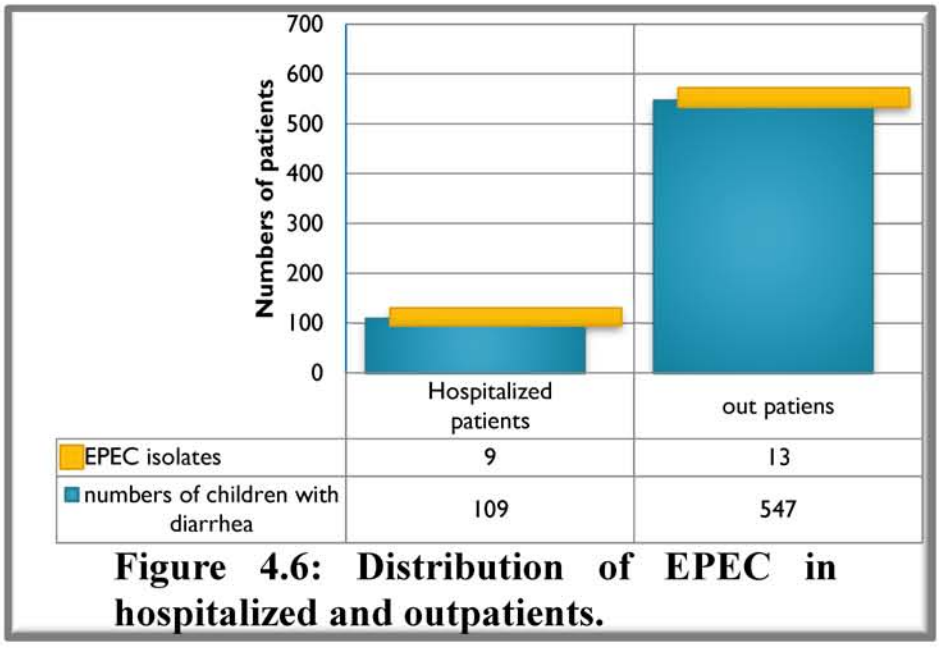


Figure 4.6: Distribution of EPEC in hospitalized and outpatients.

agreement with the present study, Alrifai *et al*. (2009) found that 25.9% of hospitalized children were acquired EPEC infections in Tikrit hospital, Iraq. Additionally, several studies have documented the spread of EPEC infection through hospitals, nurseries, and day care centers from an index causes (Bueris *et al*., 2007; Dedeić-Ljubović *et al*., 2009).

In this study, the highest number of EPEC isolates (9, 40.9%), was recovered from the 0-6 month age group, and was followed by the 19-24 month age group which produced 6 (27.3%) isolates, and the 7-12 month age group that produced 4 (18.2%) isolates. Only three isolates (13.6%) were recovered from the 13-18 month age group (Figure 4.7, Table 4.1).

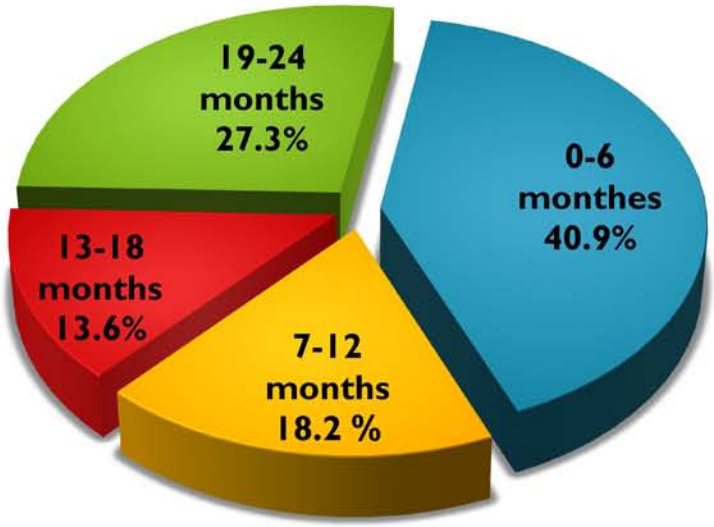


Figure 4.7: The distribution of EPEC isolates according to age groups.

The differences between these groups were found to be statistically significant ($p < 0.05$). This finding is disagreement with reports published by several authors (Jafari *et al*., 2008; ALHaj *et al*., 2008; Fujihara *et al*., 2009) where they observed that the highest incidence of gastroenteritis in children was found within the age range of 7-12 months, when weaning practice begin in many parts of the world (Iraq inclusive).

Table 4.1: Serotyping identification of EPEC isolated from children with diarrhea in Najaf

E. coli isolates	Gender	Age/month	Polyvalent	Monovalent
E1	M	17	2	O111:k58(B4)
E2	M	7	4	O44:k74(L)
E3	M	6	3	O125:k70(B15)
E4	M	12	2	O55:k59 (B5)
E5	F	6	3	O125:k70(B15)
E6	M	13	4	O44:k74(L)
E7	F	11	3	O128:k67(B12)
E8	F	2	3	O127:k63(B8)
E9	F	24	4	O44:k74(L)
E10	M	24	2	O111:k58(B4)
E11	F	24	2	O26:k60(B6)
E12	M	10	2	O111:k58(B4)
E13	M	3	2	O111:k58(B4)
E14	F	24	2	O26:k60(B6)
E15	F	6	2	O55:K59(B5)
E16	F	6	2	O55:K59(B5)
E17	M	6	2	O55:K59(B5)
E18	F	18	4	UT
E19	M	24	2	UT
E20	M	24	3	O114:k90(B)
E21	F	6	2	O55:K59(B5)
E22	F	6	2	O26:k60(B6)

En= *E. coli* isolates codes, M=male, F= female, Age per months, UT= untypeable

The WHO has considered isolates in the following twelve O serogroups to be EPEC strains (Robins-Browne *et al*., 2004; Yang *et al*., 2007): O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158. However, 8 of the 12 serogroups describe above were detected among the 22 EPEC isolates in the present study, including isolates of, Figure (4.8) shows the most common EPEC serotypes identified in the present study, the highest number of EPEC isolated belonged to polyvalent 2 sero-group with following serotypes, O55:K59(B5) (22.7%), O111:K58(B4) (18.18%), O26:K60(B6) (13.63%).

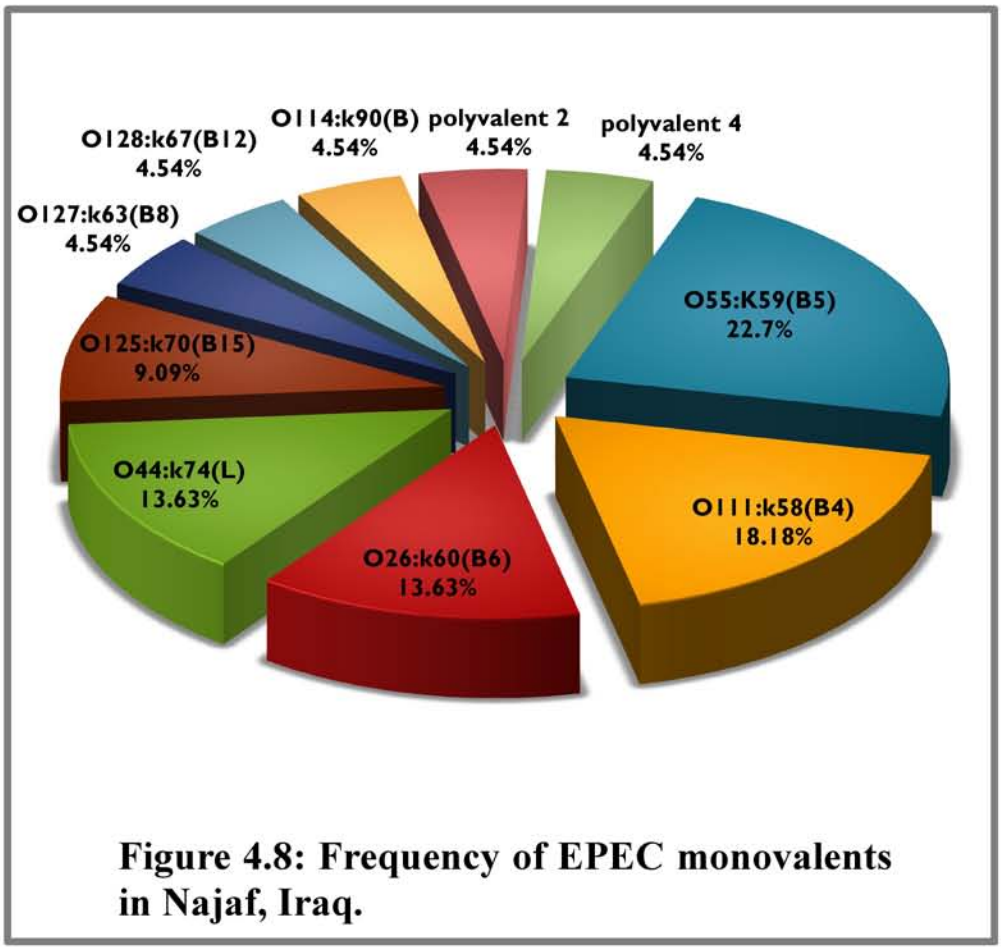


Figure 4.8: Frequency of EPEC monovalents in Najaf, Iraq.

The present study found that O55:K59(B5) was the most common EPEC isolates (22.7%) (Figure 4.8), that represented 0.8% of cases of

diarrhea in Najaf. According to our knowledge, this be a new EPEC serotype because it has not been reported previously in children with diarrhea in Najaf. In agreement with this study O55 serogroup was the most prevalent (12.6%) in Iran (Alikhani *et al*., 2006). In Romania, of the 250 *E. coli*, only three isolate demonstrated as O55, which represent 1.2% (Usein *et al*., 2009). In South Africa O55 represent 4.4% (Galane and Roux, 2001). These differences could possibly be related to hygiene, seasonality, as well as methodology issues.

Enteropathogenic *E. coli* O111:k58(B4) serotype was the second most common serotype isolates that were detected in 4 (18.2%) of the 22 EPEC isolates, constituting 0.6% of all diarrheal cases in Najaf. O111 serogroup of *E. coli* was discovered in 1945 to be the main cause of nonspecific gastroenteritis of infants and still frequently isolated from infants suffering from acute diarrheal episodes. In previous results conducted by Almohana(2004) show that the O111:K58(B4) was the most prevalent EPEC serotype (37.5%) isolated from patients with diarrhea in Najaf in the present study. However, the present results are similar to the finding of previous studies from Iran (19.18%) (Alikhani *et al*., 2006), and higher to that found in Bosnia and Herzegovina (4.34%) (Dedeić-Ljubović *et al*., 2009), and South Africa 8.3% (Galane and Roux, 2001). In Sao Paulo, Brazil, the serotypes O111 and O119 have been dominant for more than two decades (Gomes *et al*., 1991).

The serological identification in the present study led to detection of O26:k60(B6) serotype. Levine *et al*., (1987), has suggested that EPEC strains of serogroup O26 be classified as EHEC because they resemble EHEC strains rather than EPEC strains in that they produce large quantities of Verotoxins and possess a similar plasmid of molecular weight 60 x $l0^6$ that encodes a distinct adhesin. The O26:K60(B6) serotype in the present study, represent 13.6% of 22 EPEC isolates and

0.5% of all stool samples collected (Figure 4.8). This percentage resemble to that reported in South Africa (0.55%) (Galane and Roux, 2001).While these percentage was lower than that recorded in Bosnia 1.57% (Dedeić-Ljubović *et al*., 2009), in Romania 1.2% (Usein *et al*., 2009), and Iran, 1.6% (Alikhani *et al*., 2006).

The remaining EPEC isolates identified in this study were found to be belonged to serotypes O44:K74(L) (13.63%), O125:K70(B15) (9.09%), O127:K63(B8), O128:K67(B12) and O114:K90(B) (4.54% to each) (Figure 4.8). The observed result was similar to those reported previously that serogroups O44, O127 and O128 are associated with diarrhea in Najaf (Almohana, 2004). In addition, two serotypes of EPEC isolates [O125:k70(B15) and O114:k90(B)] were both newly identified in present study. Results also found that no isolate was belonged to serotypes O86, O119, O142 and O158 which may be due to variation of O serogroup between countries.

The serological investigations revealed that two *E. coli* isolates, E18 and E19, were agglutinated with polyvalent 2 and polyvalent 4, respectively but gave negative results when tested with specific monovalent antisera. Based on these criteria, the two isolates assigned into untypeable *E. coli* (Table 4.1). These two isolates represent 4.54% of the whole EPEC and 0.2% of causes of diarrhea in Najaf, separately. Similar results reported by Galane and Roux (2001) who found that 2 out of the 181 EPEC isolates gave positive agglutination with polyvalent 2 while negative agglutination with specific monovalent antisera.

After the discovery of EPEC as a cause of childhood diarrhea in 1945, EPEC was diagnosed frequently as a cause of pediatric diarrhea in developed countries over the next three decades (Afset *et al*., 2003). Then, for unknown reasons, the incidence of EPEC declined in this part

of the world (Nataro and Kaper, 1998). Detection of EPEC by serological screening for certain *E. coli* O-serogroups was established as diagnostic method more than 60 years ago, when outbreaks with traditional EPEC strains were frequent in Europe and North America (Kozub-Witkowski *et al*., 2008). The usefulness of serogrouping to define diarrheagenic *E. coli* remains controversial (Phantouamath *et al*., 2003). In the 1990s, the pathogenic factors of diarrheagenic *E. coli* were intensively studied and identified, leading to understanding of the molecular basis of EPEC pathogenicity, molecular detection methods such as DNA-hybridization and PCR were developed (Sunabe and Honma, 1998). The study reported herein stresses the importance of epidemiological survey on EPEC in Najaf. The importance of using combined methodologies (serotyping, and PCR technique) that increases diagnostic sensitivity is strongly emphasized.

