Prevalence of Diarrheagenic *Escherichia coli* in Children from Amman with and without Diarrhea Using Polymerase Chain Reaction.

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Laboratory Sciences

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July 2001

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Dedication

To

My parents, sisters and brothers for their support and encouraging me

To

My wife for helping me to overcome all the difficulties

To

My daughter Lina, with my great love.

Acknowledgement

With all the regards, I would like to express my deepest and profound acknowledgement to my supervisor Professor Asem Shehabi for his continuous and generous suggestions and encouragement. Also, I would like to express my sincere gratitude to my co-supervisor Professor Najwa Khuri-Bulos for her support and instructive suggestions throughout this work.

Sincere thanks to members of the committee Dr. Ziad Elnasser, Dr. Azmi Mahafzah, and Dr. Radi Hamed for revising the thesis and offering their valuable comments that enriched this work.

My appreciation is extended to Dr. Sameer Naji, the head of Islamic Hospital Laboratories and to the staff members of Islamic Hospital Laboratories, specially, Sameer AlHoura, Nedal AlSoufi, Usamah A. Sayed, Issa AboShama, Hisham AlDeek, Khaled AlZgaier, Mahmoud Ja'far, Mohannad Ya'coub and Majed AlMallah for their help and support they gave me.

Special acknowledgment is owed to staff members of the Genetics laboratory at the University of Jordan for their kindness and friendly understanding and cooperation. Special and sincere thanks to Zaid Abu Rabaiha, Wafa Qubbaj, Nehad Malkawi, Faten Al-Maani, and Dalia Al-Husseini.

My appreciation and thanks are extended to the staff of Microbiology laboratories both in the Jordan University Hospital and in the Department of Microbiology, Pathology, and Forensic Medicine at the Faculty of Medicine for their encouragement and support, specially, Ferial AlShudfan, Naheel Al-Dajani, and Amel Abu Alrageb.

I would like to express my gratitude to Mohammed Toma Youssef, at CDC, Atlanta, for his continuous support and encouragement and I would like also to thank Naser Sinjilawi for his help and cooperation.

My thanks are also extended to all my colleagues at the University of Jordan, faculty of Medicine for their encouragement and support, specially, Ayman Eideh, Nahed El-laha'm, khalid el-khalili, Ayman Al-Jarro, Nedal Al-Shawabkah and Mahmoud Srour.

My special deep and sincere gratitude goes to my parents, brothers and sisters.

Finally, thanks are extended to every one who aided in the completion of this work.

List of Contents

List of Tables

List of Figures

Abstract

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Acute diarrheal disease continues to be one of the major causes of morbidity and mortality in the developing world, particularly among infant and children. Diarrheagenic *E. coli* comprise a diverse group of microorganisms responsible for gastrointestinal diseases in humans. Molecular methods represent the most reliable techniques for distinguishing pathogenic from nonpathogenic *E. coli* and characterizing these pathogenic features. Therefore, it was proposed to study the prevalence of

diarrheagenic *E. coli* in children from Amman using polymerase chain reaction over a 12 – month period.

A total of 250 hospitalized patients were included in this study, of these, 133 children with gastrointestinal illnesses (diarrheal cases) and 117 children without diarrhea (control group). The age of children ranged from 0 to 13 years with mean age of 30 and 33 months for diarrheal and control groups respectively. Each stool specimen was examined by single and multiplex PCR techniques for the presence of diarrheagenic *E. coli* strains.

The PCR technique detected 9.8% Enterotoxigenic *E. coli* (ETEC), 2.9% Enteroinvassive *E. coli* (EIEC), 9.0% Enteroaggregative *E. coli* (EAggEC) and 1.5% mixed infections in children with acute diarrhea, and 11.1% ETEC, 6.0% EAggEC and 1.7% mixed infections in the children without diarrhea. The overall higher rate of EIEC isolation was statistically significant ($p < 0.05$) in diarrheal children as compared with non diarrheal controls. Enterohaemorrhagic *E. coli* (EHEC) strain was not found neither in the diarrheal cases nor in the control group. Most of diarrheagenic *E. coli* were resistant to Co-trimoxazole (60%) and ampicillin (88.7%) which are commonly used antibiotics in Jordan.

1. Introduction

Acute diarrheal disease continues to be one of the major causes of morbidity and mortality in the developing world, particularly among infants and children (Santosham *et al.*, 1995). Acute diarrhea was defined as a stool frequency of three or more unformed stools per day for less than 14 days. (Biswas *et al.*, 1996). Although diarrheal diseases are caused mostly by different etiologic agents such as *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, *Yersinia enterocolitica*, Rotavirus, *Entamoeba hystolitica*, *Giardia lamblia*, *Cryptosporidium*, diarrheagenic *Escherichia coli* is recognized to be the common cause of gastroenteritis and accounts for 30 % of the total diarrheal pathogens in some regions (Biswas *et al.*, 1996).

E. coli belongs to the family Enterobacteriaceae, tribe Escherichiae, which contains mostly motile gram-negative bacilli. *E. coli* can be recovered easily from clinical specimens on slightly selective media such as MacConkey or Eosin Methylene blue agar (EMB) at 37°C under aerobic conditions. These media are used tentatively for differentiation *E.coli* from other enteric organisms on the basis of morphology and fermention of lactose (Bettelheim, 1992). However, growth characteristics of *E. coli* on MacConkey or EMB should be used only with caution for differentiating *E. coli* colonies, because only about

90 % of *E. coli* are lactose positive; some diarrheagenic E. coli strains, including many of the enteroinvasive *E.coli* (EIEC) strains, are typically lactose negative, the indole test is positive in 99 % of *E. coli* strains. (Nataro and Kaper, 1998).

E. coli is the predominant facultative anaerobe of the human intestines. This genus colonizes the infant gastrointestinal tract within hours of life. *E. coli* usually remains harmless to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infections. Infections due to pathogenic *E.coli* may be limited to the mucosal surfaces or can be disseminated throughout the body. Three common clinical syndromes result from infection with pathogenic *E. coli* strains; urinary tract infection, sepsis / meningitis and diarrheal disease (Nataro and Kaper, 1998).

Diarrheagenic strains of *E. coli* can be divided into at least six different categories with corresponding distinct pathogenic patterns as follows: Enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAggEC). Recently a sixth category, termed diffusely adhering *E. coli* (DAEC), was discussed (Christine *et al.*, 1993). Several clinical symptoms accompany infection with diarrheagenic *E.coli* strains.

E. coli strains that produce high levels of Shiga-like toxins have been epidemiologically linked to human disease, including hemorrhagic colitis (HC), the hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenia purpura, are grouped together as enterohaemorrhagic *E.coli* (EHEC) (Hull *et al.*, 1993). Watery diahrrea is usually associated with EPEC strain, while persistent diahrrea is usually accompanied by EAggEC strain . These two strains and DAEC are characterized by their ability to produce distinct patterns of adherence to cultured epithelial cells *in vitro*. With Hep-2 or Hela cells, three patterns of adherence can be observed respectively: localized, aggregative and diffuse (Christine *et al.*, 1993).

Strains of *E. coli* that cause disease are difficult to distinguish from those that are part of normal intestinal flora of humans, there are no selective or differential cultivation methods for diagnosing or differentiating diarrheagenic strains of *E. coli*. Serological characterization of O and H antigens will identify some strains of these pathogenic groups, however, some strains are more often identified through the detection of their specific toxins or toxin genes(Olsvik and Strockbine, 1993). The toxins of these organisms have been detected by using animal; cell culture, immunologic or DNA hybridization assays. Because of their simplicity, rapidity and specificity, PCR assays for detection of pathogenic genes represent good alternatives to traditional

methods used for diagnosis of different strains of diarrheagenic *E. coli*. (Olsvik and Strockbine, 1993).

The present study aims to:

1. Determine the prevalence and epidemiology of infantile and childhood diahrrea caused by diahrreagenic *E. coli* in Amman by using single and multiplex PCR.
2. Determine antibiotic susceptibility among different strains of diahrreagenic *E. coli* in collected samples.

2. Review of Literature

2.1. Early reports: genus *Escherichia*

Theodor Escherich first described this organism which he isolated from the feces of neonates as Bacterium coli (Bettelheim, 1992).

The family Enterobacteriaceae was classified by Rahn (1937). This family currently comprises Gram negative, nonspore forming, rod shaped bacteria, size 1.1 – 1.5 μm x 2.0 – 6.0 μm, occuring singly or in pairs, capsules or microcapsules found in many strains as well as motile by peritrichous flagella or nonmotile. (Buchanan, 1974).

The majority of strains grow well on common laboratory media in the presence or absence of oxygen, and metabolism can be either oxidative or fermentative. Optimum temperature is 37ºC. Colonies on nutrient agar may be smooth (S), low convex, moist, gray, with a shiny surface and entire edge and easily dispersible in saline. Or they may be rough (R) dry and difficult to disperse well in saline. There are intermediate forms between these extremes. Mucoid and slime-producing forms occur. Chemoorganotrophic, oxidase negative, acetate can usually be used as a sole carbon source but citrate cannot be used. Glucose and other carbohydrates are fermented with the production of pyruvate which is further converted into lactic, acetic and formic acids, part of the formic acid is split by a complex

Table 1: Main biochemical characteristics of species of the genus *Escherichia*

Character	*E. coli*	*E. blattae*	*E. fergusonii*	*E. hermanii*	*E. vulneris*
Indole	98	0	98	99	0
Methyl red	99	100	100	100	100
Voges-Proskauer test	0	0	0	0	0
Citrate (Simmons)	1	50	17	1	0
Hydrogen sulfide (Triple Sugar Iron)	1	0	0	0	0
Urease	1	0	0	0	0
Phenylalanine deaminase	0	0	0	0	0
Lysine decarboxylase	90	100	95	6	85
Arginine dihydrolase	17	0	5	0	30
Ornithine decarboxylase	65	100	100	100	0
Motility (36°C)	95	0	93	99	100
Gelatin hydrolysis (22°C)	0	0	0	0	0
Growth in KCN	3	0	0	94	15
Malonate utilization	0	100	35	0	85
D-Glucose, acid	100	100	100	100	100
D-Glucose, gas	95	100	95	97	97
Fermentation of:					
Lactose	95	0	0	45	15
Sucrose	50	0	0	45	8
D-Mannitol	98	0	98	100	100
Dulcitol	60	0	60	19	0
Salicin	40	0	65	40	30
Adonitol	5	0	98	0	0
myo-Inositol	1	0	0	0	0
D-Sorbitol	94	0	0	0	1
L-Arabinose	99	100	98	100	100
Raffinose	50	0	0	40	99
L-Rhamnose	80	100	92	97	93
Maltose	95	100	96	100	100
D-Xylose	95	100	96	100	100
Trehalose	98	75	96	100	100
D-Mannose	98	100	100	100	100
Cellobiose	2	0	96	97	100
α-Methyl-D-glucoside	0	0	0	0	25
Erythritol	0	0	0	0	0
Melibiose	75	0	0	0	100
D-Arabitol	5	0	100	8	0
Glycerol	75	100	20	3	25
Mucate	95	50	0	97	78
Esculin hydrolysis	35	0	46	40	20
Tartrate (Jordan's)	95	50	96	35	2
Acetate utilization	90	0	96	78	30
Lipase (corn oil)	0	0	0	0	0
DNase at 25°C	0	0	0	0	0
Nitrate reduction	100	100	100	100	100
Oxidase (Kovac's)	0	0	0	0	0
o-Nitrophenylgalactoside hydrolysis	95	0	83	98	100
Yellow pigment	0	0	0	98	50

*Each number gives the percentage of positive reactions after 2 days incubation at 36°C.
From Farmer et al. (1985a).

hydrogenlyase system into equal amounts of CO2 and H2 (table 1). (Krieg, 1984).

2.2. Physiology and biochemistry of *E. coli*:

The biochemical reactions listed in table (1) define those activities of *E. coli* and other species of Escherichia that can be used for differentiation and characterization. However, these organisms have a far wider range of activity than those listed in table (1).

It has been suggested that *E. coli* is extremely well adapted to its environment (Koch, 1976) as it is able to survive on a relatively limited number of low molecular weight substances, which may be available transiently and at relatively low concentrations. It has been estimated that the mean generation time of *E. coli* in the intestine is 12 hours (Brock, 1971). So, *E. coli* has evolved an efficient system for survival, and for various reasons this organism has become the model on which most biochemical and genetic studies are based. The first description of the control of metabolism, was first formulated in *E. coli* (Jacob and Monod, 1961). While this system has become the paradigm of genetic control system, subsequent studies have shown that *E. coli* had a wide variety of control mechanisms in addition to the one developed by Jacob and Monod in 1961 from studies on the

utilization of lactose by *E. coli* and the induction of phage lambda. (Bettelheim, 1992).

2.3. *Escherichia coli* in the intestinal tract:

The commensally *E. coli* strains that inhabit the large intestine of all humans and warm-blooded animals comprise about 1% of the total bacterial biomass. This *E. coli* flora is in constant flux. In the neonates up to 11% of the new born babies carry *E. coli* in the oronasal cavity. Probably derived from the fecal flora of their mothers during parturition. The longer the time is between rupture of the maternal membranes and birth, the higher the percentage of babies carrying *E. coli* in the oronasal cavity (Bettelheim, 1992).