4-3 Molecular Detection of Enteropathogenic *E. coli* Isolates

Identification of diarrheagenic *E. coli* strains requires the differentiation of these organisms from non-pathogenic members of the normal flora. Although serogrouping proposed by Levine *et al*. (1985) has been carried out to define these pathogenic strains, it is now recognized that the serogrouping is not well correlated with the presence of pathogenic factors (Phantouamath *et al*., 2003). Therefore, at present, the detection of pathogenic genes by PCR may be the best way to identify the diarrheagenic *E. coli*. PCR assays using single primer set for one of the pathogenic genes have been reported elsewhere (Phantouamath *et al*., 2003; Robins-Browne *et al*., 2004). However, at least six primer sets for one strain are required to identify the diarrheagenic *E. coli* that cause diarrhea. The PCR method showed high sensitivity and specificity for identification of human diarrheagenic *E. coli* (Aranda *et al*., 2004).

The molecular methods targeting chromosomal or mobile genetic elements coding for virulence traits seem to be more reliable for EPEC identification. In Najaf, EPEC diagnosis is restricted to serological tests (Polyvalent) for classical serotypes if present. Consequently, Almohana(2004) reports on the prevalence of the various diarrheagenic *E. coli* among the causes of intestinal infections are not only few in number, but also unreliable in nature. Therefore, the goal of present study was to document the association of EPEC isolates with diarrhea in children younger than 2 years of age in Najaf, based on the PCR identification of some their intrinsic virulence. The identification process in the present study conducted by PCR assays to detect the presence of specific virulence traits or the genes encoding these traits. DNA were extracted from all 22 EPEC serologically identified by salting out method. The primers were selected according to references listed in (3-1-8-3), considering the most referenced and frequent sequences. Some primers were duplicated in different sequence to confirm the results. However, from the total isolates, 6 (27.3%) isolates were PCR-positive for at least one of the targeted virulent genes (Table 4.2).

Possession of *eaeA*, which plays an important role in intimate adhesion to intestinal epithelial cells and in producing attaching and effacing (A/E) lesions, was thought to be the primary criterion for defining isolate as EPEC in this investigation. The *eaeA* primer was subjected to all the serotyped EPEC isolates. Only 2 (9.1%) isolates belong to serotype O125:k70(B15) gave positive results (Figure 4.9, Table 4.2). The two isolates positive for *eaeA* gene. This finding goes in accordance with previous report by Ratchtrachenchai *et al*. (2004) who reported that only one of the 39 *eaeA* positive isolates belonged to O125 serogroup. Since there is no data recorded for presence of *eaeA* gene in the EPEC isolates in Najaf, the present study cannot identify the

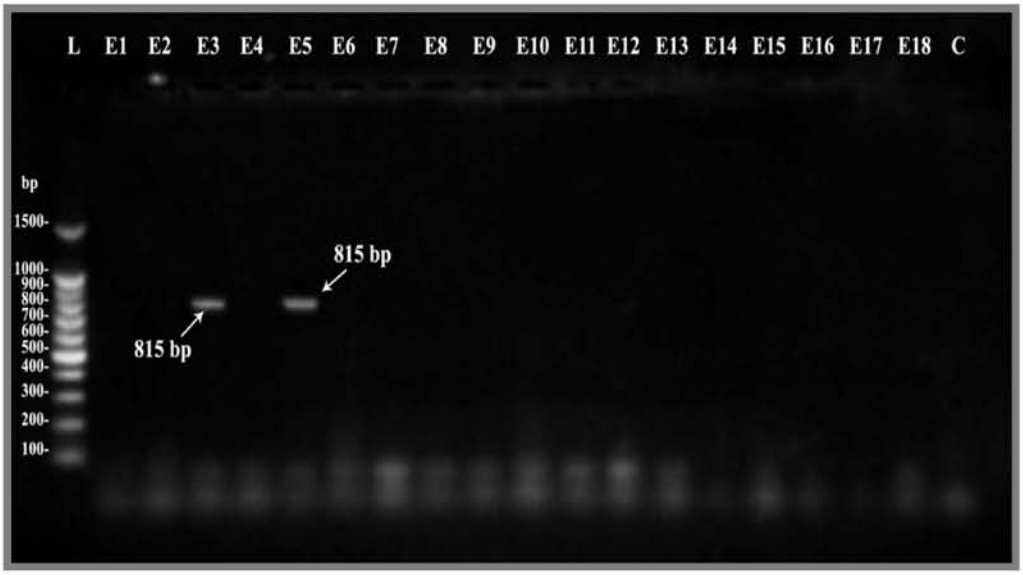


Figure 4.9: Ethidium bromide-stained agarose gel of PCR amplified products from extracted *E. coli* DNA amplified with primers *eaeA* F and *eaeA* R.

Lane (L), DNA molecular size marker(100-bp ladder)

Lane (E3), *E. coli* O125:k70(B15) show positive results with *eaeA* gene.

Lane (E5), *E. coli* O125:k70(B15) show positive results with *eaeA* gene.

Lanes (E1, 2, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19) EPEC isolates show negative results to *eaeA* gene

Lane (C), negative control

Table 4.2: Genotypic characterization of typical, atypical and non-EPEC serotypes

E. coli isolate code	EPEC		Result of PCR							Virulence category
	Polyvalent	Monovalent	*eaeA*	*bfpA*	*bfpA**	*EAF*	stx_1	stx_2	*hlyA*	
E1	2	O111:k58(B4)	-	+	+	-	-	-	-	Atypical EPEC
E2	4	O44:k74(L)	-	-	-	-	-	-	-	Non-EPEC
E3	3	O125:k70(B15)	+	+	+	+	-	-	-	Typical EPEC
E4	2	O55:k59(B5)	-	-	-	-	-	-	-	Non-EPEC
E5	3	O125:k70(B15)	+	+	+	+	-	-	-	Typical EPEC
E6	4	O44:k74 (L)	-	+	-	-	-	-	-	Atypical EPEC
E7	3	O128:k67(B12)	-	+	-	-	-	-	-	Atypical EPEC
E8	3	O127:k63 (B8)	-	-	-	-	-	-	-	Non-EPEC
E9	4	O44:k74(L)	-	-	-	-	-	-	-	Non-EPEC
E10	2	O111:k58(B4)	-	-	-	-	-	-	-	Non-EPEC
E11	2	O26:k60(B6)	-	-	-	-	-	-	-	Non-EPEC
E12	2	O111:k58(B4)	-	-	-	-	-	-	-	Non-EPEC
E13	2	O111:k58(B4)	-	+	-	-	-	-	-	Atypical EPEC
E14	2	O26:k60(B6)	-	-	-	-	-	-	-	Non-EPEC
E15	2	O55:K59(B5)	-	-	-	-	-	-	-	Non-EPEC
E16	2	O55:K59(B5)	-	-	-	-	-	-	-	Non-EPEC
E17	2	O55:K59(B5)	-	-	-	-	-	-	-	Non-EPEC
E18	4	UT	-	-	-	-	-	-	-	Non-EPEC
E19	2	UT	-	-	-	-	-	-	-	Non-EPEC
E20	3	O114:k90(B)	-	-	-	-	-	-	-	Non-EPEC
E21	2	O26:k60(B6)	-	-	-	-	-	-	-	Non-EPEC
E22	2	O111:k58(B4)	-	-	-	-	-	-	-	Non-EPEC

*bfpA**= primer mention by Obi *et al.* (2004); UT= untypeable

difference in the incidence of this virulence factor. However, the isolation frequency of EPEC having *eaeA* gene was not as high as expected in Najaf. The isolation frequency having this gene may vary tending on the district, season, child age, and so on. Notably, a high incidence of EPEC defined on the basis of *eaeA* gene was reported by Nguyen *et al*. (2005) in Hanoi, Vietnam, who found that all tested EPEC isolates were *eaeA* PCR positive. In an Iranian study, the *eaeA* gene was detected in 45 (40.5%) of the 111 EPEC examined strains (Alikhani *et al*., 2006). A lower incidence of 8.8% and 10.9% has been reported in Thailand and South Africa respectively (Ratchtrachenchai *et al*., 2004; Obi *et al*., 2004). On the other hand, Nishikawa *et al*. (2002) reported that all EPEC strains isolated from children with diarrhea did not react with *eaeA* specific primer in Japan. In another study, Phantouamath *et al*. (2003) found no strain of the 77 serological identified EPEC had the *eaeA* gene. However the results of this study concluded that EPEC isolates possess the *eaeA* gene are uncommon cause of diarrhea in Najaf.

In this work, all isolates that serotyped as EPEC were screened for the presence of bundle forming pili (type IV pili). DNA sequences for *bfpA* gene were assayed using specific primers (Afset *et al*., 2003; Obe *et al*., 2004). Of the 22 EPEC examined, only 4 (18.2%) isolates gave positive results with *bfpA* primers that equal to target product size (326 bp) (Figure 4.10). Two isolates belong to O125:k70 (B15) serotype and two isolates belong to O111:k58(B4) serotype. The ethidium bromide-stained agarose gel of PCR amplified products show other two products (200 bp) belonging to O44:k74 (L) and O128:k67(B12) (Figure 4.10) which may be considered as positive result depending on previous study conducted by Carneiro *et al*. (2006), who reported that present of 200 bp amplicons with *bfpA* primers, suggesting a deletion on this gene that could contribute for the lower efficiency with which these strains attached to

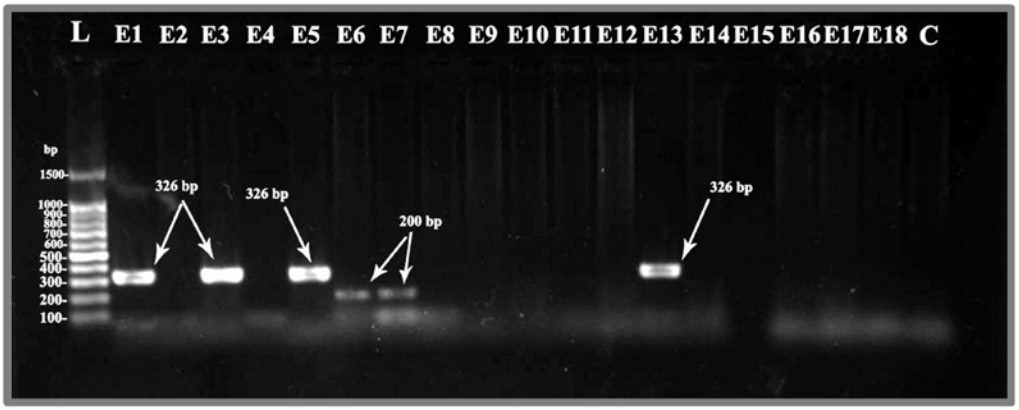


Figure 4.10: Ethidium bromide-stained agarose gel of PCR amplified products from extracted *E. coli* DNA amplified with primers *bfpA* F and *bfpA* R.

Lane (L), DNA molecular size marker(100-bp ladder)

Lane (E1), *E. coli* O111:k58(B4)show positive results with *bfpA* gene 326bp.

Lane (E3), *E. coli* O125:k70(B15) show positive results with *bfpA* gene326bp.

Lane (E5), *E. coli* O125:k70(B15) show positive results with *bfpA* gene326bp.

Lane (E6), *E. coli* O128:k67(B12) show positive results with *bfpA* gene 200bp.

Lane (E7), *E. coli* O127:k63(B8) show positive results with *bfpA* gene 200bp.

Lane (E13), *E. coli* O111:k58(B4) show positive results with *bfpA* gene 326bp.

Lanes (E2, 4, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18 and 19) EPEC isolates show negative results with *bfpA* gene

Lane (C), negative control

cells, producing a localized adherence like type phenotype. Consequently, the present study demonstrates 6 PCR amplified products with *bfpA* (27.27%) from serotypically identified EPEC. The occurrence of *bfpA* in the present study was analogous to that recorded by other studies such as, Alikhani *et al*. (2006) who found that only 35 of 111 (31.5%) strains isolated from children with diarrhea in Iran gave positive results for the *bfpA* gene, two of them belong to O111. In a study conducted in Brazil, Orlandi *et al*. (2006) reported that (34.5%) of EPEC isolate gave positive results to *bfpA*, while in South Africa, Obi *et al*. (2004) showed that the *bfpA* gene coding for EPEC was most frequently detected (22.7%) among *E. coli* isolates. On the other hand, the prevalence of *bfpA* gene observed in this study was marginally higher compared with the studies of Afset *et al*. (2003) in Norwegian children with diarrhea, who established that only one of the 450 EPEC isolate was *bfpA* gene positive (0.2%) belonging to O111:k58(B4) serotype, Blanco *et al*. (2006) in Spain demonstrated that 4.45% of EPEC isolate gave positive results to *bfpA*, and Galane and Roux (2001) in South Africa who found that 6 of 48 EPEC isolates (10.5%) were positive to *bfpA* gene.

The most regular matter in EPEC is the presence of *eaeA* alone (atypical EPEC) or with *bfpA* (typical). Unusual in this study was the detection of *bfpA* without the *eaeA* gene (Table 4.2). This observation was also documented by Kobayashi *et al*. (2000) who found that two isolates were positive to *bfpA* gene but negative to *eaeA* gene; Carneiro *et al*. (2006) reported that one strain (serogroup O125) carried the *bfpA* gene but not the *eaeA* gene still adhered in a localized adherent like pattern but as expected was incapable of eliciting the A/E lesion, and as well in study conducted in Germany by Hardegen *et al*. (2010), recorded that 5 out of the 144 EPEC isolates were *bfpA* positive and *eaeA* negative.

According to the above studies, present result revealed that the sequence variation within the intimin gene or variation in the primer sequence did not result in a failure of amplification of *eaeA* gene. It is problematic whether isolates positive for *bfpA* but not for *eaeA* should be classified as EPEC.

Another sequence mentioned by Obi *et al*. (2004) of the Bfp primer encoding *bfpA* gene, was also subjected to all the 22 EPEC serotypes. Only three isolates (13.6%) O125:k70(B15) (2 isolates), and O111:k58(B4) (1 isolate) gave positive results (Figure 4.11). The serotypes of this three isolates were (Table 4.2). The two sequences applied in this study showed variation in the results, although they are targeting the same gene and the differences in one nitrogen base. The first primer gave six amplification product visualized in gel documentation (Figure 4.10), and the second primer gave only three amplification product (Figure 4.11), this finding leads to the belief that the first sequence was more sensitive and significant for determination of *bfpA* gene.

Gomez and Kaper (1992) described a cluster of genes (*perA,B,C*) that regulate the expression of *eaeA* that are located in a 60-MDa (EAF) plasmid. The EAF plasmid also harbours the *bfpA* gene cluster and is widely distributed among EPEC. The role of EAF plasmid in promoting the virulence of EPEC was established by Levine *et al*. (1985), who showed that an EAF-negative derivative strain of EPEC, E2348/69, is markedly less virulent for adult volunteers than the wild-type strain. The same study show that an atypical EPEC strain, E128012, which intrinsically lacks EAF, is also virulent in volunteers. This observation established certain EPEC strains do not require EAF plasmid to cause disease. These intrinsically EAF-negative strain were originally called

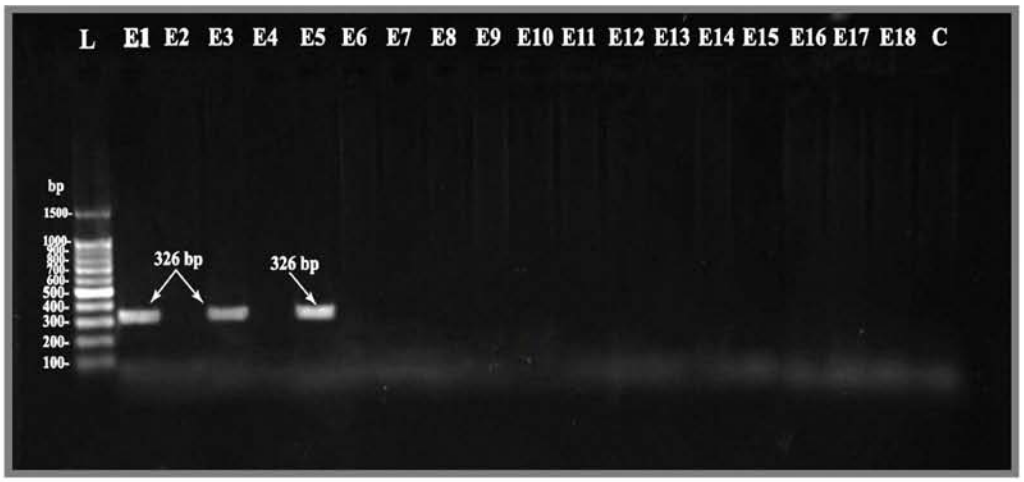


Figure 4.11: Ethidium bromide-stained agarose gel of PCR amplified products from extracted *E. coli* DNA amplified with primers *bfpA* F and *bfpA* R.

Lane (L), DNA molecular size marker (100-bp ladder)

Lane (E1), E. coli O111:k58(B4)show positive results with *bfpA* gene 326bp.

Lane (E3), *E. coli* O125:k70(B15) show positive results with *bfpA* gene326bp.

Lane (E5), *E. coli* O125:k70(B15) show positive results with *bfpA* gene326bp.

Lanes (E2, 4, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18 and 19) EPEC isolates show negative results with *bfpA* gene

Lane (C), negative control

atypical EPEC (Trabulsi *et al*., 2002; Robins-Browne *et al*., 2004). In this study, the PCR results obtained from DNA extracted from EPEC indicated that only two (9.09%) of the 22 EPEC isolates belonged to serotype O125:k70(B115) were found to harbour the *EAF* plasmid gene (Figure 4.12, Table 4.2)

Unusual in this study is the detection of *bfpA* gene in four isolates but not the *EAF* gene (Table 4.2). It's questionable whether isolates positive for *bfpA* gene but not for EAF plasmid should be classified as EPEC and whether, they actually are pathogenic. The reason for these phenomena is currently unknown. However this may be due to the absence of EAF probe from the EAF plasmid without any deleterious effects on localized adherence expression (Law, 1994). The EAF probe used to detect EPEC not recognized in some typical EPEC isolates, and the frequency of these organisms in infection may have been underestimated (Scotland *et al.*, 1991). There are no report in Iraq that can be compared with the results of the present study and concerning detection of EAF probe and plasmid in EPEC. However, the absence of the EAF plasmid in most *bfpA*-positive EPEC isolates in the present investigation was in agreement with the observation of the other workers (Tarabulsi *et al*., 2002). Similarly, Blanco *et al*. (2006) detected the EAF plasmid in only one out of the seven typical EPEC strain isolated from Switzerland. Additionally, Scaletsky *et al*. (2002) found only 17 of the 101 *bfpA*-positive EPEC isolates gave positive results with *EAF* gene. In another study by Kobayashi *et al*. (2000) recorded that two isolates of the EPEC recovered from children with diarrhea were positive to *bfpA* but negative to EAF plasmid. Based on this study observation, it can be concluded that the EAF PCR assay proved to be a nonspecific and inefficient method for the detection of EPEC isolates carrying the EAF plasmid.

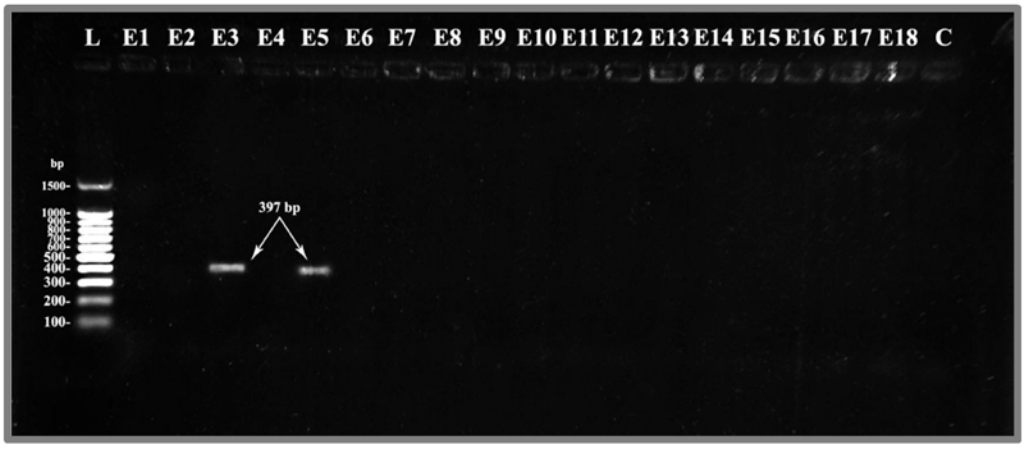


Figure 4.12: Ethidium bromide-stained agarose gel of PCR amplified products from extracted *E. coli* DNA amplified with primers *EAF* F and *EAF* R.

Lane (L), DNA molecular size marker(100-bp ladder)

Lane (E3), *E. coli* O125:k70(B15) show positive results with *EAF* gene.

Lane (E5), *E. coli* O125:k70(B15) show positive results with *EAF* gene.

Lanes (E1, 2, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19) EPEC isolates show negative results with *EAF* gene

Lane (C), negative control

Enterohemorrhagic *E. coli* producing Shiga like toxin previously named verotoxin-producing *E. coli* are responsible for bloody diarrhea, haemorrhagic colitis which may be complicated by hemolytic uremic syndrome (HUS) with some times severe neurological symptoms (Durso *et al*., 2005). EHEC strain may possess the intimin factor coded by the *eaeA* gene but their main virulence factor is Shiga like toxin (Fröhlicher *et al*., 2008). Shiga like toxin is very similar to *Shigella dysenteriae* type I toxin. The strains of Shiga toxin-producing *E. coli* (STEC) expressed only Shiga like toxin, two genes stx_1 and stx_2 have been identified (Nataro and Kaper, 1998). Confusion prevails among atypical enteropathogenic *E. coli* (EPEC) and STEC when the assay is incomplete with the detection of *eaeA* gene alone. To avoid such situation, it is recommended to detect *bfpA* as well as *stx* genes for the differential identification of EPEC and STEC, respectively (Ramamurthy, 2008). In the present study, none of the 22 EPEC serotypes tested revealed the presence of stx_1 and/or stx_2 genes by PCR method. However, the present findings disagree with previous a study that found rare occurrence of STEC (0.4%) in patients with diarrhea in Najaf (Almohana, 2004).

Enterohemolysin toxin was also investigated in this study as confirmatory test to differentiate some of atypical EPEC and to identify EHEC if present. Consequently, EHEC *hlyA* encoding gene was detected using its specific primer. None of the 22 EPEC serotypes were positive for EHEC *hlyA* gene, which is required for the production of EHEC enterohemolysin. However, in only one study in Najaf, Almohana(2004) found that six (0.7%) out of 850 *E. coli* isolated from patients with diarrhea and healthy person showed typical enterohemolytic phenotype. In another study conducted in Romania, the enterohemolysin encoding

gene was detected in one of VTEC isolates, as well as in 2 *E. coli* isolates classified as atypical EPEC (Usein *et al*., 2009).

4-4 Typical and Atypical Enteropathogenic *E. coli*

Enteropathogenic *E. coli* is a leading to cause diarrhea in many countries. Typically EPEC strain possess LEE and EAF. The LEE pathogenicity island contain the *eaeA* gene and the EAF plasmid encodes Bfp fimbria. It has been reported that the EAF plasmid can be lost during storage or even during infection, but the absence of this plasmid in certain serotypes (i.e, O111:H9 and O26:H11) seems to be a natural occurrence (Levine *et al*., 1985; Gonçalves *et al*., 1997). Kaper, (1996), proposed the designation of these strains atypical EPEC for. In the present study, 6 (27.3%) of the 22 EPEC isolates were amplified with at least one of the 7 tested virulence genes (Table 4.2). The minimum criteria for determination of the diarrheagenic *E. coli* were as follows: the presence of stx_1 and/or stx_2 for VTEC, the presence of *eaeA* and *bfpA* for 'classical' or 'typical' EPEC, and the presence of *eaeA* only or *eaeA* with *hlyA* for atypical EPEC (Afset *et al*., 2003). Based on these criteria, two isolates of the six (33.3%) were classified as typical EPEC (Table 4.2), these isolates were belonged to O125:k70(B15) serotype, obtained from infants younger than six months. Typical EPEC strains are diarrheagenic *E. coli* historically associated with outbreaks of infantile diarrhea, particularly during the 1940s and 1950s. Although large outbreaks of infant diarrhea due to typical EPEC have largely disappeared from industrialized countries (Blanco *et al*., 2006), typical EPEC strains remain an important cause of potentially fetal infant diarrhea in developing countries (Bueris *et al*., 2007). In Brazil, up to the 1990s, typical EPEC was the main cause of acute diarrhea in children younger than one year old, of low socio-economic status (Gomes *et al*., 1991;Trabulsi *et al*., 2002). In Iran,

Alikhani *et al*. (2006), reported that the typical EPEC strains continue to be an important cause of diarrhea in children, mostly encountered among the strains belonging to serogroups O55 and O86.

In a meeting on EPEC held in 1995, a consensus definition of atypical EPEC was established, namely, that they are *EAF*$^-$ (*bfpA*$^-$), *eaeA*$^+$ strains that promote attaching and effacing lesions (Gomes *et al*., 2004). The present study demonstrated unusual detection of four isolates carrying *bfpA* gene without the *eaeA* gene (Table 4.2). Hardegen *et al*. (2010) detected five isolates which were positive for EAF plasmid only, and reported this question, whether *E. coli* strains positive for EAF plasmid but not for *eaeA* should be classified as EPEC. Alikhani *et al*. (2006) reported that, the present of only one determinates (*eaeA* or *bfpA*) used to identify EPEC as atypical. Therefore, these four isolates 66.7% recovered in the present study were classified as atypical EPEC. The atypical EPEC isolates belonged to O111:k58(B4) (2 isolates), O44:k74(L) (1 isolate), O128:k67(B12) (1 isolate) serotypes, obtained from children with diarrhea aged from 3 months to 17 months in Najaf. In agreement with Al-Gallas *et al*. (2007) in Tunisia, atypical EPEC represented 66.7% of isolates. In Tanzania the frequency of diarrhea caused by EPEC was 5.3%, atypical EPEC represented 62.5% of isolates (Vargas *et al*., 2004), as well as in Peru, the frequency of diarrhea caused by EPEC was 3.9%, atypical EPEC representing 88.9% of isolates (Barletta *et al*., 2007). The highest frequency of atypical EPEC isolates reported in Vietnam that all EPEC isolates were identified as atypical EPEC (Ochoaa *et al*., 2008). Atypical EPEC appear to be more closely related to STEC and as such organism are considered emerging pathogens (Beutin *et al*., 2003). Outbreaks of adult gastroenteritis due to atypical EPEC have been reported by Bueris *et al*. (2007). Typical EPEC are

rarely found in animals and can be isolated from human only (Fröhlicher *et al*., 2008), and thus humans appear to be the unique living reservoir for these organisms. It is likely that, the frequency of typical-EPEC has been influenced by the recently improved public health measures, such as more efficient control of hospital infections and implementation of sanitary conditions, more than the frequency of the atypical EPEC and EHEC which are hosted by humans and animals (Trabulsi *et al*., 2002).