2.4. Categories of diarrheagenic *E. coli*:

E. coli strains known to cause human diarrhea are now grouped into the following categories:

2.4.1. Enterotoxigenic *E. coli* (ETEC):

In 1970, strains of *E. coli* serotype O148.H28 were found to be involved in the condition known as traveler's diarrhea (Bettelheim, 1992). Toxogenic *E. coli* have also been associated with a condition known as

"non–Vibrio cholera–like diarrhea". A culture filtrate of a strain of *E. coli* O15:H11 isolated from the small intestine of a patient with this condition produced effects in the ligated rabbit ileal loop test and in the adenyl cyclase system similar to those of *V. cholera* preparations. Two types of enterotoxins produced by enterotoxigenic *E. coli* were identified using the rabbit ileal loop test. The heat-stable enterotoxin (ST) survives boiling for 30 minutes, while the heat-labile enterotoxin (LT) is destroyed by such treatment. ETEC is defined as the *E. coli* strains that elaborate at least one member of two defined groups of enterotoxins: ST and LT (Nataro and kaper, 1998). STa is associated with diarrheal disease in both humans and animals and STb is associated primarily with diarrhea in piglets. LT-I enterotoxins are related to diarrheal disease in both humans and animals while LT-II has been associated only with animal diseases. ETEC strains isolated from humans produce STa only, LT-I or both (Sears and Kaper, 1996).

2.4.2. Enteropathogenic *E. coli* (EPEC):

EPEC includes *E. coli* with traditional EPEC O:H serotypes that possess adherence factor (EAF) plasmid and belong to the localized adherent (LA) group (Germani *et al.*, 1994). Levine, 1987, proposed classification of EPEC strains in two groups: Class I (serogroup O55, O86, O111, O119, O125, O126, O127, O128 and O142), which usually express localized adherence and possess EAF plasmid, and Class II (serogroup O44, O112 and O114), which neither exhibit LA nor possess EAF plasmid (Sinjilawi, 1998).

EPEC do not produce enterotoxins and lack of Shiga-like toxins (SLT) and invasiveness properties in epithelial cells. Characteristically, they attach in large numbers to the small intestine, causing a localized effacement of the microvilli. These attaching and effacing (A/E) lesions are characterized by close adhesion to the enterocyte membrane (Giamanco *et al.*, 1996). The adhesive properties of EPEC have been examined *in vitro* with human duodenal biopsy material and cell cultures. In Hep-2 cells a localized adherence (LA) pattern was clearly differentiated from two other patterns; diffuse and aggregative adherence (figure 1). The LA pattern has been observed in many EPEC and enterohemorrhagic *E. coli* (EHEC) strains (Giamanco *et al.*, 1996).

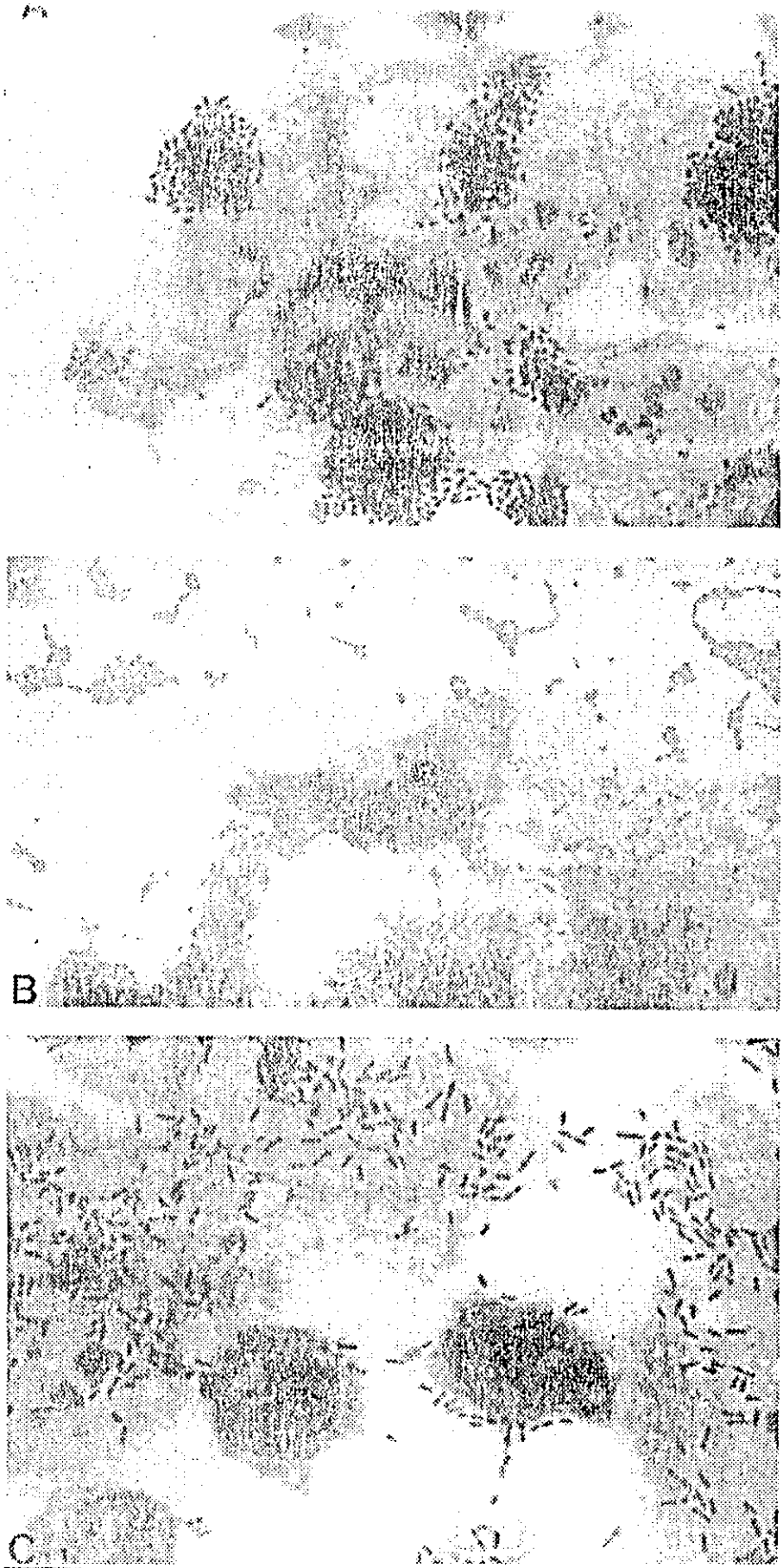

Figure 1: The three Hep2 adherence patterns manifested by diarrheagenic *E. coli*. (A) Localized adherence. (B) Aggregative adherence. (C) Diffuse adherence.(from Nataro and Kaper, 1998)

Recent studies have shown that not all EPEC serotypes harbor EAF plasmids and that the EAF-negative EPEC strains also adhere to Hep-2 cells, even if in a weaker pattern and cause A/E lesions and actin accumulation (Giamanco *et al.*, 1996). Molecular analysis of EPEC pathogenesis has progressed and enabled several loci potentially involved in virulence to be identified. From these analyses, it has been shown that both chromosomal and plasmid-encoded virulence determinants are involved in EPEC pathogenesis. The *eae* gene cluster (stands for EPEC attaching and effacing) necessary for attaching and effacing is located on the chromosome. One of the cluster's genes (*eae A*) encodes for a 94-kDa outer membrane protein involved in the intimate adhesion of bacteria to the cell membrane and has been called intimin. The *bfp A* gene, located on a large plasmid, which encodes the bundle-forming pili (BFP), is also an important gene for EPEC virulence (Franke *et al.*, 1994).

2.4.3. Enteroinvasive *E. coli* (EIEC):

EIEC strains were first shown to be capable of causing diarrhea in volunteer studies conducted by DuPont *et al.* in 1971. EIEC strains are biochemically, genetically and pathogenetically related closely to *Shigella* spp.; like *Shigella* spp. EIEC strains are genetically lysine decarboxylase negative, non motile, and lactose negative (Bettelheim, 1992). EIEC infections are characterized by a period of watery diarrhea that precedes the onset of scanty dysenteric stools containing blood and mucus. Nataro and Kaper, 1998, have cloned and sequenced a plasmid-borne gene from EIEC (designated *sen*), which encodes a novel protein with a predicted size of 63-kDa. A mutation in the *sen* gene causes a significant diminution of the enterotoxic activity of the parent strain. A role for enterotoxins is unproven, but their presence may explain the characteristic watery diarrhea attributed to EIEC.

2.4.4. Enteroaggregative *E. coli* (EAggEC):

Enteroaggregative *E. coli*, the most recently recognized category of diarrheagenic *E. coli*, adheres to tissue culture cells (Hep-2 or HeLa cells) as well as to glass or plastic cover slips in a stacked – brick like pattern (Haider *et al.*, 1992). EAggEC is associated with persistent, watery diarrhea in young children. The pathogenesis of EAggEC is not fully understood, some EAggEC strains posses plasmid encoded, D-mannose-resistant hemagglutinin (MRHA) and adhesive fimbriae called aggregative adherence fimbriae I (AAF/I) (Yamamoto *et al.*, 1997). EAggEC strains characteristically enhance mucus secretion from the mucosa with trapping of the bacteria in a bacterium-mucus biofilm (Nataro and Kaper, 1998).

2.4.5. Enterohemorrhagic *E. coli* (EHEC):

The recognition of EHEC as a distinct class of pathogenic *E. coli* resulted from two key epidemiological observations. The first was the 1983 report by Riley *et al.*, who investigated two outbreaks of a distinctive gastrointestinal illness characterized by severe crampy abdominal pain, watery diarrhea followed by grossly bloody diarrhea, and little or no fever. This illness was designated hemorrhagic colitis (HC). Stool cultures from these patients yielded a previously rarely

isolated *E. coli* serotype O157:H7. The second key observation was made by Karmali *et al.*, also in 1983, who reported the association of sporadic cases of hemolytic uremic syndrome (HUS) with fecal cytotoxin and cytotoxin producing *E. coli* in stool. HUS (defined by the triad of acute renal failure, thrombocytopenia, and microangiopathic. Hemolytic anemia was already known to be preceded typically by a bloody diarrheal illness indistinguishable from HC (Nataro and Kaper, 1998).

The cytotoxin assay used by Karmali *et al.*, demonstrated that some strains of *E. coli* produced a striking, irreversible cytopathic effect on cultured Vero cells which were derived from a normal adult African green monkey (Bettelheim, 1992). At the same time, O'Brien *et al.*, reported that extracts of certain *E. coli* strains were cytotoxic for HeLa cells and that this cytotoxic activity could be neutralized by antitoxin prepared against crude *Shigella dysenteriae* 1 Shiga toxin (Stx). Also, O'Brien *et al.* subsequently showed that Shiga-like toxin and the vero-cytotoxin were the same toxin and that the O157:H7 strains described by Riley *et al.* produced this toxin independently (Riley, 1987).

E. coli O157:H7 strains are sorbitol negative, ornithine and lysine decarboxylase positive. Another characteristic of *E. coli* O157:H7

that distinguishes it from most other *E. coli* serotypes is the inability to produce β-glucuronidase (GUD), which hydrolyses 4-methyl-umbelliferyl-D-glucuronide (MUG) and related substrates. GUD enzyme encoded by *Uid A* gene in *E. coli* other than wild type *E. coli* O157:H7 strains (Karch *et al.*, 1993). Most of the work on pathogenic factors of *E. coli* O157:H7 has focused on the SLT, which are encoded on the Bacteriophage inserted into the chromosome. Additional potential virulence factors are encoded in the chromosome and on a ca.60Mda plasmid found in all EHEC strains. SLTs have been divided into two groups on the basis of their immunologic characteristics: SLT-I, which cross-react with antibody directed against Shiga toxin, and SLT-II, which is not immunologically cross – reactive with polyclonal anti-Shiga toxin antibody (Hull *et al.*, 1993). EHEC strains also have *eae A* genes that produce the same histopathology that has been seen with EPEC strains (Nataro and Kaper, 1998).

2.4.6. Diffusely adherent *E. coli* (DAEC):

The term "Diffusely adherent *E. coli*" was initially used to refer to any Hep-2-adherent *E. coli* strains that did not form EPEC-like microcolonies. With the discovery of EAEC, most authors now

recognize DAEC as an independent category of potentially diarrheagenic *E. coli*. Although recent epidemiological studies have shown a high prevalence of diffusely adhering *E. coli* (DAEC) strains isolated from diarrheal stools, their involvement is still a matter of debate (Poitrineau *et al.*, 1995). DAEC strains were the main etiological agent of diarrheal cases among hospitalized patients in France, this suggest that these strains may be important diarrheal pathogens in the developed world (Pointrineau *et al.*, 1993).