In total, 16 of 22 (72.7%) isolates recovered from children with diarrhea were negative to all virulence factors genes applied in this study. Consequently, the incidence of EPEC in Najaf was 0.9% according to molecular techniques. The frequency of EPEC in Najaf was lower than that conducted in Iran by Alikhani *et al*. (2006) who reported that EPEC isolates were 18.2% from children with diarrhea, and Jafari *et al*. (2009) who found that, 70 (12.6%)of 555 isolates obtained from patient with diarrhea were identified as EPEC, as well as, 14.3% in Brazil, 10.6% in Chili, and 5.2% in Tunisia (Scaletsky *et al*., 2002; Vidal *et al*., 2005; Al-Gallas *et al*., 2007).

According to the results of the present study, it is concluded that the serotyping of *E. coli* is not likely correspond to the pathogenic factors. Significant difference were found between the serotyping method and molecular techniques in detection of EPEC ($P<0.05$). The molecular methods was more specific but slower than serotyping method. The possession of EPEC-related O and K antigens is no longer deemed an essential characteristic of true pathogenic EPEC strains (Carneiro *et al*., 2006). Based on these findings, the usefulness of serotyping to define diarrheagenic *E. coli* remains controversial. It is important to clarify this issue, given that the serotyping method is still used in clinical laboratories to define the serotypes of *E. coli.*

4-5 Antibiotics Susceptibility Results

4-5-1 Improve Surveillance of Antibiotic Resistance

A variety of antibiotics have been used to treat infection caused by EPEC and have proved useful in many cases, but multiple antibiotic resistances are common among EPEC (Brenner *et al*, 2004). Many strains of EPEC are known to harbour mobile elements that encode antibiotic resistance and can be transferred among themselves or to other bacterial species (Martí, 2005) to establish multiple antibiotic resistances. The widespread use of antibiotics has been identified as a major factor accounting for the increased incidence of antibiotics resistance (Eom *et al*., 2002). Detection and monitoring of multi-antibiotic resistant EPEC is important to substantiate the choice of antibiotics for the treatment of infections cause by this organism.

The isolates belonging to EPEC serotypes were tested by disc diffusion method (Bauer *et al*., 1966) and were interpreted according to Clinical Laboratory Standard Institute guidelines (CLSI, 2007) as susceptible, intermediate or resistant.

Out of the 22 EPEC isolates, 2 (9.1%) isolates were resistant to 2 antibiotics, 20 (90.9%) isolates were resistant to 3 or more antibiotics, but no one isolate was susceptible to all antibiotics tested (Figure 4.13). Present data showed that the incidence of resistance to most antibiotics tested for EPEC isolates is high in Najaf.

A strain of EPEC is considered as a multidrug resistant if it were resistant to at least three antibiotic classes (Eom *et al*., 2002). However, the present study revealed that a high percentage of EPEC isolates (90.9%) were multidrug resistant showing resistance to a minimum of three classes of the antibiotics tested. Almohana(2004) analyzed 37 EPEC isolates obtained from patient with diarrhea in Najaf, the reported percentage of multidrug resistance (56.8%) was lower than those found in

the present study. This percentage was higher in another study conducted by Hadi (2008), who reported that 82.6% of *E. coli* isolates in Najaf were multidrug resistance. In contrast, Al-Hilli (2010) found that all *E. coli* isolate obtained from Merjan Teaching Hospital in Hilla, Iraq, were considered as multidrug resistant. These results confirmed data reported by other authors, indicating that EPEC are frequently and increasingly demonstrating multiple resistances to the antibiotics (Fei *et al*., 2003). However, the high occurrence of multidrug resistant isolates of EPEC may be due to the widespread use of antibiotics. The continuous used of even a single antibiotic over a period of weeks or months will select bacteria resistant to different kind of antibiotics in addition to the one in use (Livermore., 2007). Transference of resistance determinants by mobile genetic elements including plasmids, transposons, and gene cassettes in integrons between and across different bacterial species with relative ease important factor that can contribute to the increase of multiresistant bacteria (Livermore, 2007).

Multiple antibiotics resistance can occur even in the absence of plasmid or transposon. The study published by Levy (1992) showed that plasmid- and trasposon-free *E. coli* was resistant up to seven types of antibiotics. This higher level of resistance may be caused by initial mutation located in a single site on the *E. coli* chromosome. Since none of the EPEC isolates used in this study had been examined for their ability to transfer their antibiotic resistance genes, it's not possible with certainly to determine if the antibiotic resistance was plasmid-mediated. The casual antibiotics usage, without antibiotic sensitivity testing, is the most important factor promoting the emergence of multidrug resistance which lead to selection and dissemination of antibiotic resistant pathogens in clinical medicine (Neu, 1992).

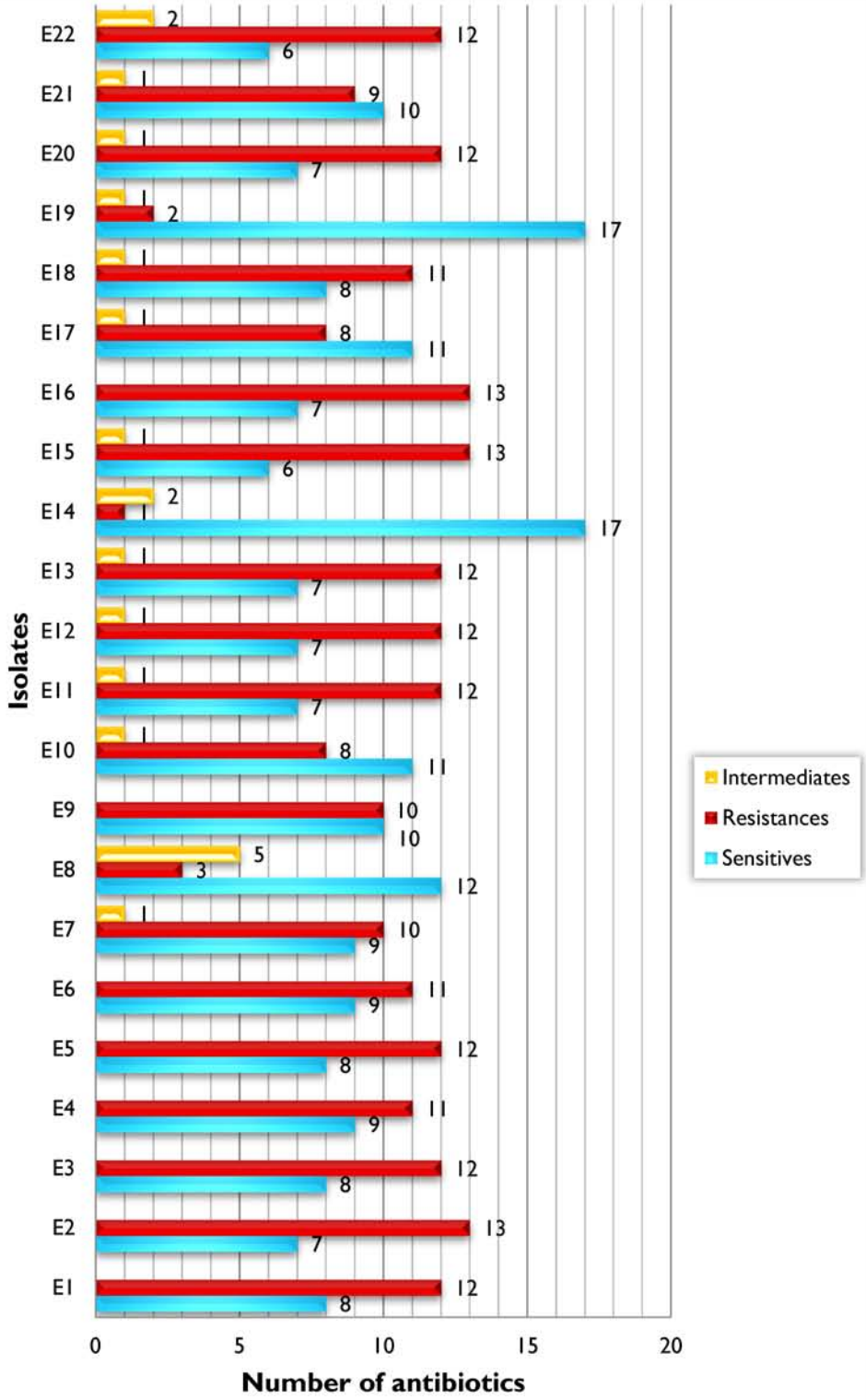


Figure 4.13: Antibiotic susceptibility of 22 EPEC isolates.

High rates of resistance were recorded for carbenicillin (90.9%), amoxicillin, piperacillin, and amoxyclav (86.4% each) (Figure 4.14). Several reports have indicated that these drugs were also less effective against *E. coli* and other bacterial agents isolated in Najaf (Almohana, 2004; Hadi, 2008), largely because they are inexpensive and can be obtained easily without a medical prescription, resistance is probably due to indiscriminate antibiotics usage (drug abuse) which could result in plasmid-mediated antibiotic resistance found to be common in *E. coli* (Taneja *et al*., 2008). According to this result, the above antibiotics should not be used for treatment diarrhea and other disease caused by *E. coli* isolate in Najaf hospitals. Therefore, local information about antibiotics resistance should be used in clinical management and treatment guideline should be update routinely.

Antibiotic susceptibility testing of isolates revealed high rate of resistance to third generation cephalosporins including ceftazidime (72.7%), cefotaxime (68.2%), and ceftriaxone (59.1%) (Figure 4.14). Although the cephalosporins are not indicated to routinely to treat diarrhea (Nguyen *et al*., 2005), the current study have tested the susceptibility of EPEC isolates to these antibiotics, since they could be empirically or incidentally used. Most parenteral third-generation cephalosporins, e.g. cefotaxime, are administrated in a hospital setting (DuPont and Herbert, 2005). However, it might be possible that this high level of resistance to third-generation cephalosporin in present study was most probably due to acquisition of β-lactamases which encodes by *bla* genes by these isolates , possibly during therapy.

Results of this study revealed high frequency of resistant to cefoxitin (90.9%), equal to carbenicillin (Figure 4.14). Reduced susceptibility to cefoxitin in the *E. coli* may be an indicator to AmpC activity, but cefoxitin resistance may also be mediated by alterations in

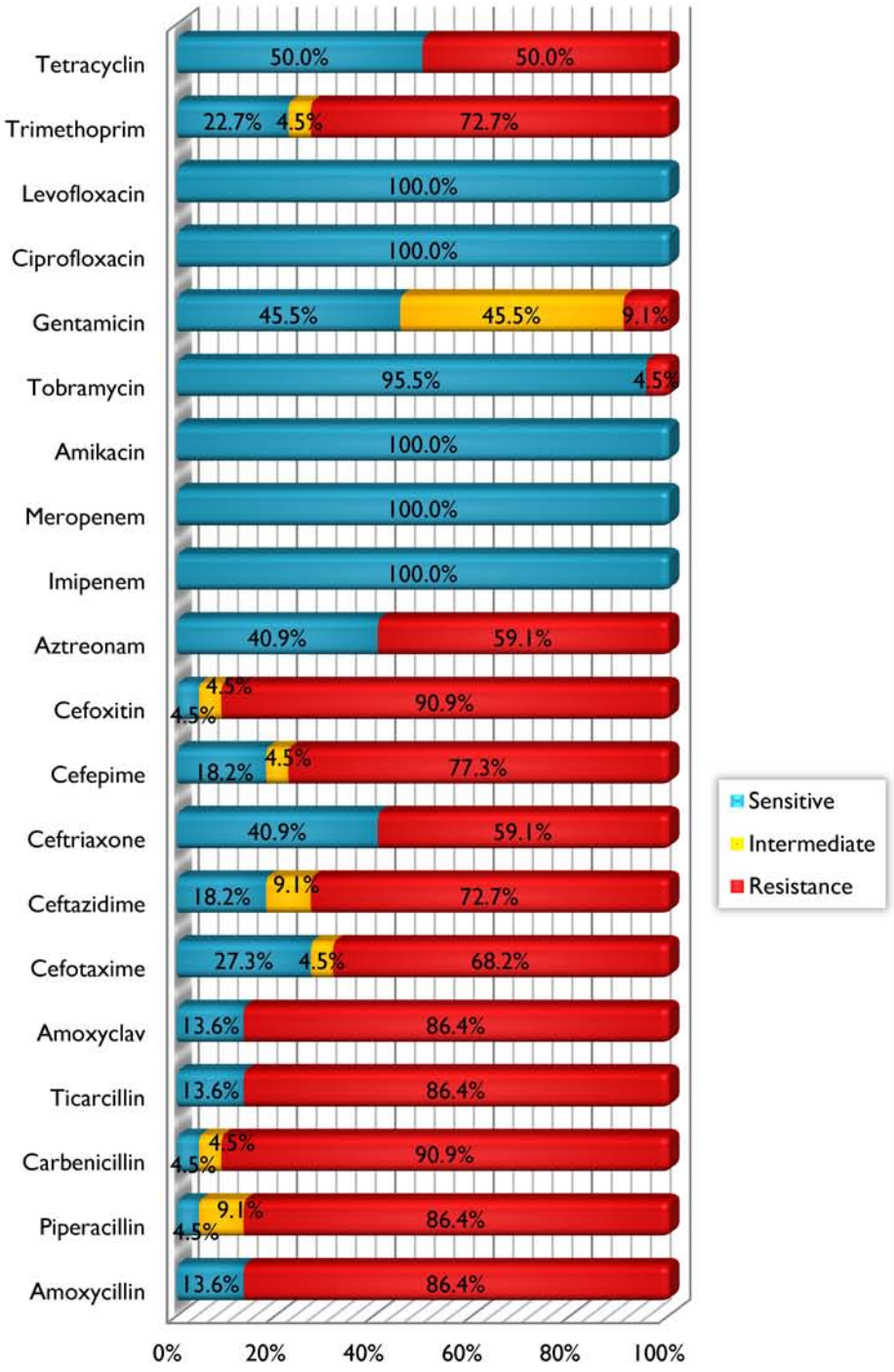


Figure 4.14: Frequency of antibiotics susceptibility of 22 EPEC isolates in Najaf.

outer membrane permeability (Tan *et al*., 2009).