2.5. Pathogenesis of diarrheagenic *E. coli* strains:

Like most mucosal pathogens, *E. coli* can be considered to follow a requisite strategy of infection (i) colonization of a mucosal site, (ii) evasion of host defenses, (iii) multiplication, and (iv) host damage. The most highly conserved feature of diarrheagenic *E. coli* strains is their ability to colonize the intestinal mucosal surface despite peristalsis and competition for nutrients by the indigenous flora of the gut (Sears and Kaper, 1996). The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains, including nonpathogenic *E. coli*. However, diarrheagenic *E. coli* posses specific fimbrial antigens that enhance their intestinal colonization and allow adherence to the small bowel mucosa, a site that is not normally

colonized. Once colonization is established, the pathogenetic strategies of the diarrheagenic *E. coli* strains exhibit remarkable variety. Three general methods have been described by which *E. coli* may cause diarrhea: (i) enterotoxin production (ETEC and EAEC), (ii) invasion (EIEC), and/or (iii) intimate adherence with membrane signaling (EPEC and EHEC). The over all pathogenic schemes of diarrheagenic *E. coli* are summarized in figure (2) (Nataro and Kaper, 1998).

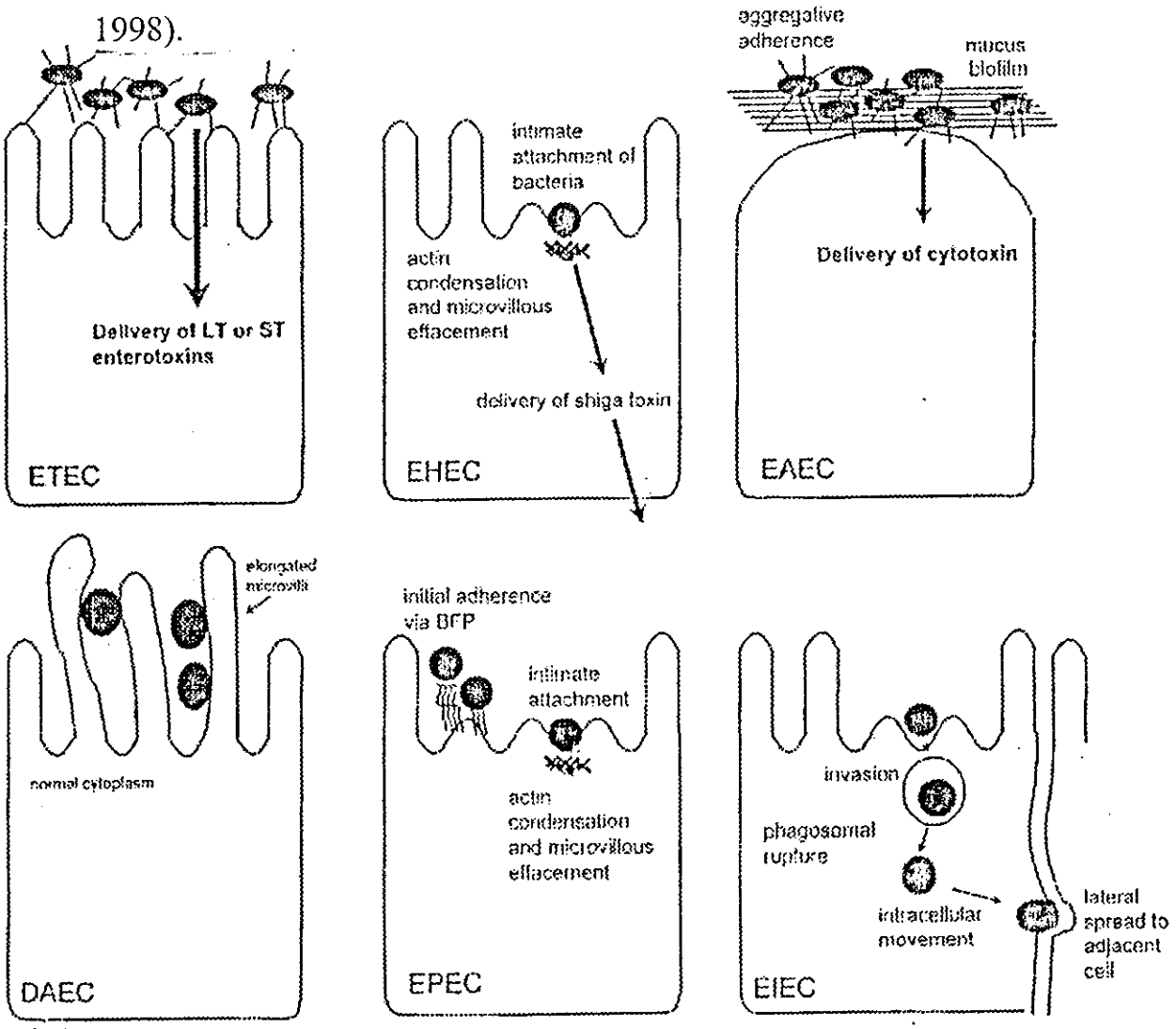

Figure 2: Pathogenic schemes of diarrheagenic *E. coli*. The six recognized categories of diarrheagenic *E. coli* each have unique features in their interaction with eukaryotic cells.

2.5.1. The pathogenesis of Enterotoxigenic *E. coli*:

ETEC causes diarrhea by producing enterotoxins, LT-I is expressed by these *E. coli* strains, this toxin is an oligomeric toxin composed of one 28 kDa A subunit and five identical 11.5 kDa B subunits. The B subunits bind strongly to the ganglioside GM1 receptor of the host cell membrane. The A subunit is responsible for the enzymatic activity of the toxin and is proteolytically cleaved to yield A1 and A2 peptides Joined by disulphide bond. After binding to the host cell membranes, the toxin is endocytosed and translocated through the cell in a process involving trans- Golgi vesicular transport. The cellular target of LT is adenylate cyclase located on the basolateral membrane of polarized intestinal epithelial cells. The A1 peptide has an ADP-ribosyltransferase activity and acts by transferring an ADP-ribosyl moiety from NAD to the alpha subunit of the GTP-binding protein, (Gs), which stimulates adenylate cyclase activity. ADP-ribosylation of Gsα subunit results in adenylate cyclase being permanently activated leading to increased levels of cyclic AMP (cAMP). cAMP dependent protein kinase (A kinase) is thereby activated leading to supranormal phosphorylation of chloride channels located in the apical epithelial cell membranes. The major chloride channel activated by LT and CT is CFTR (cystic fibrosis

transmembrane conductance regulator). The net result is stimulation of Cl^- secretion from secretory crypt cells and inhibition of NaCl absorption by villus tip cells. The increased luminal ion content draws water passively through the paracellular pathway, resulting in osmotic diarrhea (Sears and Kaper, 1996). LT-II increases intracellular cAMP levels by similar mechanism to those involved with LT-I, but LT-II uses ganglioside GD1 as its receptor rather than GM1. As noted above, there is no evidence that LT-II is associated with human or animal disease (Bangaerts *et al.*, 1985).

Escherichia coli heat stable enterotoxin (STa) is a cysteine-rich, 18 or 19 – amino acid peptide with a molecular size of ca. 2 kDa. The major receptor for STa is a membrane-spanning enzyme called guanylate cyclase C (GC-C). GC-C is located in the apical membrane of intestinal epithelial cells. Binding of STa to GC-C stimulates GC activity, leading to increased intracellular cGMP levels. This activity leads ultimately to stimulation of chloride secretion and/or inhibition of sodium chloride absorption, resulting in net intestinal fluid secretion (Victor *et al.*, 1991).

STb is associated primarily with ETEC strains isolated from pigs, although some human ETEC isolates expressing STb have been reported. Unlike STa, STb induces histologic damage in the intestinal

epithelium, consisting of loss of villus epithelial cells and partial villus atrophy. The receptor of STb is unknown. To cause diarrhea, ETEC strains must first adhere to small bowel enterocytes, an event mediated by surface fimbria (CFA) also called pili. CFA genes are usually encoded on plasmids, which typically also encode the enterotoxins, ST and/or LT (Nataro and Kaper, 1998).

2.5.2. The pathogenesis of Enteropathogenic *E. coli*:

The hallmark of infection due to EPEC is the attaching-and-effacing (A/E), this striking phenotype is characterized by effacement of microvilli and intimate adherence between the bacterium and the epithelial membrane. Marked cytoskeletal changes, including accumulation of polymerized actin, are seen directly beneath the adherent bacteria. The bacteria sometimes sit upon a pedestal-like structure. These pedestal structures can extend up to 10 μm out from the epithelial cell in pseudopode-like structures (Nataro and Kaper, 1998).

Multiple steps are involved in producing the characteristic A/E histopathology. In 1992, Donnenberg and Kaper proposed a three-stage model of EPEC pathogenesis consisting of (i) localized adherence, (ii) signal transduction, and (iii) intimate adherence. Experiments showed that the ability of EPEC strains to adhere in a

localized pattern was dependent on the presence of a 60-MDa plasmid. Loss of this plasmid led to loss of the LA phenotype. Adherence of EPEC to epithelial cells induces a variety of signal transduction pathways in the eukaryotic cell. The bacterial genes responsible for this signal transduction activity are encoded on a 35 kb pathogenicity island called the locus of enterocyte effacement (LEE). These signals start with inducing tyrosine protein kinase activity, possibly in the receptor itself. This enzyme then begins to phosphorylate a variety of proteins, one of which may be phospholipase C, which then generates the secondary messengers inositol-1,4,5-triphosphate and diacylglycerol. Inositol-1,4,5-triphosphate causes release of calcium from calmodulin stores. The increase in the intracellular calcium levels activates an actin binding protein, villin, which becomes an actin severing protein. This causes breakdown of the structural integrity of the actin cytoskeleton, which leads to microvilli destruction. The increase in calcium ions concentration results in the phosphorylation of several epithelial cell proteins on serine and threonine residues, the most prominent of which is myosin light chain and protein kinase C (pkc) (Sinjilawi, 1998). Activation of pkc induces rapid changes in intestinal water and electrolyte secretion *in vivo* and *in vitro*, and phosphorylation of myosin light chain can lead to increased permeability of tight-junction, thereby suggesting

additional potential mechanisms of diarrhea due to EPEC (Law, 1994). The phosphorylated cytoskeleton proteins aggregate under EPEC in an organized manner, and intimin (which is encoded by *eae A* gene) may serve as an organizer for aggregation of proteins. The most prominent change in the small intestinal mucosa is a reduction in the absorptive surface area due to destruction off microvilli, which could reduce the activity of mucosa and release of mucosal enzymes such as disaccharidases and peptidases. These alterations would prevent absorption of salt and water, which result in the development of diarrhea (Nataro and Kaper, 1998).

2.5.3. The pathogenesis of Enteroinvasive *E. coli*:

The current model of *Shigella* and EIEC pathogenesis comprises epithelial cell penetration, lysis of the endocytic vacuole, intracellular multiplication, directional movement through the cytoplasm and extension into adjacent epithelial cells. When the infection is severe, this sequence of events elicits a strong inflammatory reaction, which is manifested grossly as ulceration. The site of *Shigella* and EIEC infection is the colonic mucosa.

Genes necessary for invasiveness are carried on a 140-MDa plasmid (p Inv). P Inv plasmid carries *ipa* gene cluster of secreted effector proteins which are necessary for full pathogenicity. The Ipa

proteins (Ipa A to Ipa D) are secreted proteins, of which Ipa B, Ipa C, and Ipa D are effectors of the invasion phenotype. Ipa C has been shown to promote the uptake of EIEC into the eukaryotic cell, whereas Ipa B is thought to function in the lysis of the phagocytic vacuole and in the induction of apoptosis in macrophages. Finally Ipa D encodes for invasion plasmid antigen H (Ipa H) (Nataro and Kaper, 1998).

2.5.4. The pathogenesis of Enteroaggregative *E. coli*:

EAggEC strains enhance mucus secretion from the mucosa, with trapping of the bacteria in a bacterium-mucus biofilm. The formation of a heavy biofilm may be related to the diarrheagenicity of the organism and to its ability to cause persistent colonization and diarrhea (Haider *et al.*, 1992). Experiments suggest that EAggEC infection is accompanied by cytotoxic effects on the intestinal mucosa, resulting in a destructive lesion. The lesion is characterized by shortening of the villi, hemorrhagic necrosis of the villous tips, and a mild inflammatory response with edema and mononuclear infiltration of the submucosa (Yamamoto *et al.*, 1997). A three-stage model has been described for EAggEC pathogenesis. Stage I, involves initial adherence to the intestinal mucosa and/or the mucus layer, this adherence is facilitated by aggregative adherence fimbriae I (AAF/I)

and aggregative adherence fimbriae II (AAF/II) that are encoded by 60-MDa plasmid called PCVD432. Stage-II involves enhanced mucus hypersecretion, where the EAggEC is embedded. Stage-II involves elaboration of an EAggEC cytotoxin, which results in enterocyte damage (Haider *et al.*, 1992). EAggEC heat-stable enterotoxin (EAST1) is another toxin that stimulates net intestinal secretion by stimulating elevation of cGMP levels (Sears and Kaper, 1996). The *ast A* gene, which codes for EAST1 enterotoxin was identified in certain diarrheagenic *E. coli* (e.g., in 41% EAggEC, 41% ETEC, 100% EHEC, and 22% of EPEC strains) but was not detected in EIEC, *Yersinia enterocolitica*, or *V. cholera* non-O1 strains (Savarino *et al.*, 1996).