Result also found that, all 22 EPEC isolates showed 100% susceptible to amikacin, while susceptibility to tobramycin and gentamicin was detected in 95.5% and 45.5% isolates, respectively (Figure 4.14). The percentages of aminoglycosides susceptibility described in present study agree with those reported by Almohana(2004) in Najaf and Al-Hilli (2010) in Hilla , who found that vast majority of *E. coli* isolates were susceptible to amikacin. On the other hand, a high prevalence of resistance to gentamicin (71.4%) and amikacin (28.5%) was seen in Sao Paulo, Brazil (de Paula and Marin, 2008).

Although resistance to ciprofloxacin and levofloxacin has emerged among enteric organisms, all the 22 EPEC isolates tested in this study were susceptible to these drugs. The same finding has been reported in many other studies in different parts of the world (El Metwally *et al*., 2007; carattoli *et al*, 2005; de Paula and Marin, 2008). Resistance to ciprofloxacin was detected in 8.6% of *E. coli* isolates collected from patients with diarrhea in Italy (Tumbarello *et al*, 2007). While in USA, ciprofloxacin and levofloxacin resistance rates rise from 6 to 11% between 1998 and 2001(Livermore, 2004). However, ciprofloxacin and other quinolones are not approving for children because of the risk of damage to immature joints (Bhattacharya and Sur, 2003). On the other hand, ciprofloxacin is still the drug of choice for the treatment of traveler's diarrhea as reported by other investigators (Ericsson *et al*., 2003; Dupont, 2006). However, if quinolone drugs are used widely as the first choice of treatment of diarrhea, especially in Iraq, without effective control of usage of antibiotics is not effectively controlled, a rapid emergence of antibiotics resistance most likely will occur.

In agreement with Hadi (2008) and Al-Hilli (2010), this study demonstrated that all (100%) EPEC isolates were susceptible to penems class of antibiotics (imipenem and meropenem) (Figure 4.14). The high efficiency of these antibiotics may be due to rarely usage in Najaf.

The increase of antibiotics resistance in EPEC isolates is often related to the overdose and mistreatment of the antibiotics prescribed. Heavy and widespread use of antibiotics in hospital doe

s not only force the emergence of antibiotic resistance, but also promotes selection of drug-resistant organisms in the hospital environment (Benèiæ *et al*., 2001). Antibiotic choices are important considerations in reducing resistance. Resistance to a class of antibiotics is not necessarily initiated by exposure to that same class (Thomas, 2007). Iraq is one of the developing countries where antibiotics sold over the counter, an attitude that encourages self-medication. In other hand, remarked that during period of time a group of antibiotics become more usage than other antibiotics without susceptibility tests which may lead to variability in resistance to these antibiotics. Pongpech *et al*. (2008) reported that, the persistent exposure of bacteria to a multitude of β-lactams has induced dynamic changes in terms of increasing production of β-lactamases and mutations in their restricted spectrum enzymes to become ESBLs. The high prevalence of ESBL-production among *Enterobacteriaceae* was commonly observed in *Klebsiella pneumoniae* and *E. coli* in Asia.

4-5-2 Extended spectrum β-lactamases (ESBLs) production

4-5-2-1 Third generation cephalosporins and aztreonam resistance

In this study, the 22 EPEC isolates were farther characterized for their ESBLs production. Initial screening for reduced susceptibility to

cefotaxime, ceftazidime, ceftriaxone and aztreonam was done by the standard Kirby-Bauer disk diffusion method (Bauer *et al*, 1966).

The results of susceptibility to these antibiotic were as follows: cefotaxime 15 (68.2%), ceftazidime 16 (72.7%), ceftriaxone 13 (59.1%) and aztreonam 13 (59.1%) (Figure 4.14). Besides, the present study revealed that all the 22 EPEC isolates were resistant to at least one of these β-lactam antibiotics. The high rates of resistance might be as markers for the production of ESBLs by these isolates. However, there are very few reports of ESBL production by diarrheagenic *E. coli* (Sonnevend *et al*., 2006; Albert *et al*., 2009). In Najaf, children with invasive diarrhea might be treated with third-generation cephalosporin, in view of the fact that the majority of EPEC isolates in this study were resistant to third-generation cephalosporins. Therefore, the prevalence of resistance to this class of antibiotics should be continuously monitored to detect any increase in resistance rates that could affect treatment with these antibiotics.

Most probably, access to use antibiotics without a physician's prescription in many countries has resulted in the emergence of new variants of β-lactamases. Concentrated use of third-generation cephalosporins in Iraq may be the most prominent risk factor for emergence of ESBL-producing pathogens. Siegel (2008) reported that other risk factors may increase resistance to third generation cephalosporins including prolonged antibiotic exposure, severe chronic illness, prior infections, prolonged hospital stay, residence in a long-term care facility, and an indwelling catheter.

4-5-2-2 Double-Disk Synergy Test (DDST)

Among 22 EPEC isolates 3 (13.63%) (E7, E8, E14) isolates were found to be ESBL producers (Figure 4.15). The study reported that the frequency of ESBL-producing isolates was as expected comparing with previous study conducted in Najaf by Hadi (2008) who revealed that 15.8% of isolates belonging to *E. coli* were ESBL producers. But it was lower than a study accomplished in Hilla by Al-Hilli (2010) who indicates that ESBL production was confirmed in 36/82 (43.9%) of *E. coli*. In the United States, the occurrence of ESBLs in *Enterobacteriaceae* ranges from 0 to 25%, and the national average is 3% (Das *et al*., 2001). In India, among 87 *E. coli* isolates 14 (16.1%) were found ESBL producers (Datta *et al*., 2004).

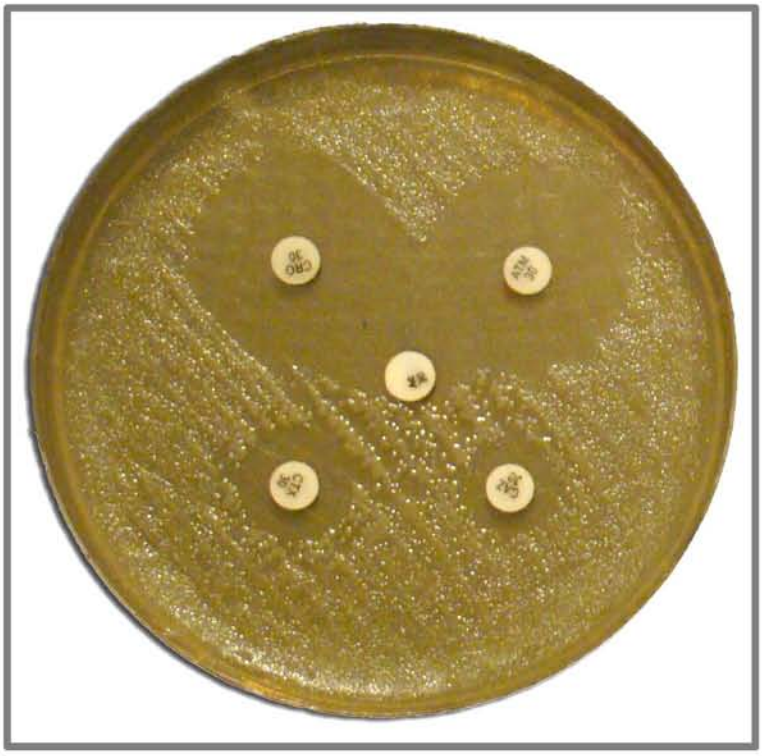


Figure 4.15: Disk Approximation Test for detection of ESBL in EPEC, E15 isolate. **AC= Amoxi-clav; ATM= Aztreonam; CTX= Cefotaxime; CRO= Ceftriaxone; CAZ= Ceftazidime.**

There is a number of instances whereby the screening tests are positive but the confirmatory tests are negative or indeterminate (Steward *el al*., 2001). Nonetheless, in this investigation not all screened positive EPEC isolates were found to be ESBL producers. Therefore, other mechanisms of resistance are requires to explain these results. On the other hand, 86.4% of EPEC were found to be resistance to amoxi-clav in this study. Since clavulanate inhibits the ESBLs, reducing the level of resistance to the cephalosporins and thereby increasing the zone of inhibition for the disc diffusion tests (Datta *et al*., 2004). Consequently, amoxi-clav resistance could actually give false negative for ESBL producer. Resistance against β-lactamase inhibitors occurs mainly by several mechanisms: hyperproduction of β-lactamases, production of β-lactamases resistant to inhibitors, and chromosomal cephalosporinases (Espinasse *et al*., 1997). The effectiveness of inhibitor may be reduced in the presence of multiple ESBLs in the bacteria (Chanawong *et al*., 2002).

In this study, DDST test is a trustworthy, suitable and reasonably priced method of screening for ESBLs. However, this test can lack sensitivity because of problems of optimal disk spacing, the inability of clavulanate to inhibit all ESBL, the inability of test to detect ESBL in isolates that also produce chromosomal cephalosporinase, and the loss of clavulanate disk potency during storage (Moland and Thomoson, 1994).

4-5-2-3 Molecular technique for detection of ESBLs

The results of this study revealed that of 22 EPEC isolates analyzed, 18 (81.8%) isolates yielded amplification products with TEM-PCR specific primers (Table 4.3, Figure 4.16). The frequency of TEM enzyme in this study was higher than reported by Hadi (2008) who found that, 14 (66.6%) out of 21 *E. coli* isolates in Najaf were able to yield amplification products with TEM-PCR specific primers. In Portugal,

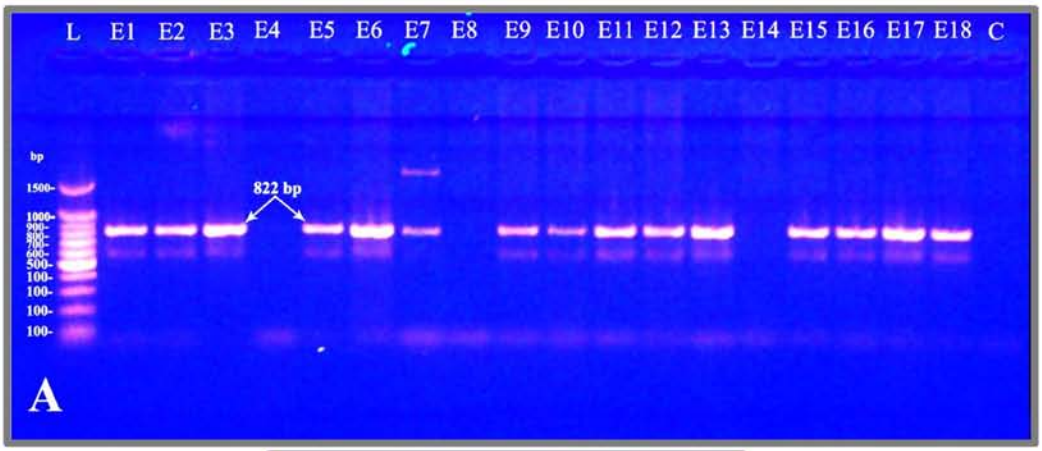
bla$_{TEM}$ gene was identified in 101 (85%) (Mendonca *et al*., 2007). A review conducted by Bradford (2001) reported that up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. This enzyme has the ability to hydrolyze penicillin's and early cephalosporins such as cephalothin and cephaloridine.

The results in this study also showed that all (100%) EPEC isolates yielded amplification products with SHV-PCR specific primers (Table 4.3, Figure 4.17). The present study demonstrated a notable increased in the frequency of *bla*$_{SHV}$ gene in Najaf compared with previous result conducted by Hadi (2008) who revealed that 3 (14.3%) out of the 21 β-lactamase-producing *E. coli* were positive for *bla*$_{SHV}$ gene. This results was also much higher than reported by Pongpech *et al*. (2008) who found that, 3 (8%) of the 37 confirmed ESBL producing *E. coli* in Thailand had SHV types. In Spain, Machado *et al*. (2005) reported that PCR experiments detected *bla*$_{SHV}$ genes in17% of *E. coli* isolates with an ESBL phenotype. Tasl and Bahar (2005) demonstrated that 15.1% of *E. coli* isolates from clinical samples in Turkey were able to produce SHV enzymes depending on PCR test.

In this study, 17 (77.3%) of the 22 EPEC harboured a *bla*$_{CTX-M}$ β-lactamase gene as determined by PCR (Table 4.3, Figure 4.18).The results was higher than those previously reported in Hilla by Al-Hilli (2010) who found that 50% of the phenotypic ESBL-producing *E. coli* isolates had CTX-M type. However, this group of enzymes constitute a novel and rapidly growing family of plasmid-mediated ESBLs that are currently replacing mutant TEM or SHV ESBL families and which are much greater penetration into *E. coli*. They have become the most prevalent type of ESBLs described during the last 5 years, especially from certain European and South America countries (Livermore, 2007). In the Middle East area, reports from Lebanon and Kuwait pointed out

Table 4.3: Distribution of *bla* genes in EPEC isolates

Isolate Designation	Serotype	*bla* gene									
		TEM	SHV	CTX-M	OXA	RER	VEB	GES	AmpC	IMP	VIM
E1	O111:k58(B4)	+	+	+	-	-	-	-	-	-	-
E2	O44:k74(L)	+	+	+	-	-	-	-	-	-	-
E3	O125:k70(B15)	+	+	+	-	-	-	-	-	-	-
E4	O55:k59(B5)	-	+	+	-	-	-	-	-	-	-
E5	O125:k70(B15)	+	+	+	-	-	-	-	-	-	-
E6	O44:k74(L)	+	+	+	-	-	-	-	-	-	-
E7	O128:k67(B12)	+	+	+	+	-	-	-	-	-	-
E8	O127:k63(B8)	-	+	+	-	-	-	-	-	-	-
E9	O44:k74(L)	+	+	-	-	-	-	-	-	-	-
E10	O111:k58(B4)	+	+	+	-	-	-	-	-	-	-
E11	O26:k60 (B6)	+	+	+	-	-	-	-	-	-	-
E12	O111:k58(B4)	+	+	-	-	-	-	-	+	-	-
E13	O111:k58(B4)	+	+	+	-	-	-	-	-	-	-
E14	O26:k60(B6)	-	+	-	-	-	-	-	-	-	-
E15	O55:K59(B5)	+	+	+	-	-	-	-	-	-	-
E16	O55:K59(B5)	+	+	+	-	-	-	-	-	-	-
E17	O55:K59(B5)	+	+	+	-	-	-	-	-	-	-
E18	UT (polyvalent 4)	+	+	+	-	-	-	-	-	-	-
E19	UT (polyvalent 2)	-	+	-	-	-	-	-	+	-	-
E20	O114:k90(B)	+	+	+	-	-	-	-	-	-	-
E21	O55:K59(B5)	+	+	-	-	-	-	-	-	-	-
E22	O26:k60(B6)	+	+	+	-	-	-	-	-	-	-
Sum		**18**	**22**	**17**	**1**	**0**	**0**	**0**	**2**	**0**	**0**



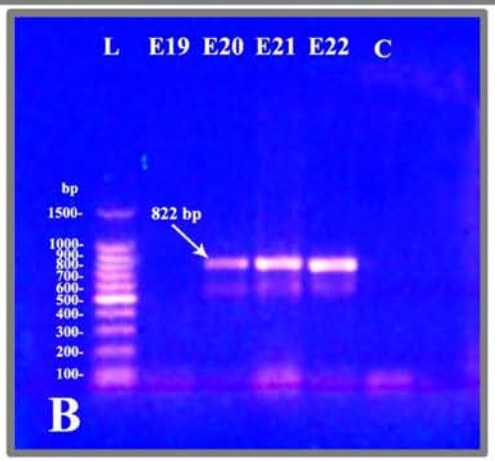


Figure 4.16: Ethidium bromide-stained agarose gel of PCR amplified products from extracted EPEC DNA amplified with primers *bla*$_{TEM}$ F and *bla*$_{TEM}$ R.

A.

Lane (L), DNA molecular size marker (100-bp ladder)

Lanes (E1), (E2), (E3), (E5), (E6), (E7), (E9), (E10), (E11), (E12), (E13), (E15), (E16), (E17) and (E18) show positive results with *bla*$_{TEM}$ gene.

Lanes (E4, 8, and 14) EPEC isolates show negative results with *bla*$_{TEM}$ gene

Lane (C), negative control

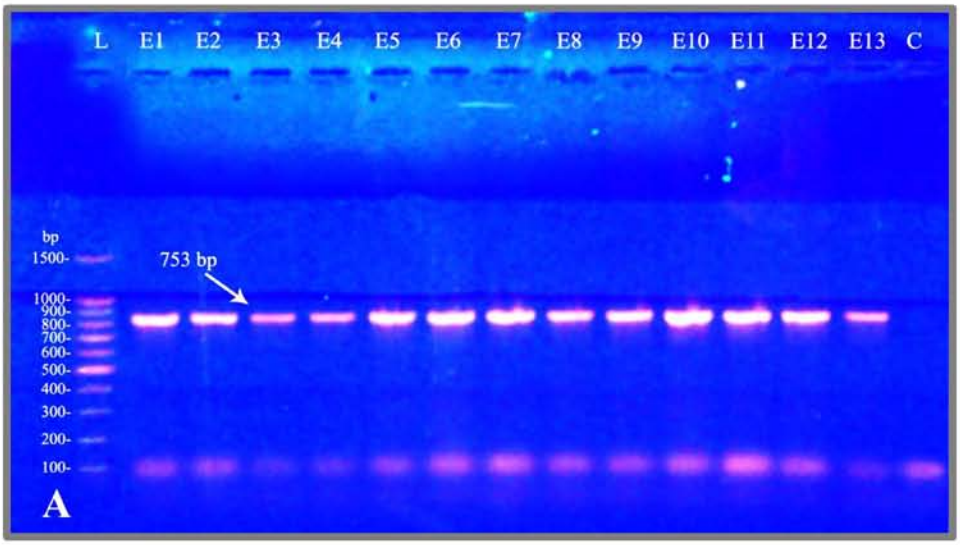
B.

Lane (L), DNA molecular size marker (100-bp ladder)

Lanes (E20), (E21) and (E22) show positive results with *bla*$_{TEM}$ gene.

Lane (E19) EPEC isolates show negative results with *bla*$_{TEM}$ gene

Lane (C), negative control



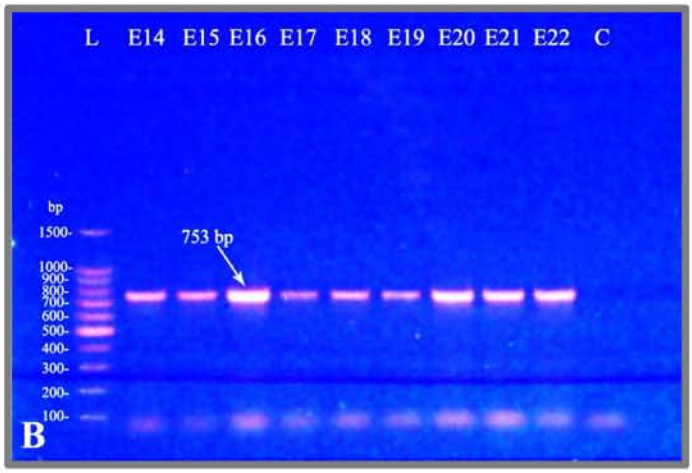


Figure 4.17: Ethidium bromide-stained agarose gel of PCR amplified products from extracted EPEC DNA amplified with primers *bla*$_{SHV}$ F and *bla*$_{SHV}$ R.

A.

Lane (L), DNA molecular size marker(100-bp ladder)

Lanes (E1), (E2), (E3), (E4), (E5), (E6), (E7), (E8), (E9), (E10), (E11), (E12) and (E13 show positive results with *bla*$_{SHV}$ gene.

Lane (C), negative control

B.

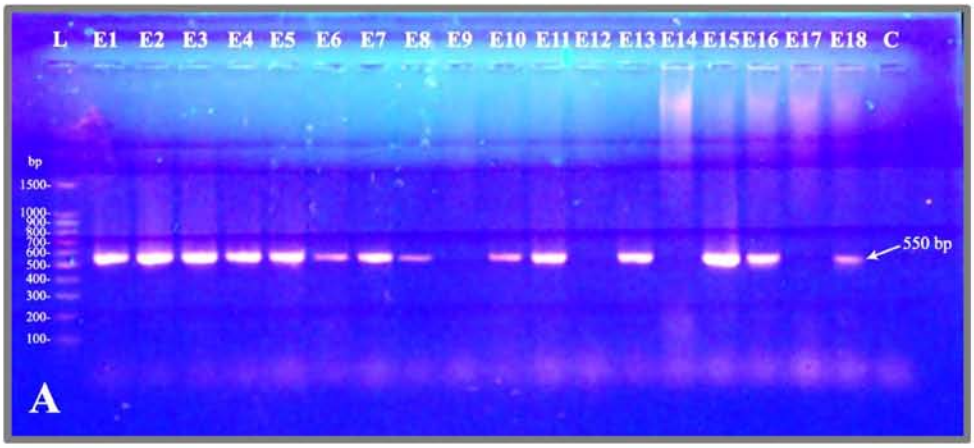
Lane (L), DNA molecular size marker(100-bp ladder)

Lanes (E14), (E15), (E16), (E17), (E18), (E19), (E20), (E21) and (E22) show positive results with *bla*$_{SHV}$ gene.

Lane (C), negative control

that CTX-M is the predominant ESBL in *E. coli* (Moubareck *et a*l., 2005; Poirel *et al*., 2005). The main reason for the prevalence of CTX-M β-lactamases in Najaf may be the wide spread use of certain third generation cephalosporins. Antibiotic selective pressure probably contributes to the increasing prevalence of cefotaxime and ceftriaxone hydrolyzing CTX-M β-lactamases in clinical setting (Wei *et al*., 2005). However, to the best of our knowledge this is first report done with detection of CTX-M β-lactamase in Najaf. This work also corroborated that this enzyme is one of the most common worldwide.

Results also revealed that, only one isolates (4.54%) of EPEC carried bla_{OXA} gene (Table 4.3, Figure 4.19). Al-Hilli (2010) found that all 82 *E. coli* and *Klebsiella* spp. isolates from Merjan teaching hospital in Hilla city were negative in OXA-PCR. While, Karami and Hannoun (2008) reported that out of 32 ampicillin resistant *E. coli* isolates in Sweden, only one isolate (3%) harboured the bla_{OXA}. In a study done in Thailand, *bla* genes encoding OXA were found in 8.1% of ESBL-producing *E. coli* isolates (Kiratisin *et al*., 2008). OXA enzymes are regarded as OXA-type ESBLs and have been discovered mainly in *Pseudomonas aeruginosa* in specimens from Turkey and France (Harada *et al*., 2008). These β-lactamases are classified into class D in the Ambler scheme and were placed in group 2d in the Bush-Jacoby-Medeiros functional scheme (Walther-Rasmussen and Hoiby, 2006). The OXA enzymes are characterized by their high hydrolytic activity against oxacillin and cloxacillin and are poorly inhibited by clavulanic acid. Extension of the hydrolytic spectrum of oxacillinases to oxyimino cephalosporins has been reported in OXA-2 and OXA-10 extended-spectrum derivatives (Bhattacharjee *et al*., 2007). Some OXA-type β-lactamases have carbapenemase activity, augmented in clinical



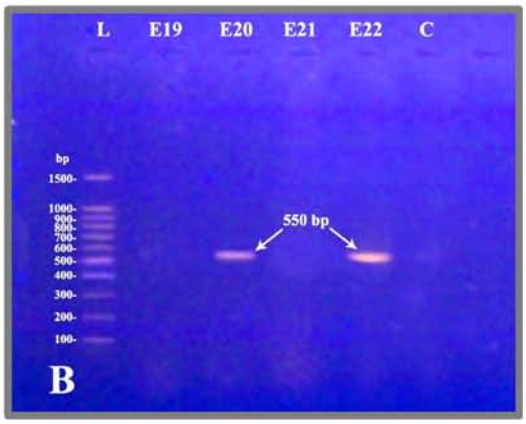


Figure 4.18: Ethidium bromide-stained agarose gel of PCR amplified products from extracted EPEC DNA amplified with primers *bla*$_{CTX-M}$ F and *bla*$_{CTX-M}$ R.

A.

Lane (L), DNA molecular size marker(100-bp ladder)

Lanes (E1), (E2), (E3), (E5), (E6), (E7), (E8), (E10), (E11), (E13), (E15) and (E18) show positive results with *bla*$_{CTX-M}$ gene.

Lanes (E9, 12, 14 and 17) EPEC isolates show negative results with *bla*$_{CTX-M}$ gene

Lane (C), negative control

B.

Lane (L), DNA molecular size marker(100-bp ladder)

Lanes (E20) and (E22) show positive results with *bla*$_{CTX-M}$ gene.

Lanes (E19 and E21) EPEC isolates show negative results with *bla*$_{CTX-M}$ gene

Lane (C), negative control

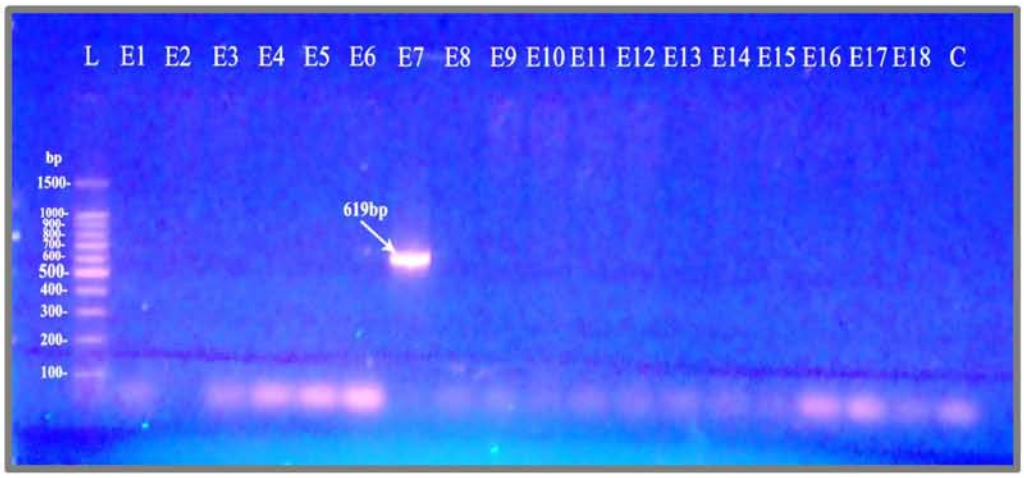


Figure 4.19: Ethidium bromide-stained agarose gel of PCR amplified products from extracted EPEC DNA amplified with primers *bla*$_{OXA}$F and *bla*$_{OXA}$ R.