A third toxin produced by EAggEC is a heat labile protein (120-KDa) that stimulates an increase in intracellular calcium levels (Nataro and Kaper, 1998).

2.5.5. The pathogenesis of Enterohemorrhagic *E. coli*:

EPEC, EHEC induces a host inflammatory response that is apparently linked to the A/E histopathology, which is characterized by hemorrhage and edema in the lamina propria. Colonic biopsy specimens from many patients show focal necrosis and infiltration of neutrophils.

The major virulence factor, and a defining characteristic of EHEC, is Stx. This toxin contains a single A subunit associated with a pentamer of B subunits. The single 32-KDa A subunit is proteolytically nicked to yield a ca. 28-KDa peptide (A1) and a 4-KDa peptide (A2); these peptides remain linked by disulfide bonds. The A1 peptide contains the enzymatic activity, and the A2 peptide serves to bind the A subunit to a pentamer of five identical 7.7 KDa B subunits. The B pentamer binds the toxin to a specific glycolipid receptor, globotriaosylceramide or Gb3, which is present on the surface of eukaryotic cells. While Gb3 is the main receptor for Stx. After binding, the holotoxin is endocytosed through coated pits, and is transported to the Golgi apparatus and then to endoplasmic reticulum. The A subunit is translocated to the cytoplasm, where it acts on the 60s ribosomal subunits, specifically, the A1 peptide is a N-glycosidase that removes a single adenine residue from the 28S rRNA of eukaryotic ribosomes, thereby inhibiting protein synthesis. The resulting disruption of protein synthesis leads to the death of renal endothelial cells, intestinal epithelial cells, or any cells possessing the Gb3 receptor. Stx produced in the intestine is assumed to translocate to the blood stream, probably through a transcellular, rather than a paracellular, pathway. Damage of the intestinal epithelium by Stx could also aid translocation of the toxin to the blood stream.

(Sinjilawi, 1998) This possibility is supported by the fact that patients with bloody diarrhea due to *E. coli* O157:H7 are more likely to develop HUS than are those with non-bloody diarrhea. The reach of Stx to the blood stream will damage the glomerular endothelial cells, leading to narrowing of capillary lumina and occlusion of the glomerular microvasculature with platelets and fibrin. The decreased glomerular filtration rate is presumably responsible for the acute renal failure that is typical of HUS. Epidemiological data suggest that Stx_2 is more important than Stx_1 in development of HUS (Nataro and Kaper, 1998).

In the intestinal disease, one possible mechanism for fluid secretion in response to Stx involves the selective killing of absorptive villus tip intestinal cells by Stx. The Gb3 receptor is present in much higher concentration in villus cells than in the secretory crypt cells and so the death of absorptive cells and preservation of secretory crypt cells could shift the usual balance of intestinal absorption and secretion toward net secretion. The available evidence therefore suggests that unlike LT or CT, Stx does not increase active secretion of Cl- ions (Riley, 1987).

2.6. Epidemiology of diarrheagenic *E. coli* strains:

ETEC strains are associated with two major clinical syndromes: weanling diarrhea among children in the developing world, and traveler's diarrhea. ETEC disease is determined by mucosal immunity to ETEC infection and by infectious dose, because the infection requires a relatively high infectious dose (Nataro and Kaper, 1998). Epidemiologic investigations have implicated contaminated food and water as the most common vehicles for ETEC infection. Person-to-person transmission was not found to occur during a study of ETEC-infected volunteers (Stacy – Phipps *et al.*, 1995).

Although ETEC infection occurs most frequently in infants, immunologically naive adults are susceptible. Indeed, ETEC is the predominant etiologic agent causing traveler's diarrhea among adults from the developed world visiting areas where ETEC infection is endemic (Nataro and Kaper, 1998).

EPEC infection is primarily a disease of infants younger than two years. The reason for the relative resistance of adults and older children is not known, but loss of specific receptors with age is one possibility. As with other diarrheagenic *E. coli* strains, EPEC transmission is fecal-oral, with contaminated hands, contaminated

weaning foods or formula. The reservoir of EPEC infection is thought to be symptomatic or asymptomatic children and asymptomatic adult carriers, including mothers and persons who handle infants. Numerous studies have documented the spread through hospitals, nurseries, and day care centers (Mayatepek *et al.*, 1993). In contrast to the limited importance of EPEC in the developed countries, EPEC is a major cause of infant diarrhea in the developing countries. Studies in Brazil, Mexico and south Africa have shown that 30 to 40% of infant diarrhea, particularly in the 0 to 6 month age group can be attributed to EPEC (Nataro and Kaper, 1998).

The important features of EHEC epidemiology include a reservoir in the intestinal tract of cattle and other animals, transmission by a wide variety of food items; with beef as being a major vehicle of infection, and a very low infectious dose, enabling high rates of attack and of person-to-person transmission (Beutin *et al.*, 1993).

A growing number of studies have supported the association of EAggEC with diarrhea in developing populations, most prominently in association with persistent diarrhea ($\geq$ 14 days). Eslava *et al.*, have described two outbreaks of EAggEC persistent diarrhea occurring in a Mexico city Hospital; infants who died in these outbreaks were found

to have developed necrotic lesions of the ileal mucosa (Haider *et al.*, 1992).

Many EIEC strains are probably misidentified as *Shigella* spp, or non-pathogenic *E. coli* strains. Documented EIEC outbreaks are usually food borne or water borne, although person-to-person transmission does occur. The infective dose of EIEC in volunteers is higher than that for *Shigella* spp, and thus the potential for person-to-person transmission is lessened (Pointrineau *et al.*, 1995).

2.7. Clinical considerations of the infections due to diarrheagenic *E. coli* strains:

The clinical characteristic of ETEC disease is that the illness is abrupt in onset with a short incubation period (14 to 50 hr.). The diarrhea is watery, usually without blood, mucus, pus, fever and vomiting may be present in a minority of patients. ETEC diarrhea may be mild, brief, and self-limiting or may result in severe purging similar to that seen in *V. cholera* infection (Levine, 1987;and Riley, 1987). The corner stone of management of ETEC infection is to maintain a normal hydration status. Oral rehydration and parenteral rehydration therapy are often life-saving in infants and children with ETEC diarrhea. For travelers to the developing world, bismuth subsalicylate or loperamide is effective in decreasing the severity of diarrhea. Oral

vaccines against ETEC are being developed and now they are available for prevention of disease (Nataro and Kaper, 1998).

EPEC causes primarily acute diarrhea, and the infection can often be quite severe (Law, 1994). EPEC infections are characterized by profuse watery diarrhea, vomiting, malaise, and low-moderate fever (Levine, 1987; Riley, 1987). There may be also edema, neutrophils infiltrate, and a moderate disordered arrangement of enterocytes in the intestinal mucosa (Law, 1994). As with other diarrheal pathogens, the primary goal of treatment of EPEC diarrhea is to prevent dehydration by correcting fluid and electrolyte imbalances. Bismuth subsalicylate, specific bovine anti-EPEC, and milk immunoglobulins may also be used. There are no vaccines currently available to prevent disease due to EPEC (Franke *et al.*, 1994).

The incubation period of EHEC diarrhea is usually 3 to 4 days. The initial complaint is usually non-bloody diarrhea, preceded by crampy abdominal pain and short-lived fever in many patients. Vomiting occurs in about half of the patients during the period of non-bloody diarrhea. Within 1 or 2 days, the diarrhea becomes bloody and the patient experiences increased abdominal pain (Riley, 1987). HUS is defined by a triad of hemolytic anemia, thrombocytopenia, and renal failure; the initial clinical manifestations include oligouria or

anuria, edema, pallor, and sometimes seizures (Nataro and Kaper, 1998). Treatment of EHEC disease is limited largely to supportive care. The use of antibiotics may be harmful for two potential reasons: first, lysis of bacteria by some antibiotics leads to increased release of toxin, at least *in vitro*; second, antibiotic therapy could kill other intracolonic bacteria, thereby increasing the systemic absorption of toxin. There are no currently available vaccines to prevent disease due to EHEC (Riley, 1987).

EAggEC is clinically associated with watery, mucoid, and secretory diarrheal disease, low-grade to absence of fever, little to no vomiting, and without blood or fecal leukocytes (Bhan *et al.*, 1989).

2.8. Isolation and identification of diarrheagenic *E. coli* strains:

Culturing stools for most categories of diarrheagenic *E. coli* should be performed in cases of persistent diarrhea, especially in travelers, children and immunocompromised patients as well as in outbreak situations. The identification of isolates is done by one or more of the following methods:

2.8.1. Biochemical methods;

There is no biochemical test used for specific differentiation between diarrheagenic *E. coli* strains, except a few biochemical tests used for the differentiation of EHEC strains such as O157: H7 strain which are sorbitol negative, ornithine and lysine decarboxylase positive, and also their inability to produce β-glucuronidase (Riley, 1987).

2.8.2. Serotyping:

Serotyping of *E. coli* has played a central place in the history of these pathogens. In 1944, Kauffman proposed a scheme for the classification of *E. coli*, which is still used in a modified form today. *E. coli* strains are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) antigens. Serotype of an isolate is defined as specific combination between O and H antigens (table 2).

Table 2: Serotypes caracteristics of the diarrheagenic *E. coli* categories.

ETEC		EPEC	EIEC	EHEC	EAggEC
O6:H16	O128ac:H7	O55:H6	O28ac:NM	O26:H11	O3:H2
O8:NM[a]	O128ac:H12	O55:NM	O29:NM	O26:H32	O15:H18
O8:H9	O128ac:H21	O86:H34	O112ac:NM	O26:NM	O44:H18
O11:H27	O139:H28	O86:H2	O115:NM	O55:H7	O77:H18
O15:H11	O148:H28	O111:H2	O124:NM	O111ab:H8	O86:NM
O20:NM	O149:H4	O111:H12	O124:H7	O111ab:NM	O111:H21
O25:NM	O149:H10	O111:NM	O124:H30	O113:H21	O127:H2
O25:H42	O153:H45	O114:NM	O135:NM	O117:H14	
O27:H7	O159:H4	O114:H2	O136:NM	O157:H7	
O27:H20	O159:H20	O119:H6	O143:NM		
O63:H12	O159:H34	O119:NM	O144:NM		
O73:H45	O166:H27	O125:H6	O144:H25		
O78:H11	O167:H5	O125ac:H21	O152:NM		
O78:H12	O168:H16	O126:H2	O159:H2		
O85:H7	O169:NM	O126:H27	O159:NM		
O114:H21	O173:NM	O126:NM	O164:NM		
O114:H49		O127:NM	O167:NM		
O115:H21		O127:H6	O167:H4		
O115:NM		O127:H9	O167:H5		
O115:H40		O127:H21			
O115:H51		O128ab:H2			
O126:H9		O142:H6			
O127:H8		O158:H23			

a; NM: nonmotile.

*: Reviewed by Nataro and Kaper, 1998.

Serotyping markers correlate some times very closely with specific strains of diarrheagenic *E. coli*, however, these markers are rarely sufficient for characterization of diarrheagenic strains because of its limited sensitivity and specificity. Serotyping is tedious and expensive and is performed reliably only by a small number of reference laboratories (Bettleheim, 1992).

2.8.3. Tissue culture assays:

In this technique a wide variety of non-intestinal epithelial cell lines such as **Chinese hamster ovary** (CHO), vero cells, Y-1 adrenal cells, HeLa cells, or Hep-2 cells have been used to study the activity of a particular toxin by observing a change in the shape of the cells or by cytotoxicity in response to treatment by enteric toxin (Johnson and Liord, 1998). In addition, the study of the mode of adherence is used to distinguish between EPEC, EAggEC, and DAEC strains (Haider *et al.*, 1992). In response to heat-labile enterotoxins of *E. coli*, CHO cells elongate while Y-1 adrenal cells become round (Sears and Kaper, 1996). Tissue culture techniques currently used for the detection of ETEC are nonspecific and insensitive and thus, not used for routine diagnosis (Victor *et al.*, 1991).

2.8.4. Infant mouse model:

The most commonly used test for STa is the infant mouse assay, however, this test is expensive, laborious, and requires animal facilities with trained personnel. It is, therefore limited to few laboratories (Starvic *et al.*, 1992). The principle of the test is based on the study of the effect of culture filtrates of certain bacterial strains, or ingestion of bacterial strains themselves or isogenic bacterial strains, then observe the clinical symptoms on mice or take muscle biopsy and study it under the microscope (Law, 1994).

2.8.5. Enzyme linked immunosorbent assay (ELISA):

When compared with the infant mouse assay for the identification of diarrheagenic *E. coli*, ELISA was shown to be more sensitive. ELISA can be used to examine sera from patients with HUS for antibodies against *E. coli* O157. ELISA tests with *E. coli* O157 lipopolysaccharide (LPS) have been used successfully to provide serological evidence of infection with *E. coli* O157 (Chart *et al.*, 1991). In another study, a variant of the microtiter GM1-ELISA for *E. coli* LT was studied and was 4 – 8 times more sensitive than the vero cell monolayer assay. The sensitivity of this ELISA and CHO cell monolayer assay were identical (Bangaerts *et al.*, 1985).

2.8.6. Fluorescence techniques:

An *in vitro* test to determine the ability of EPEC and EHEC to produce A/E lesion. The test uses fluorescene isothiocyanate – labeled phalloidin (a fungal toxin that binds to filamentous actin) to detect polymerized actin derived from localized cytoskeletal breakdown in cell cultures to which EPEC organisms attach (Law, 1994).

2.8.7. Molecular techniques:

Diarrheagenic *E. coli* strains were among the first pathogens for which molecular diagnostic methods were developed. Indeed, molecular methods remain the popular and most reliable techniques for differentiating diarrheagenic strains from non-pathogenic members of the stool flora and distinguishing one category from another (Olsvik and Strockbine, 1993)

The *E. coli* genome is a single circular DNA molecule consisting of about $4*10^6$ base pairs with a molecular weight of $4*10^9$ and a total length of 1.4 mm. Since this DNA is packed into a cell of about 2.0μm in length (Bettelheim, 1992). The molecular techniques that are used for differentiating diarrheagenic strains of *E. coli* are:

2.8.7.1. Nucleic acid probes:

They are segments of DNA or RNA that have been labeled with enzymes, antigenic substrates, chemiluminescent moieties, or radioisotopes and can bind with high specificity to complementary sequences of nucleic acids. Nucleic acid – based probes themselves can be of two types polynucleotide or oligonucleotide. DNA fragment (polynucleotide) probes may be derived from genes that encode a particular phenotype. Oligonucleotide probes derived from the DNA sequence of a target gene. Oligonucleotide probes have the advantage of faster or often cleaner results than those generated by polynucleotide methods (Fred and Elizabeth, 1993). Specimen preparation for nucleic acid probe methods include inoculation of purified cultures onto agar plates to produce "colony" blots, after incubation, the bacterial growth is transferred to nitrocellulose or Whatman filter paper for hybridization, the bacterial growth on the paper can be lysed, denatured, and hybridized with the probe *in situ*, and then a radiographic image is generated by exposure to X-ray film (Fred and Elizabeth, 1993).

2.8.7.2. Polymerase chain reaction (PCR)

The best – studied and most widely used target amplification technique is PCR (Persing, 1993). In the 8 years since its first description by scientist at the Cetus Corporation, PCR has developed into a main stay technique in many molecular biology laboratories. This method uses repeated cycles of oligonucleotide – directed DNA synthesis to perform *in vitro* replication of target nucleic sequences. The oligonucleotides whose sequence is determined by the target nucleic acid are synthesized to be complementary to their annealing sites with the two different strands forward and reverse strand, each cycle of PCR consists of three steps: (i) a denaturation step, in which the target DNA is incubated at high temperature so that the target strands are melted apart and thus made accessible to hybridization by specific oligonucleotide primers, (ii) an annealing step, in which the reaction mixture is cooled to allow the primers to anneal to their complementary target sequences; and (iii) an extension reaction, usually done at an intermediate temperature, in which the primers are extended on the DNA template by a DNA polymerase. These three incubation steps are linked in what is referred to as thermal cycle. A typical PCR protocol comprises 30 to 50 thermal cycles. Each time a cycle is completed there is a theoretical doubling of the target

sequence. Thus, repeating the thermal cycle results in a geometric accumulation of amplified target sequences (Michael and David, 1990 and Persing, 1993).

2.8.7.2.1. Multiplex PCR

In multiplex PCR, multiple primer pairs specific for different targets are included in the same amplification reaction. Coamplification of different target serves several purposes (i) large regions of the DNA sequence can be scanned for alteration (ii) unrelated segments of the target genome can be tested (iii) cost – effective panel of tests for multiple pathogens from single specimen can be developed (Persing, 1993).

2.8.7.2.2. Nested PCR.

In this PCR, nested sets of PCR primers are used and is known as nested amplification. In a typical nested –amplification protocol, a first round of amplification is performed with a single primer pair for 15-30 cycles. This step produces an amplification product, which is transferred to a new reaction tube for a second round of amplification by using a second primer pair that is specific for the internal sequence amplified by the first primer pair, the second amplification usually

proceeds for an additional 15-30 cycles, after which the products are detected by gel electrophoresis (Persing, 1993)

Diagnosis of diarrheagenic *E. coli* by PCR:

The sensitivity of EAF PCR in diagnosis of EPEC determines the sensitivity of PCR, serial diluted broth cultures of E 2348/69 were subjected to amplification. A visible band was seen with even 10 bacteria, this result was also obtained when these bacteria were mixed with 10^8 organisms of EAF PCR – negative strains, and also all EHEC, EIEC, and ETEC strains revealed negative EAF PCR results (Frank *et al.*, 1994).

Victor *et al.* (1991) have described improved PCR technique for amplification of the subunit of LT gene directly without prior extraction by optimizing the amplification conditions. Their procedure was inexpensive, sensitive, and simple. It has been reported that preselection of bacteria on plates is better than using stool samples because *Taq* polymerase can be inhibited in the presence of raw biological samples, and other PCR inhibitors which are found in stool including bile salts, bilirubin, urobilinogens, polysaccharides and large amounts of irrelevant DNA (Stacy – Phipps *et al.*, 1995).

Finally, the PCR method is simple, rapid and inexpensive for the diagnosis of diarrheagenic *E. coli*, therefore suitable for application in a developing – country field setting including Jordan (Yussef *et al.*, 1996).

3. Materials and Methods:

3.1.1. Materials and Reagents: -

- MacConkey agar – Oxoid, Unipath Ltd., Hampshire, England.
- Brain Heart infusion broth – Oxoid, Unipath Ltd., Hampshire, England.
- Absolute Ethanol – Labscan Ltd. Dublin, Ireland.
- Glycerol – Merk, Germany.
- Cryotubes – AB gene House, Epsom, UK.
- PCR buffer: 100 mM Tris-HCl, 500 mM KCl, (GIBCO-BRL, Gaithersburg, USA), stored at -20°C in 100 to 300 µL aliquots.
- MgCl2 (50 mM), GIBCO-BRL, Gaithersburg, USA.
- Deoxynucleoside triphosphates (dNTPs) / 100mM each; GIBCO-BRL: 5mM each nucleotide in sterile distilled water, stored at -20°C in 100 – 300 µL aliquots.
- Primers (50 µM in distilled water), stored separately at -20°C in aliquots of 10 – 50 µL (Table 3).
- Taq polymerase (5U/µL), GIBCO – BRL, UK.
- DNA marker ø x –174 – RF DNA HaeIII Digest (500 µg/mL) AB gene House, Epsom, UK.
- DNA marker 1 kb ladder, AB gene House, Epsom, UK.
- 0.2 mL eppendorf tubes, AB genes House, Epsom, UK.

- LE agarose, Promega, USA.
- TBE buffer (Boric acid, Trisma base and EDTA), Promega, USA.
- Nuclease free water, Promega, USA.
- Loading Die, Promega, USA.
- Ethidium Bromide, Sigma, USA.
- Sterile (autoclaved) water, stored at room temperature in 10 – 20 mL volumes, with fresh aliquot used for each assay.
- Normal Saline.
- API 20E kit, system for identification of gram – negative bacteria.

3.1.2.Equipments:

- Incubator.
- Thermal Cycler, MJ Research - INC, USA.
- Laminar Flow Cabinet.
- Electrophoresis Tank, Biorad, USA.
- Power supply, Biorad, USA.
- Autoclave.
- UV Camera, Monitor & Printer, UVP, USA.

Table 3: Oligonucleotide primers used in the detection of diarrheagenic *E. coli*.

Gene/ pathogen	Primer	Primer sequence	Concentration (μM)	Expected amplified product (bp)
SLTI/ EHEC	NS1 NS2	CAG TTA ATG TGC TGG CGA AG CAC AGA CTG CGT CAG TGA GG	2.0 2.0	475
SLTII/ EHEC	NS5 NS7	CTT CGG TAT CCT ATT CCC GG CGC TGC AGC TGT ATT ACT TTC	2.0 2.0	863
LTI/ ETEC	LTIB1 LTIB2	TCT CTA TGT GCA TAC GGA GC CCA TAC TGA TTG CCG CAA T	2.0 2.0	320
STa/ ETEC	STI-1 STI-2	TTA ATA GCA CCC GGT ACA AGC AGG CCT GAC TCT TCA AAA GAG AAA ATT AC	4.0 4.0	147
*ipa*H/ EIEC	IPAH8B IPAH15B	GTT CCT TGA CCG CCT TTC CGA GCC GGT CCA CCC TCT GAG	0.4 0.4	620
O157 *uid*A/ EHEC	*uid* A1 *uid* A2	GCG AAA ACT GTG GAA TTG GG TGA TGC TCC ATA ACT TCC TG	0.4 0.4	252
PCVD432 / EAggEC	PCVD432 Start Stop	 CTG GCG AAA GAC TGT ATC AT CAA TGT ATA GAA ATC CGC TGT T	 0.4 0.4	630

3.2. Methods

3.2.1. Sampling:

A total of 250 stool samples were collected from hospitalized patients who were admitted to pediatric departments of the Jordan University Hospital (JUH) and Islamic Hospital (IH) over a one – year period from January through December 2000. Ten to 14 stool samples were collected each month from both diarrheal and control patients. Stool samples were collected from children below 13-year old and from both sexes (145 male and 105 female).

Fresh stool samples were collected in sterile leak-proof plastic containers and cultured as soon as possible as recommended (Keskimaki *et al.*, 2000) or transported on ice within 4 hours of collection to the microbiology laboratory at JUH and IH where they were diluted by 3 ml of saline and cultured directly on MacConkey agar plates and incubated at 37°C for 18 – 24 hours.

Ten colonies that morphologically resemble *E. coli* either lactose fermenter or lactose nonfermenter were isolated from MacConkey plates and inoculated in one mL Brain heart infusion broth containing 25% glycerol (Ramotear *et al.*, 1995), then stored at –70°C for PCR detection of diarrheagenic *E. coli*.

A number of lactose fermenter *E. coli* isolated colonies were randomly selected and all *E. coli* colonies that are non-lactose fermenter

from different stool cultures were subcultured on MacConkey agar and tested by API 20E kit to ensure their identity as *Escherichia coli*.

3.2.1.1. Clinical Cases and Controls

Out of the 250 collected stool samples, there were 133 stool samples obtained from children suffering from acute diarrhea, and 117 stool samples that were collected from children admitted to the hospital for reasons other than diarrhea were selected as control group with age and sex matching the diarrheal group.

About half of the diarrheal stool samples were collected from children in the emergency room, before the administration of any drug as recorded by the attending physician. There were 15 samples sent to the bacteriology laboratory with a request from physicians for detection of diarrheagenic *E. coli* or their toxins.

3.2.1.2. Collection of Clinical Data:

For both clinical cases and control groups, the age, sex, name, length of hospitalization, and date of collection of the specimen were recorded in a questionnaire shown in appendix I. For the diarrheal specimens gross appearance of the stool (watery, mucoid, soft, or bloody), signs and symptoms of the patient due to diarrhea were recorded, in addition to the antibiotic treatment. Also, microscopic examination of diarrheal samples was done for detecting the presence of red blood cells and white blood cells.

3.2.2.PCR Detection of Diarrheagenic *E. coli*: -

3.2.2.1. Sample preparation:

Frozen *E. coli* isolates were inoculated in Brain heart infusion broth and incubated at 37°C for 2 – 3 hours for refreshment. After their subculture on MacConkey agar plates and incubation at 37°C for 24 hours, a heavy bacterial suspension was prepared from 10 *E. coli* colonies in 300-µL sterile distilled water and was boiled for 15 minutes using PCR Thermocycler to release template DNA (Tornieporth *et al.*, 1995), then the mixture was spined at 3000 rpm for 15 seconds to precipitate undesired substances.

3.2.2.2. Master Mix preparation:

Three preparation mixtures for PCR have been used as follows:

The first, Master Mix A, was directed for multiplex PCR in which multiple primer pairs specific for different targets were included in the same amplification reaction, this PCR master mix A was able to detect LT-I, STa of ETEC, *ipa*H of EIEC, and SLT-I and SLT-II of EHEC in any given cultured stool sample at the same time. In addition to these primers, the mixture contains 50 mM KCl, 10 mM Tris-HCl [8.3 pH], 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleoside triphosphate, 1 unit Taq polymerase, and sterile distilled water to complete the reaction mixture volume up to 25µL (Tornieporth *et al.*, 1995) (Table 4)

Table 4: Master Mix A.

Master mix A	Volume (µL)
10 X PCR buffer	2.5
50 mM $MgCl_2$	0.75
DNTPs 10 X (5mM)	1
Primers	
$LTIB_1$, LTI-2 (2µM)	1
STI-1, LTI-2 (4µM)	1
SLT-I NS_1, SLT-I NS_2(2 µM)	1
SLT-II NS_5, SLT-II NS_7(2 µM)	1
IPAH 15 B, IPAH 8 B (0.4 µM)	1
Taq polymerase	0.2
DNA template	2
H_2O	13.55

The second, Master Mix B (Table 5) was able to detect only one strain of *E. coli* by amplification of PCVD432 gene of EAggEC.

Table 5:Master Mix B.