Lane (L), DNA molecular size marker (100-bp ladder)

Lane (E7), show positive results with *bla*$_{OXA}$ gene.

Lanes (E1, E2, E3, E4, E5, E6, E8, E9, E10, E11, E12, E13, E14, E15, E16, E17 and 18) EPEC isolates show negative results with *bla*$_{OXA}$ gene

Lane (C), negative control

isolates by additional resistance mechanisms, such as impermeability or efflux (Jacoby and Munoz-Price, 2005). Since, there was no resistance to carbapenem class antibiotics in EPEC isolates in the present study (Figure 4.14), the study suggested that OXA-type β-lactamases detected may be belonged to ESBL enzymes.

The present study revealed that no ESBL genes, including bla_{VEB}, bla_{PER} and bla_{GES} were identified in 22 EPEC isolates. However, in Thailand, Kiratisin *et al*. (2008) found that 8.5% of ESBL-producing *E. coli* were encode bla_{VEB} genes.

The present study concluded that, the incidence of ESBL was very high in Najaf; all hospitals and clinical laboratories, do not screen for ESBL producing *Enterobacteriaceae*, although they are increasingly found in the community and hospitals and associated with treatment failure. However, many risk factors may play important role in the increasing frequency of ESBLs, include long hospital stays, prolonged stays in intensive care units, severe primary disease, intubation, urinary tract or vascular catheterization, and repeated administration of antibiotics, especially of third or fourth generation cephalosporins. ESBL enzyme had in the meantime also become a relevant problem in outpatient care given the frequent prescription of quinolones and third generation cephalosporins in outpatient care (Reinthaler *et al*., 2010).

4-5-3 AmpC β-lactamase production

4-5-3-1 Cefoxitin susceptibility

The results indicated that 20 (90.9%) isolates yielded cefoxitin zone diameter less than 18 mm, these isolates may be AmpC producers (Figure 4.14, Table 4.4). The frequency of cefoxitin resistance in the present study was higher than previously recorded in Iraq by Al-Hilli

(2010) who found that 8/18 (44.4%) of *E. coli* isolates were resistance to cefoxitin, as well as higher than other studies in Spain (4.8%) (Oteo et al.,2010), France (77.5%) (Corvec et al., 2010) and Korea (9.4%) (Yoo et al., 2010).

4-5-3-2 Modified Three-Dimensional Test (MTDT)

Modified three-dimensional test has been described for testing AmpC enzymes in Gram-negative bacterial isolates (Taneja *et al*., 2008). Coudron *et al*. (2000) reported that the MTDT did not reveal false negative results and only 3.6% of the 28 AmpC harbouring *E. coli* and *Klebsiella* spp. isolates was false positive. This suggests that the technique can be used for routine screening of the AmpC enzyme in a clinical laboratory.

Enhanced growth of the surface organism at the point where the slit intersected the zone of inhibition was considered a positive three-dimensional test result and was interpreted as evidence for the presence of AmpC β-lactamase. The present study revealed that out of 20 cefoxitin resistance EPEC isolates, AmpC β-lactamase production was confirmed by MTDT in 4 (20%) isolates (Figure 4.20). The remaining cefoxitin

resistant isolates (16/20) were non-AmpC β-lactamase producers (Table 4.4). This result indicated that even though the screening methods that use cefoxitin for the detection AmpC-producing isolates are easily performed, they are not accurate. However, in a previous study in Hilla, Al-Hilli (2010) tested 40 *E. coli* isolates from Merjan teaching hospital and found that 5 (12.5%) contained AmpC-β-lactamases. In another study, Black *et al*. (2005) estimated that 44 (31%) of the 140 cefoxitin-insusceptible clinical isolates yielded positive AmpC with MTDT. In study conducted by Coudron *et al*, (2000), AmpC-β-lactamases were

Table 4.4: AmpC β-lactamases production in EPEC isolates

EPEC isolate Code No.	Cefoxitin resistance isolates	AmpC detected with		
		3DT	AmpC Disk test	Disc antagonism test
E1	+	-	-	-
E2	+	+	+	-
E3	+	+	+	-
E4	+	-	-	-
E5	+	-	-	-
E6	+	-	-	-
E7	+	-	-	-
E8	+	-	-	-
E9	+	-	-	-
E10	+	-	-	-
E11	-	-	-	-
E12	+	+	+	-
E13	+	-	-	-
E14	-	-	-	-
E15	+	-	-	-
E16	+	-	-	-
E17	+	-	-	-
E18	+	-	-	-
E19	+	+	+	-
E20	+	-	-	-
E21	+	-	-	-
E22	+	-	-	-
Total	20(90.9%)	4(18.18%)	4(18.18%)	0

produced by 35.5% of cefoxitin resistant *E. coli* isolates.

A limitation of methods used to detect the AmpC enzyme is that an increasing number of clinical isolates that have multiple β-lactamases, which in turn can make inhibition patterns complex and difficult to interpret (Coudron *et al*., 2000).

A slit beginning 3 mm from the edge of a cefoxitin disk must be cut in the agar with a sterile scalpel blade in an outward radial direction; cutting this slit is a fastidious and time-consuming procedure. In addition,

Figure 4.20: Modified three-dimensional test to detect AmpC β-lactamase. Growth of *E. coli* ATCC 25922 strain around slits containing AmpC enzyme extractions of EPEC E19 test isolate, exhibit clear distortion of the zone of inhibition of cefoxitin.

filling the slits homogeneously without overflow onto agar surface is problematic. These problems were also recorded by Silva Dias *et al*. (2008).

To date, several modifications of the three dimensional test to detect AmpCs were tried, but no satisfactory technique has been established (Silva Dias *et al*., 2008). In routine laboratory, three

dimensional tests for detection of AmpC are not feasible as it is cumbersome and time consuming (Taneja *et al*., 2008).

4-5-3-3 AmpC Disk Test

The origin of AmpC in *E. coli* is chromosomal; although recently, plasmidic AmpC has also been isolated. Detection of any type of AmpC β-lactamase is a challenge to clinical microbiologists. There are no guidelines in place for efficient detection by CLSI guidelines (Vandana and Honnavar, 2009). It is difficult in *E. coli* to distinguish phenotypically plasmid-mediated AmpC producers from isolates overproducing chromosomal enzymes at high levels (Corvec *et al*., 2010). The AmpC disk test showed reliably detected plasmid mediated AmpC β-lactamases and also high-level production of chromosomally mediated AmpC β-lactamases in *E. coli* (Black *et al*., 2005). Therefore, the AmpC disk test was an easier, reliable and rapid method of detection of isolates that harbour AmpC β-lactamases, and provide confirmatory or alternative test for detection AmpC enzyme in *E. coli* isolate. This test discriminates between cefoxitin-resistance caused by cephamycinase activity in test-positive strains and other causes (e.g. loss of permeability or altered drug target) in test-negative strains (Schønning *et al*., 2007).

The present study demonstrated that 4 (20%) of the 20 cefoxitin resistance EPEC isolates, were positive by extraction procedure (MTDT), and also showed positive results by AmpC disk test. Indentation indicating strong AmpC producer was observed in 2 EPEC isolates (E12, E19), whereas flattening (weak AmpC) was observed in 2 isolates (E2, E3) (Table 4.4, Figure 4.21). In previous study in Iraq, Al-Hilli (2010) reported that 12.5% of *E. coli* revealed positive results by AmpC disk test. In similar studies, Singhal *et al*. (2005) found that AmpC β-

Figure 4.21: AmpC disk test to detect AmpC β-lactamase production in *E. coli* E19 (strong positive result) (FOX; cefoxitin)

lactamases was confirmed in 36% (22/61) of *E. coli* and *Klebsiella* spp. isolates from tertiary care hospitals by the 3DT, and all the 22 AmpC producer isolates were positive by AmpC disk test, and Schønning *et al.* (2007) who revealed that 47/68 *E. coli* and *Klebsiella* spp. tested AmpC disk positive and were classified as AmpC producing. The result of present study revealed that 16 (80%) EPEC isolates resistant to cefoxitin were non AmpC producer may be due to other mechanisms than β-lactamases, such as reduced outer membrane permeability (porin mutations). It is questionable whether the latter positive results of this test are truly AmpC producing or the positive AmpC disk test is the result of cephamycinase activity of the ESBL present is presently unknown.

4-5-3-4 Disc Antagonism Test

AmpC β-lactamases are of two types plasmid-mediated and chromosomal or inducible AmpC. Chromosomal AmpC enzymes are seen in some microorganisms and are typically inducible by β-lactam antibiotics such as cefoxitin and imipenem but poorly induced (any) by the third- or fourth-generation cephalosporins (Hemalatha *et al*., 2007). In many enterobacterial species, the expression of the chromosomal *ampC* gene is low and inducible. In *E. coli*, the natural chromosomal AmpC is constitutively produced at a very low level because of a weak promoter as well as a transcriptional attenuator, which results in the up regulation of naturally occurring chromosomal AmpC β-lactamase production (Bogaerts *et al*., 2010).

In this study, screening for the inducible AmpC β-lactamase was done by the disc antagonism test, a modified double disk approximation method (MDDM). Results demonstrates no blunting of the ceftazidime zone adjacent to cefoxitin disc among the 20 cefoxitin resistance EPEC isolates (Table 4.4). While, in previous study conducted by Al-Hilli (2010) found that 1/40 (2.5%) of *E. coli* isolates were positive for inducible AmpC β-lactamases. Singhal *et al*. (2005) reported that three of 61 *E. coli* isolates also showed blunting of the inhibition zone of either ceftazidime or cefotaxime adjacent to cefoxitin disk were considered as presumptive AmpC producers. In a another study, Taneja *et al*. (2008), found that among 205 highly drug resistant uropathogenic isolates, no *E. coli* isolate was positive for inducible AmpC β-lactamases producer.

Gram-negative bacteria that lack an inducible AmpC enzyme (such as *K. pneumoniae, E. coli, Salmonella* spp. and *P. mirabilis*) may acquire plasmids resulting in a stably derepressed resistance phenotype, since most plasmid-mediated *ampC* genes are expressed constitutively, even in

the presence of a complete chromosomal system for induction (Thomas, 2007).

4-5-3-5 Molecular detection of AmpC β-lactamase

There is no standardized method (such as synergy test for ESBL) to easily detect AmpC enzymes. Particularly, it is difficult in *E. coli* to distinguish phenotypically plasmid-mediated AmpC producers from isolates overproducing chromosomal enzymes at high levels. Additionally, a strain with a plasmid-mediated AmpC enzyme can also produce other β-lactamases, such as ESBL, which may complicate the detection of the AmpC phenotype (Sidjabat *et al*., 2009; Corvec *et al*., 2010).

Polymerase chain reaction assay was used in this study for the detection of bla_{AmpC} gene that proved useful as a rapid screening tool to distinguish cefoxitin-resistant non-AmpC producers from cefoxitin-resistant AmpC producers in EPEC isolates from children with diarrhea in Najaf. Results demonstrated that 2 (9.1%) isolates were amplified with bla_{AmpC} primers (Table 4.3, Figure 4.22). These two isolates showed cefoxitin resistance in cefoxitin susceptibility test (Figure 4.14). The PCR assay confirms only 2 of the 4 AmpC producer isolates previously identified in the three dimensional and AmpC tests. Cefoxitin resistance in non-AmpC β-lactamase producers may be due to some other resistance mechanisms. Hernandez-Alles *et al*. (2000) demonstrated that the interruption of a porin gene by insertion sequences is a common type of mutation that causes loss or decrease of outer membrane porin expression and increased cefoxitin resistance in *E. coli* and *Klebsiella* spp. Lack of understanding or the resource to crab the spread of β-lactam resistance mechanisms is responsible for a continuous failure to response appropriately to prevent the rapid dissemination of pathogens passing the

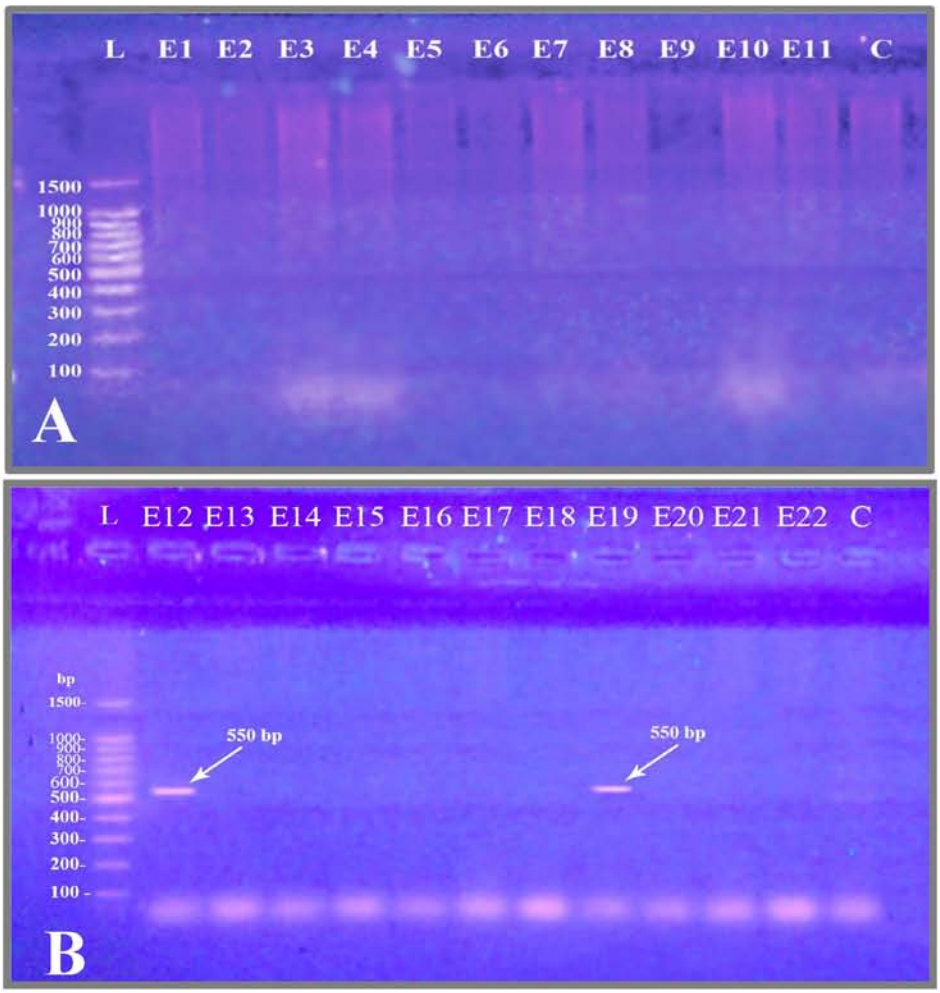


Figure 4.22: Ethidium bromide-stained agarose gel of PCR amplified products from extracted EPEC DNA amplified with primers *bla*$_{AmpC}$ F and *bla*$_{AmpC}$ R.