Master Mix B	Volume (µL)
10 X PCR buffer	2.5
50 mM $MgCl_2$	0.75
DNTPs 10 X (5mM)	1
PevD432 start, pevD432 slip (0.4µM)	1
Taq polymerase	0.2
DNA template	2
H_2O	17.55

The third, Master mix C was designed to detect EHEC (other than O157: H7) by amplification of *uid A* gene of EHEC (Table 6).

Table 6:Master Mix C.

Master Mix C	Volume (µL)
10 X PCR buffer	2.5
50 mM $MgCl_2$	0.75
dNTPs 10 X (5mM)	1
Uid A_1, UidA_2 (0.4 µM)	1
Taq polymerase	0.2
DNA template	2
H_2O	17.55

Master Mix should not be frozen, because the DNA polymerase is inactivated by freezing thawing and primers are more stable in a concentrated form.

Table 6:Master Mix C.

Master Mix C	Volume (µL)
10 X PCR buffer	2.5
50 mM $MgCl_2$	0.75
dNTPs 10 X (5mM)	1
Uid A_1, UidA_2 (0.4 µM)	1
Taq polymerase	0.2
DNA template	2
H_2O	17.55

Master Mix should not be frozen, because the DNA polymerase is inactivated by freezing – thawing and primers are more stable in a concentrated form.

3.2.2.3. PCR Amplification:

Several trials of PCR optimization have been done by changing the primers concentration, $MgCl_2$ concentration, annealing temperature and template concentration, to get best separation and resolution of the desired band and reduce non-specific amplification (Figure 3).

The PCR assays for the target genes were performed by using programmable thermocycler with the following cycling parameters: first, at 95°C for 30 seconds in order to denature DNA, then at 55°C for 1 minute to anneal the primers on the template, and finally at 72°C for one minute to extend the annealed primers. The cycle was repeated 35 times and final extension was performed at 72°C for 10 minutes. Tubes were hold at 4°C when the cycles ended (Meqdam *et al.*, 1997).

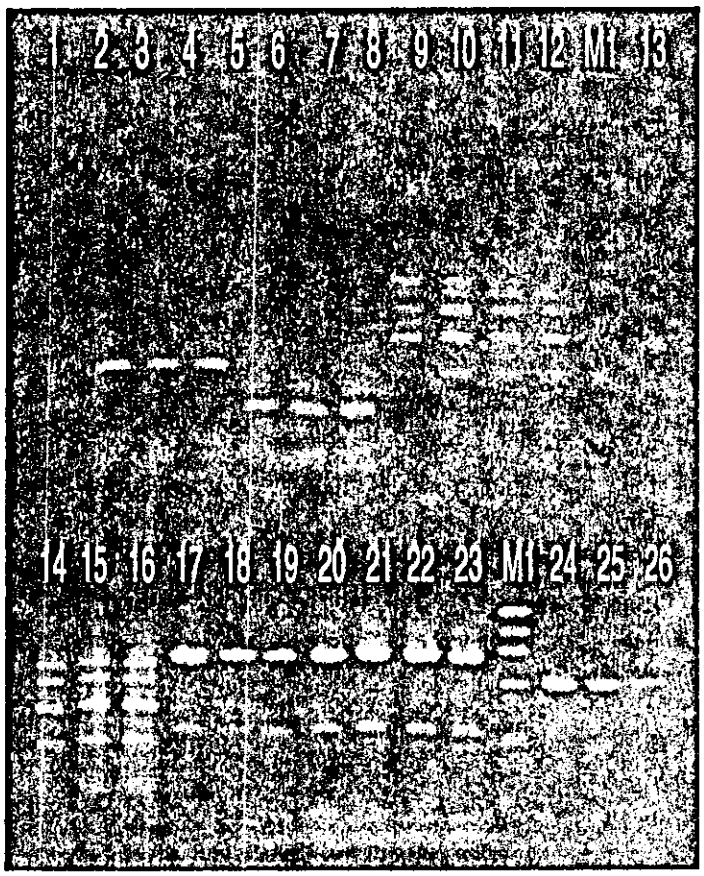

Figure 3: Primers optimization. Agarose gel electrophoresis for PCR products of the *E. coli* reference strains. Lane 1 negatine control, lane 2 to lane 4: Reference strain H10407 (STa^+ - 147 bp), lane 5 to lane 7: Reference strain H10407 ($LT\text{-}I^+$ - 320 bp), lane 9 to lane 12 and lane 14 to lane 16: Reference strain ZB4 ($SLT\text{-}I^+$ - 475 bp), lane 17 to lane 23: Reference strain C4193-1 ($SLT\text{-}II^+$ - 863 bp), lane 24 to lane 26: Reference strain EDL1284 ($ipaH^+$ - 620 bp), M1: DNA marker ØX-174 Hae III.

➢ **Quality Control: -**

Two negative controls and a positive control have been used in each trial:

- **Negative Control 1**: PCR test without any added DNA template to detect contamination of PCR reagents.
- **Negative Control 2**: PCR test with DNA from *E. coli* strain which is negative for the target gene amplified by the primers in that reaction.
- **Positive Controls**:
  1. Heat – stable enterotoxin (STa) producing ETEC strain TX_1.
  2. LT1 and STa enterotoxin producing ETEC strain H 10407.
  3. SLT-I and SLT-II producing EHEC strain C4193-1.
  4. EIEC strain EDL 1284 containing *ipa*H plasmid.
  5. EAggEC strain 3591-78 containing PCVD432 plasmid.
  6. ZB4 strain containing *ipa*H plasmid and SLT-I producer.

3.2.2.4. Detection of PCR products:

10X TBE buffer was prepared by dissolving 55gm Boric acid, 108 gm Trisma base, and 7.4 gm EDTA in 1 liter distilled water. This buffer was used to prepare 1.5% agarose. The PCR products were analyzed by agarose gel electrophoresis run for 2 hours at 150V using horizontal electrophoresis apparatus. After the electrophoresis was completed the gel was stained for 15 minutes in ethidium bromide (0.2 gm/ml), destained for 15 min in tap water, then visualized under ultraviolet light. ΦX 174 - RF DNA HaeIII digest and 1 kb ladder, was used as molecular mass marker (Yu-Litsai *et al.*, 1993).

3.2.3. Antibiotic Susceptibility:

All positive isolates for one or more diarrheagenic *E. coli* strains from diarrheal samples and control samples were tested for susceptibility to most common antibiotics used for treatment of diarrhea or urinary tract infection in Jordan.

Four to five colonies were picked from fresh culture of *E. coli* and suspended in 5 ml Mueller–Hinton broth for 1 to 2 hours, a cotton swab absorbed by this suspension was used to spread the bacteria on Mueller–Hinton agar. The following commercially available antibiotic discs (Mast diagnostics, Merseyside, UK) were used at the following concentration:

ampicillin (10 μg), cefuroxime (30 μg), cotrimoxazole (25 μg), gentamicin (10 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), and tetracycline (25 μg).

Mueller–Hinton agar plates were incubated at 37°C for 24 hours, and inhibition zones were measured and recorded as susceptible or resistant according to NCCLs guidelines, 1997. *E. coli* ATCC 25922 strain was included for control.

3.2.4.Statistical Analysis:

All data were entered in a computer database and analyzed using the SPSS® statistical computer software to make frequency distributions, cross – tabulations and to calculate (χ^2) values. The correlation between results have been considered statistically significant when the p value was less than 0.05.

4. Results

4.1. Epidemiology and clinical considerations of the patients with diarrhea and without diarrhea (controls):

During the 12 – months study period (January – December 2000), 133 patients with diarrhea and 117 patients without diarrhea (controls) were randomly selected among children hospitalized in the pediatric section of the JUH and IH. The age range of the children was 0 to 13 years with mean age of 30 months and 33 months for diarrheal and control groups, respectively. All diarrheal cases were clinically diagnosed as gastroenteritis, whereas the controls were diagnosed to have respiratory infections. The percentage of males (57.9%) and females (42.1%) in the diarrhea group matches the control group ratios (58.1% males and 41.9% females) without a significant difference between the two groups ($p>0.05$) (Tables 7 and 8).

Table 7: Distribution and frequencies of clinical cases and controls according to age.

Cases	Age						Total
	Less than one year	1 – 3	3 – 5	5 – 7	7 – 10	10 – 13	
Diarrhea Number (%)	58 (43.6)	34 (25.6)	15 (11.3)	12 (9.0)	10 (7.5)	4 (3.0)	133
Control Number (%)	33 (28.2)	40 (34.2)	5 (4.3)	15 (12.8)	11 (9.4)	13 (11.1)	117
Total	91	74	20	27	21	17	250

Table 8: Distribution and frequencies of clinical cases and controls according to sex.

Cases	Sex		Total
	Male	Female	
Diarrhea Number (%)	77 (57.9)	56 (42.1)	133
Control Number (%)	68 (58.1)	49 (41.9)	117
Total	145	105	**250**

Recorded clinical symptoms, such as fever, vomiting, abdominal pain, and dehydration were significantly more frequent in the children hospitalized for diarrhea than in controls ($p<0.05$). Table 9 shows the results of stool examination, frequency of diarrhea, and symptoms of diarrheal children.

Table 9: Stool examination and other characteristics of diarrheal children.

Type	Number of patients (percent)
Frequency of diarrhea (Number/day)	
3-5	38 (28.6)
5-8	70 (52.6)
8-10	15 (11.3)
>10	10 (7.5)
Macroscopic examination	
Mucoid stool	69 (51.9)
Bloody stool	2 (1.5)
Watery stool	46 (34.6)
Soft stool	12 (9.0)
Mucoid bloody stool	4 (3.0)
Microscopic examination	
Moderate presence of RBC's	32 (24.1)
Numerous and abundant RBC's	14 (10.5)
No RBC's seen	87 (65.4)
Moderate presence of WBC's	58 (43.6)
Numerous and abundant WBC's	29 (21.8)
No WBC's seen	46 (34.6)
Signs and symptoms	
Vomiting only	33 (24.8)
Fever only	28 (21.1)
Vomiting and fever	48 (36.1)
No signs	24 (18.0)

Abundant: more than 50 cells / HPF.
Numerous: less than 50 cells/HPF and more than 25 cells/HPF.
Moderate: between 10-25 cells/HPF

4.2. Prevalence of diarrheagenic *E. coli* and their virulence genes in diarrheal and control children:

A total of 250 Stool specimens were collected and cultured to detect diarrheagenic *E. coli* by PCR using oligonucleotide primers (mixture A) that are designed to simultaneously amplify LT-1, STa of ETEC, ipaH of EIEC, or SLT-I and SLT-II of EHEC in a single PCR tube if they are present (figure 4). The PCR reaction mixture B was able to detect PCVD432 of EAggEC (figure 5). PCR reaction mixture C was able to detect *uid A* of EHEC other than wild type O_{157}: H_7 (figure 6). Each *E. coli* isolate was analyzed by using the three primer mixtures. Figures 7,8 and 9 show the agarose gel electrophoresis of PCR-amplified DNA products of *E. coli* isolates from control samples after it was analyzed by PCR reaction mixture A, B and C respectively. All 250 *E. coli* isolates from stool samples were examined for ETEC, EAggEC, EIEC, and EHEC. Of these 250 specimens, 53 (21.2%) were positive for one or more of diarrheagenic *E. coli*. Diarrheagenic *E. coli* strains were detected in 31 (23.3%) patients of diarrheal group and in 22 (18.8%) patients of the control group, this finding was statistically not significant ($p>0.05$). Among the patients with diarrhea there was 13 (9.8%) *E. coli* isolates positive for ETEC [LT-I / STa or both], 4 (3.0%) for EIEC [ipaH], and 12 (9.0%) for EAggEC [PCVD432]. Among the control group 13 (11.1%) *E. coli* isolates were

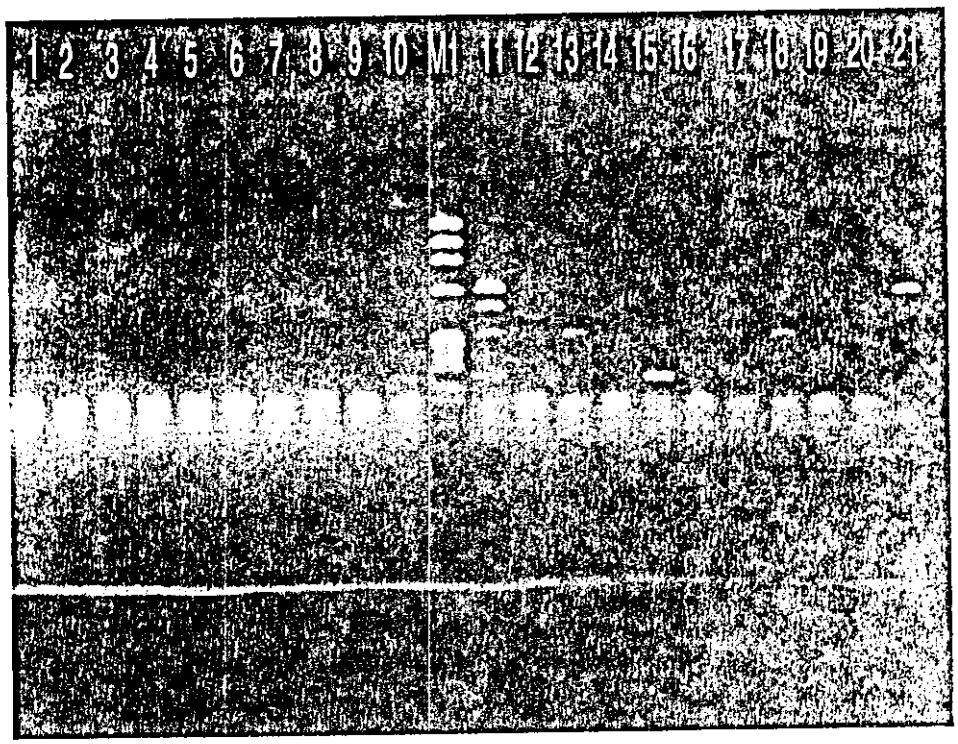

Figure 4: Agarose gel electrophoresis for PCR product of *E. coli* isolates recovered from diarrheal samples analyzed by master mix A (primer mix: LT-I + STa + ipaH + SLT-I + SLT-II), lane 1: Negative control 1 (no DNA template), lane 2 – lane 10: Negative samples, M1: DNA marker (ØX-174 Hae III), lane 11: Positive control (Reference strains: H10407 (STa^+-147 bp, $LT\text{-}I^+$-320 bp), ZB4 ($SLT\text{-}I^+$-475 bp), EDL1284 ($ipaH^+$-620 bp)), lane 12: negative control 2 (template negative for the target gene), lane 13: LT-I positive, lane 15: ($LT\text{-}I^+$, STa^+), lane 18: $LT\text{-}I^+$, lane 21: $ipaH^+$.

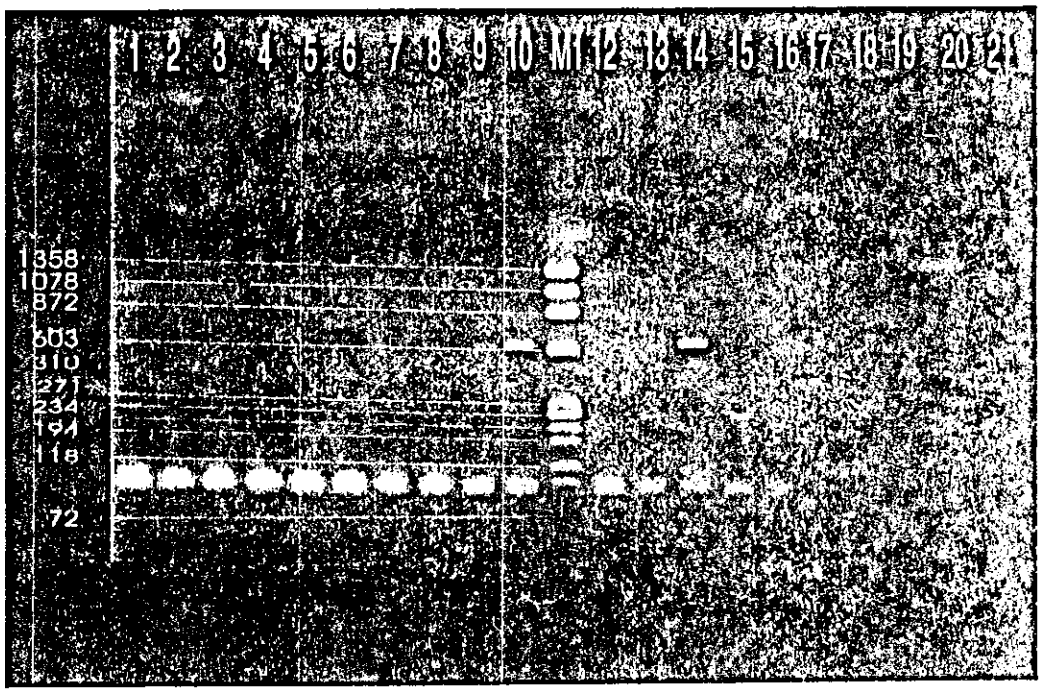

Figure 5: Agarose gel electrophoresis for PCR product of *E. coli* isolates recovered from diarrheal samples analyzed by master mix B (primer: PCVD432), lane 1: Negative control 1, lane 2 – lane 9: Negative samples, Lane 10: Reference strain (3591 – 78 (PCVD432^{1} 630 bp)), lane 12: Negative control 2, lane 14: PCVD432^{1} sample, M1: DNA marker (ΦX 174 Hae III).

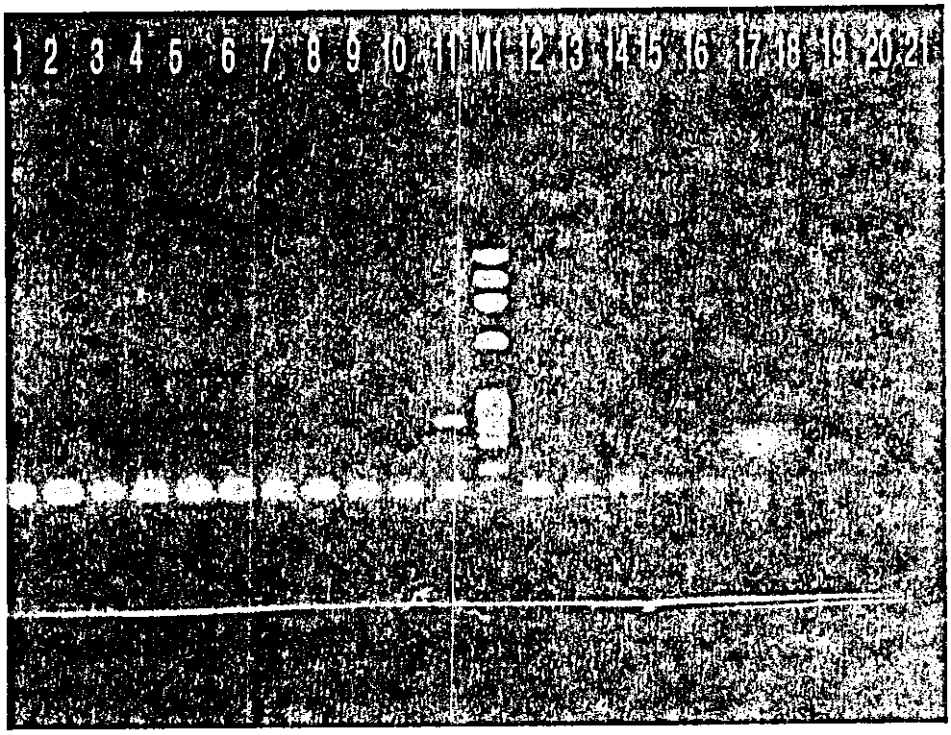

Figure 6: Agarose gel electrophoresis for PCR product of *E. coli* isolates recovered from diarrheal samples analyzed by master mix C (primer: *uid* A), lane 1: Negative control 1, lane 2 – lane 10: Negative samples, Lane 11: Reference strain (4193 – 1 (*uid* A^+ – 252 bp)), M1: DNA marker (ØX – 174 Hae III).

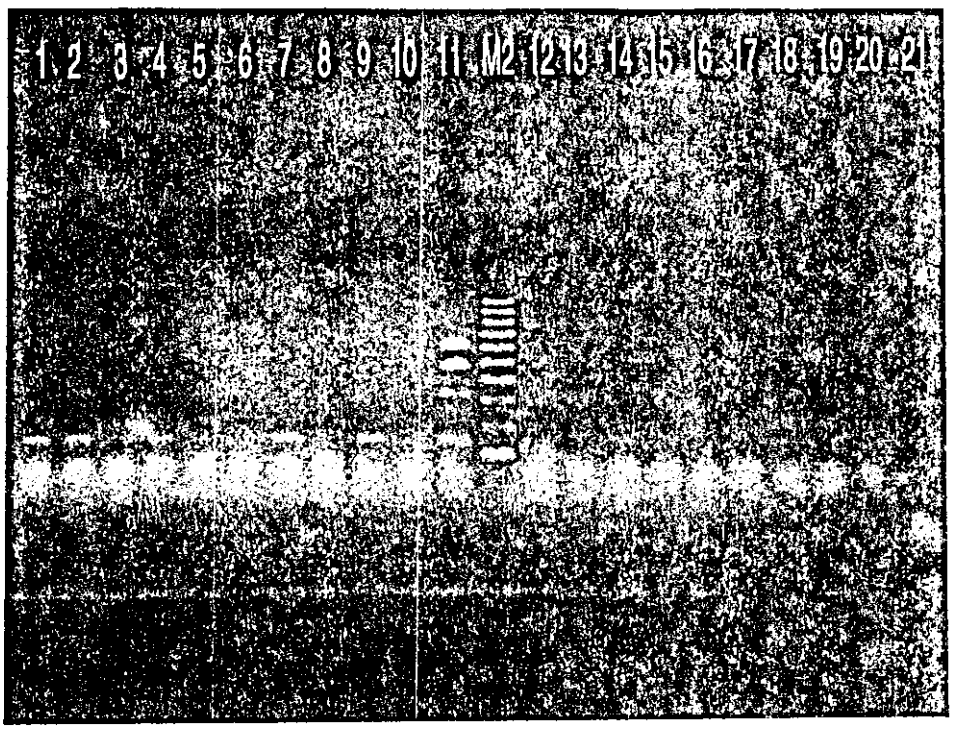

Figure 7: Agarose gel electrophoresis for PCR product of *E. coli* isolates recovered from control samples analyzed by master mix A (primer mix: LT-I + STa + ipaH + SLT-I + SLT-II), lanes 1,2,4,7, and 9:(STa^+ - 147 bp), lane 2: ($LT\text{-}I^+$ - 320 bp), lane 10: Negative control 1, M2: DNA marker (100 bp digest), lane 12 – lane21: Negative samples, lane 11: Positive control (Reference strains: H10407 (STa^+-147 bp, $LT\text{-}I^+$-320 bp), ZB4 ($SLT\text{-}I^+$-475 bp), EDL1284 ($ipaH^+$-620 bp)).

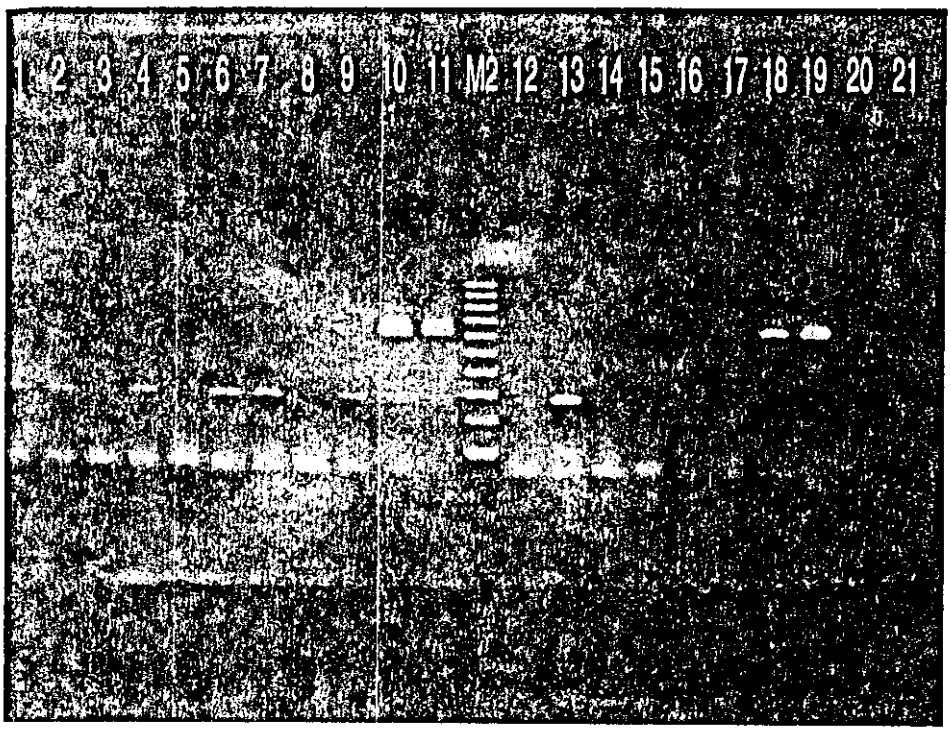

Figure 8: Agarose gel electrophoresis for PCR product of *E. coli* isolates recovered from control samples analyzed by master mix B (primer: PCVD432), lane 1 – lane 9: Negative samples, Lane 11: Positive control (Reference strain: 3591 – 78 ($PCVD432^{+}$ – 630 bp)), lane 12: Negative control 2, lane 10, lane 18 and lane 19: $PCVD432^{+}$ samples, M2: DNA marker (1 Kb ladder).