A.

Lane (L), DNA molecular size marker (100-bp ladder)

Lanes (E1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) EPEC isolates show negative results with *bla*$_{AmpC}$ gene

Lane (C), negative control

B.

Lane (L), DNA molecular size marker (100-bp ladder)

Lanes (E12) and (E19) show positive results with *bla*$_{AmpC}$ gene.

Lanes (E13, 14, 15, 16, 17, 18, 20, 21, and 22) EPEC isolates show negative results with *bla*$_{AmpC}$ gene

Lane (C), negative control

β-lactamases. In the developing countries, including Iraq, many clinical laboratories are not fully aware of the importance of ESBLs and AmpC and how to detect them. In Najaf, little attention has been paid to β-lactamases producing isolates. Therefore, there is an increase demand to investigate the role of these isolates in community and hospitals infections.

4-5-4 Molecular Detection of Carbapenemases Production

The introduction of carbapenems into clinical practice represented a great advance for the treatment of serious bacterial infections caused by β-lactam resistant bacteria (Saderi *et al*., 2008). However, the present study was undertaken to detect metallo β-lactamases in isolates of EPEC taken from children with diarrhea using PCR assay. All the 22 EPEC isolates were examined with bla_{IMP} and bla_{VIM} specific primers. The PCR assay demonstrated that no amplification with MBLs primers was happened confirming the susceptibility test of antibiotics that found all EPEC isolates were susceptible to these antibiotics (Figure 4.14). Meropenem and imipenem have been traditionally used to treat multidrug-resistant pathogens and remained the drugs of choice for the treatment of infections caused by these organisms (DeRyke *et al*., 2007). Its wide antibacterial spectrum and great β-lactamase stability make imipenem an option for monotherapy in serious bacterial infections (Benèiæ *et al*., 2001), but problems associated with their use are on the rise imipenem-resistant bacterial strains occur after increased use of imipenem. Bacterial resistance to imipenem arises from the production of carbapenemases capable of hydrolyzing the carbapenem nucleus, and from alteration in the porin channels in the bacterial cell walls, thereby reducing the permeability of the drug (Benèiæ *et al*., 2001).

Conclusions and Recommendations

Conclusions

According to the results obtained in this study, the following conclusions have been reached at:

1-The frequency of EPEC isolates in Najaf was lower than what has been suspected.

2- The usefulness of serotyping to define factual EPEC remains debatable.

3- The investigations, including the use of molecular technique and serotyping, are necessary to allow precise identification and epidemiological study of these pathogens.

4- The molecular detection provides definite identification of virulence factors that are not affected by phenotypic behavior.

5- Atypical EPEC are more prevalent than typical EPEC which may indicate that animals are the reservoir of these organisms in the area of the study.

6- The predominant resistant genes are blaTEM, blaSHV and blaCTX-M among EPEC isolated in this study.

7- The vast majority of the tested EPEC isolates were multidrug resistant, therefore, such organisms represent a serious therapeutic challenge.

8- The prevalence of ESBL-producing isolates is high, and the carbapenems, fluoroquinolone and aminoglycosides are the most effective against β-lactams resistance EPEC isolates *in vitro*.

Recommendations

1- Further studies are needed to determine the neighbor joining phylogenetic tree among EPEC isolates in Najaf.

2- This study emphasizes the need to continued surveillance of β-lactamases- producing species of Gram-negative and Gram-positive bacteria in other hospitals in Iraq, which will be helpful in monitoring antimicrobial resistance and to guide intervention to minimize its occurrence.

3- Extended-spectrum β-lactamases detection should be carried out in all routine tests in all Iraqi hospital laboratories.

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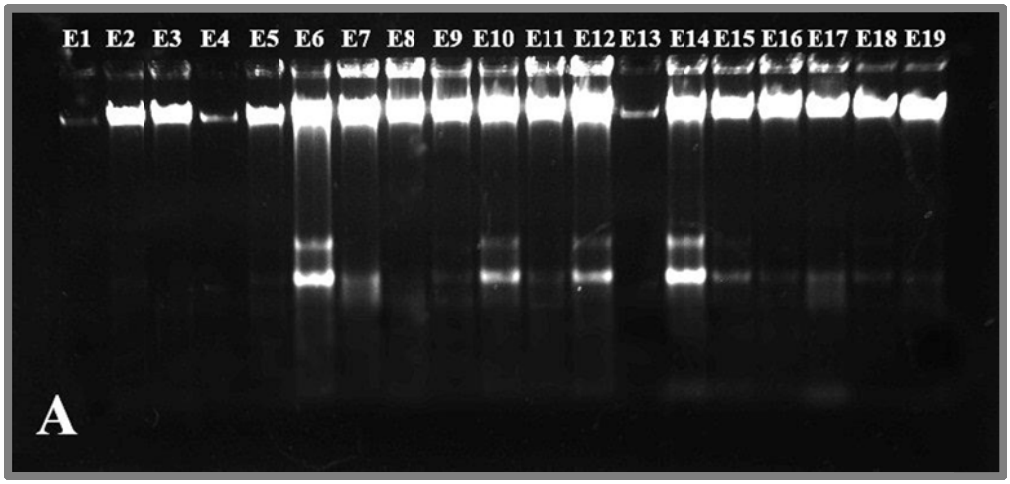
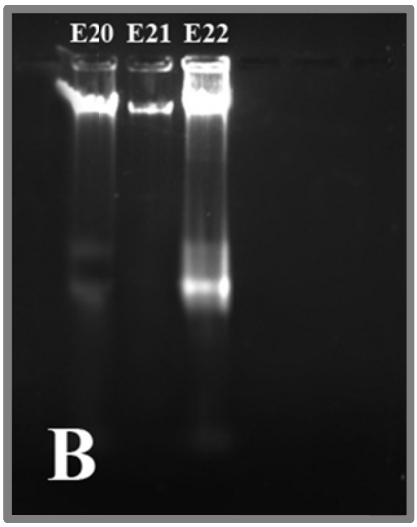
Appendix





Plasmid profile of EPEC isolates from children with diarrhea in Najaf

الخلاصة

مازال الاسهال هو احد اكثر الامراض المسببة للوفيات بين الاطفال دون عمر السنتين في الدول النامية، ومن اهم العوامل المسببة للإسهال بين الاطفال هو الاصابة ببكتريا *Escherichia coli* المعوية الممرضة (EPEC).

ان الهدف من هذه الدراسة هو التحري عن بكتريا EPEC بوصفها المسبب لحالات الاسهال بين الاطفال دون عمر السنتين، وتوصيف جيناتها المسؤولة عن خواصها الامراضية، بالإضافة الى تحديد حساسيتها تجاه المضادات الحيوية.

تم خلال فترة الدراسة، اختبار جميع عزلات بكتريا *E.* coli في قدرتها على التلازن مع مصول EPEC المضادة المتعددة (Polyvalent) والاحادية (Monovalent)، كما تم التحري عن المورثات التي قد تتميز بها هذه البكتريا وتتضمن (*eaeA*، *bfpA*، stx_1، stx_2، EHEC *hlyA*، وبلازميد EAF) والمورثات المسؤولة عن المقاومة لمضادات البيتالاكتام وتشمل (bla_{TEM}، bla_{SHV}، bla_{CTX-M}، bla_{OXA}، bla_{GES}، bla_{VEB}، bla_{PER}، bla_{AmpC}، bla_{VIM}، bla_{IMP}) بواسطة تقنية سلسلة تفاعل انزيم البلمرة (PCR)، بالإضافة الى دراسة النمط المظهري تجاه المضادات الحيوية.

جمعت 656 عينة خروج مأخوذة من مصابين بالإسهال و 54 عينة خروج من اطفال لا يعانون من الاسهال. أظهرت نتائج الفحوصات ان ٦٥٦/٢٢ طفل (3.35%) يعانون من الاسهال نتيجة اصابتهم بـ EPEC، بينما لم يتم عزلها من الاطفال الذين لا يعانون من الإسهال. توزعت معظم عزلات EPEC المشخصة المصل المضاد المتعدد ٢ (polyvalent 2) حيث تضمن المصول المضادة الاحادية التالية: O55:K59(B5) (22.7%)، O111:K58(B4) (18.18%)، O26:K60(B6) (13.63%)، يليه المصل المضاد المتعدد ٣ (polyvalent 3) وتضمن المصول المضادة الاحادية التالية: O125:K70(B15) (9.09%)، O128:K67(B12) (4.54%)، O127:K63(B8) (4.54%)، O114:K90(B) (4.54%)، ثم المصل المضاد المتعدد ٤ (polyvalent 4) وتضمن المصل المضاد الاحادي: O44:K74(L) (13.63%). تم اختبار جميع العزلات للتأكد من احتوائها على مورثات المحددات المرضية، اذ اظهرت عزلتين (9%) احتواءها على موروثة *eaeA* ، كما اظهرت 6 عزلات احتوائها على موروثة *bfpA* (27.27%)، وعزلتين على موروثة *EAF*، بينما لم تعطي عزلات EPEC نتيجة

موجبة مع البادئات الخاصة بالمورثات stx_1، stx_2، *hlyA* EHEC. صنفت عزلات بكتريا EPEC الى قياسية (Typical) (*eaeA*$^+$، *bfpA*$^+$) وغير قياسية (Atypical) (*eaeA*$^+$، *bfpA*$^-$). شخصت بكتريا EPEC القياسية في عزلتين (33.3%) تعود الى النوع المصلي O125:K70(B15). بينما شخصت بكتريا EPEC غير القياسية في 4 عزلات (66.7%) اثنان منها تعود الى النوع المصلي O111:k58(B4)، عزلة واحدة تعود الى O44:K74(L) وواحدة تعود الى O128:K67(B12).

ابدت 20 عزلة (90.9%) مقاومة متعددة للمضادات الحيوية من خلال استخدام ٢٠ نوع من المضاد الحيوي، بينما كانت جميع العزلات حساسة الى imipenem، meropenem، amikacin، ciprofloxacin و levofloxacin.

ابدت جميع العزلات مقاومة لمضاد حيوي واحد على الاقل، ومن ثم تم فحص قابلية العزلات على انتاج انزيمات البيتالاكتاميز واسعة الطيف (ESBLs) باستخدام فحص ثنائي الاقراص المتعاضد (double-disk synergy test)، اظهرت الدراسة وجود 3 (13.6%) عزلات لها القابلية على انتاج هذه الانزيمات. بينما اكدته تقنية سلسلة تفاعل انزيم البلمرة حمل 18 (18.8%)، 22 (100%)، 17 (77.3%)، و 1 (4.54%) عزلة بكتريا EPEC لمورثات bla_{TEM}، bla_{SHV}، bla_{CTX-M} و bla_{OXA} على التوالي. اظهرت 4 عزلات (18.2%) قدرتها على انتاج انزيم البيتالاكتاميز نوع AmpC باستخدام اختبار ثلاثي الابعاد المحور (three-dimensional) وقرص AmpC (AmpC disc)، بينما اكدت تقنية سلسلة تفاعل انزيم البلمرة إمكانية انتاج انزيم AmpC في عينتين فقط (9%).

وزارة التعليم العالي والبحث العلمي
جامعة الكوفة
كلية الطب

التواجد والتوصيف الجزيئي للأنماط المصلية لبكتريا إيشيريشيا القولون المعوية الممرضة (EPEC) المعزولة من الاطفال المصابين بالإسهال في النجف

رسالة تقدم بها إلى

مجلس كلية الطب/ جامعة الكوفة جزءاً من متطلبات نيل درجة الماجستير

في علم الأحياء المجهرية الطبية

الطالب
سامر عبد الصاحب محمد حسين الهلالي
بكالوريوس علوم حياة/ جامعة الكوفة (1997)

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2010 ميلادي