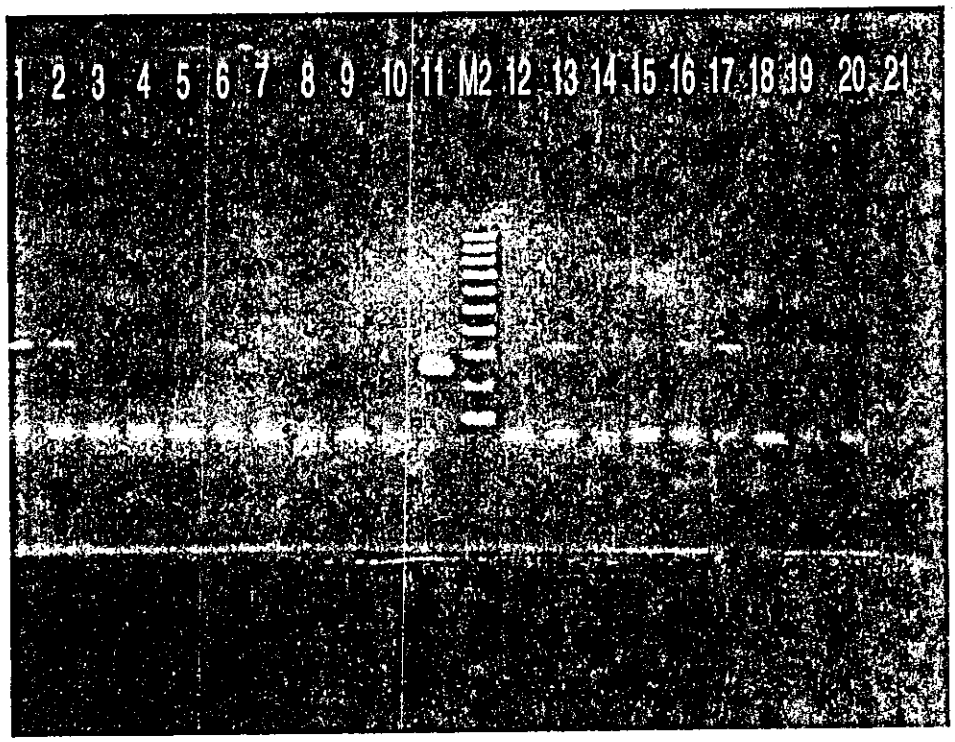

Figure 9: Agarose gel electrophoresis for PCR product of *E. coli* isolates recovered from diarrheal samples analyzed by master mix C (primer: *uid* A), lane 1 – lane 10 and lane 13 – lane 21: Negative samples, Lane 11: Positive control (Reference strain: (4193 – 1, *uid* A^+ – 252 bp)), lane 12: Negative control 1, M: DNA marker (1 Kb ladder).

positive for ETEC, 7 (6.0%) for EAggEC. Mixed infections with two categories of diarrheagenic *E. coli* strains were detected in 2 (1.5%) of the diarrheal group and in 2 (1.7%) of the control group (Table 10).

Table 10: **Frequency and significance of diarrheagenic *E. coli* isolated from diarrheal and control samples.**

Category of *E. coli*	Patients No. (%)		p-value
	With diarrhea	Without diarrhea	
ETEC	13 (9.8)	13 (11.1)	0.7
EIEC	4 (2.9)	0	0.045
EHEC	0	0	---
EAggEC	12 (9.0)	7 (6.0)	0.3
Mixed infection	2 (1.5)	2 (1.7)	---
Total positive	31 (23.3)	22 (18.8)	---
Total negative	102 (76.7)	95 (81.2)	---
Total	133	117	---

p-value < 0.05 significant.
p-value > 0.05 nonsignificant.

There is no significant difference in the rate of isolation of ETEC and EAggEC between cases and control children. EHEC strains were not found neither in stool specimens with diarrhea nor in control specimens. ETEC was the most common diarrheagenic *E. coli* detected in both diarrheal stool specimens (41.9%) and in control stool specimens (59.0%).

The rate of EIEC isolation was statistically significant (p= 0.045) in diarrheal children (2.9%) as compared with nondiarrheal control children (Null). All mixed infections were caused by *E. coli* strain known as PCVD432 + STa.

The PCR results with oligonucleotide primers specific for the virulence factors of diarrheagenic *E. coli* are listed in table 11.

Table 11: **Distribution of diarrheagenic *E. coli* virulence factors among control and diarrheal samples.**

Virulence factor	Patients No. (%)	
	With diarrhea	Without diarrhea
ETEC		
LT-I	4 (3.0)	1 (0.9)
STa	8 (6.0)	11 (9.4)
LT-I/STa	1 (0.8)	1 (0.9)
EIEC		
IpaH	4 (3.0)	----
EAggEC		
PCVD432	12 (9.0)	7 (6.0)
EHEC		
SLT-I	---	---
SLT-II	---	---
nid A	---	---
Mixed		
PCVD432 + STa	2 (1.5)	2 (1.7)
Total positive	31 (23)	22 (18.8)
Total negative	102 (76.7)	95 (81.2)
Total	133	117

4.3. Prevalence of diarrheagenic *E. coli* in clinical cases according to different variables:

4.3.1.Prevalence of diarrheagenic *E. coli* in clinical cases and controls according to age:

Table 12 shows that more than half of the positive samples for diarrheagenic *E. coli* strains (54.1%) were isolated from children less than 3 years old. EIEC was not found in the stool specimens of patients less than 3 years old. However, there was a statistically significant correlation between prevalence of diarrheagenic *E. coli* and age group (p=0.001). Since 34% of isolates were found in children aged less than one year.

Table 12: **Prevalence of diarrheagenic *E. coli* in clinical and control samples according to age.**

Category of *E. coli*	Group	Age groups (%)						Total
		Less than one year	1 – 3	3 – 5	5 – 7	7 – 10	10 – 13	
ETEC	Diarrhea	8 (6.0)	4 (3.0)	--	1 (0.8)	--	--	13
	Control	2 (1.8)	4 (3.4)	1 (0.9)	2 (1.7)	2 (1.7)	2 (1.7)	13
EIEC	Diarrhea	--	--	1 (0.8)	--	3 (2.3)	--	4
	Control	--	--	--	--	--	--	--
EAggEC	Diarrhea	6 (4.5)	1 (0.8)	3 (2.3)	--	1 (0.8)	1 (0.8)	12
	Control	--	2 (1.7)	1 (0.9)	2 (1.7)	--	2 (1.7)	7
EHEC	Diarrhea	--	--	--	--	--	--	--
	Control	--	--	--	--	--	--	--
Mixed	Diarrhea	1 (0.8)	--	--	--	1 (0.8)	--	2
	Control	1 (0.9)	--	--	--	--	1 (0.9)	2
Total no.(%)		18 (34)	11 (20.1)	6 (11.3)	5 (9.4)	7 (13.2)	6 (11.3)	53 (100)

4.3.2.Prevalence of diarrheagenic *E. coli* in clinical cases and controls according to sex:

Table 13 demonstrates that the prevalence of diarrheagenic *E. coli* in the stools of diarrheal and control children was (52.8%) for males and (47.2%) for females. The results indicate that there is no significant difference between the sex of the patient and prevalence of diarrheagenic *E. coli* infections ($p>0.05$).

Table 13: **Prevalence of diarrheagenic *E. coli* in clinical and control samples according to sex.**

Category	Group	Sex		Total
		Male (%)	Female (%)	
ETEC	Diarrhea	8 (6.0)	5 (3.8)	13
	Control	9 (7.7)	4 (3.4)	13
EIEC	Diarrhea	2 (1.5)	2 (1.5)	4
	Control	--	--	--
EAggEC	Diarrhea	5 (3.8)	7 (5.3)	12
	Control	2 (1.7)	5 (4.3)	7
EHEC	Diarrhea	--	--	--
	Control	--	--	--
Mixed	Diarrhea	1 (0.8)	1 (0.8)	2
	Control	1 (0.9)	1 (0.9)	2
Total no. (%)		28 (52.8)	25 (47.2)	53

4.3.3. Prevalence of diarrheagenic *E. coli* in clinical cases as associated with the presence of RBC's in the stool specimens:

Table 14 shows that most of diarrheagenic *E. coli* is not associated with blood in the stool (58%), except for EIEC which is mostly associated with blood as shown in the Table 13, however, there was no statistically significant correlation between bloody stool and diarrheagenic *E. coli* infection ($p>0.05$).

Table 14: **Prevalence of diarrheagenic *E. coli* in clinical cases as associated with presence of blood in stool.**

E. coli isolates (no.31)	Erythrocytes			
	Nil	Less than 10 cell / HPF	10 – 20 cells/HPF	Abundant
ETEC (13)	11 (8.3)	1 (0.8)	--	1 (0.8)
EIEC (4)	--	1 (0.8)	--	3 (2.3)
EAggEC (12)	5 (3.8)	4 (3.0)	1 (0.8)	2 (1.5)
Mixed (2)	2 (2.3)	--	--	--
Total no. (%)	18 (58)	6 (19.4)	1 (3.2)	6 (19.4)

4.3.4.Prevalence of diarrheagenic *E. coli* in clinical cases as associated with the frequency of diarrhea:

Table 15 demonstrates that there was no statistically significant correlation between frequency of diarrhea and diarrheagenic *E. coli* infections ($p>0.05$).

Table 15: **Prevalence of diarrheagenic *E. coli* according to frequency of diarrhea in days.**

***E. coli* isolates**	**Frequency of diarrhea/day (%)**			
(Total no. 31)	**3 – 5**	**5 – 8**	**8 – 10**	**> 10**
ETEC (13)	2 (1.5)	11 (8.3)	--	--
EIEC (4)	2 (1.5)	1 (0.8)	1 (0.8)	--
EAggEC (12)	--	10 (7.5)	2 (1.5)	--
Mixed (2)	1 (0.8)	1 (0.8)	--	--
Total	5	23	3	--

4.3.5.Distribution of diarrheagenic *E. coli* in clinical cases among the months of study period:

This study shows that positive cases for diarrheagenic *E. coli* strains were distributed over the year with a slightly increase in isolates during the summer months as shown in table 16.

Table 16: Distribution of positive cases among the study period.

Study period	No. of positive cases
January	1
February	2
March	2
April	1
May	3
June	5
July	4
August	3
September	2
October	2
November	4
December	2

4.4. Antibiotic susceptibility:

The antibiotic susceptibility results of the 53 diarrheagenic *E. coli* isolates recovered from both patients and controls are shown in Table 16. Most of diarrheagenic *E. coli* isolates were susceptible for nitrofurantoin (94.3%), gentamicin (88.7%) norfloxacin (86.8%), and nalidixixic acid (83%), respectively, but these isolates were resistant to ampicillin (88.7%) and to less extent to cotrimoxazole (60.4%) and tetracycline (45.3%), respectively.

Table 17: **Antibiotic susceptibility results of diarrheagenic *E. coli* isolates from diarrheal and control samples**

Pattern of susceptibility	Type of antibiotic							
	Amp	Cef	Cotr	Gent	NA.	Nitro.	Norf.	Tet.
Susceptible No. (%)	6 (11.3)	44 (83)	21 (39.6)	47 (88.7)	44 (83)	50 (94.3)	46 (86.8)	24 (45.3)
Resistant No. (%)	47 (88.7)	9 (17)	32 (60.4)	6 (11.3)	9 (17)	3 (5.7)	7 (13.2)	29 (54.7)

Ampicillin (Amp), Cefuroxime (Cef), Cotrimoxazole (Cotr), Gentamicin (Gent), Nalidixic Acid (N.A.), Nitrofurantoin (Nitro.), Norfloxacin (Norf), Tetracyclin (Tet)

When the pattern of susceptibility between *E. coli* isolates recovered from diarrheal samples compared with those recovered from control samples, a significant statistical correlation was found in the pattern of their susceptibility to tested antibiotics. Most of *E. coli* isolates from control stool samples were susceptible to ampicilin , cotrimoxazole and tetracycline ($p < 0.05$), whereas all diarrheagenic *E. coli* isolates that

were resistant to nalidixic acid, and nitrofurantoin were isolated from diarrheal stool samples ($p<0.05$).

5. Discussion

5.1. Prevalence of diarrheagenic *E. coli* in clinical cases and controls:

Diarrhea is a common cause of morbidity and mortality among infant and children in developing countries (Guerrant *et a.l*, 1990), including certain Arab countries (WHO report, 1997).

Numerous epidemiological studies have demonstrated that diarrheagenic *E. coli* strains are important cause of diarrhea in children worldwide (Nataro and Kaper, 1998). Diarrheagenic *E. coli* strains were among the first pathogens for which molecular diagnostic methods were established. Molecular methods, especially PCR, are currently considered the most reliable techniques for differentiating diarrheagenic *E. coli* strains from nonpathogenic members of the stool flora and for distinguishing among *E. coli* enteropathogens (Nataro and Kaper, 1998).

It is well established that diarrhea is mostly caused by different causative agents such as *Salmonella species*, *Shigella species*, *Campylobacter species*, *Yersinia Enterocolitica*, *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium* and others. literature review showed that few studies were published during the last ten years on bacterial causes of diarrhea in Jordanian children (Youssef *et al.*, 2000; Malkawi and Youssef, 1998; Shehabi, 1995).

The present study was carried out in Amman to determine the prevalence of diarrheagenic *E. coli* in children with and without diarrhea by using single and multiplex PCR. This study has investigated the occurrence of diarrheagenic *E. coli* in the stools of children hospitalized at Jordan University hospital (JUH) and Islamic hospital (IH) during a 12-month period of 2000. Clinical features and age of these patients were recorded. A total of 250 randomly selected children (0 to 13 year old) were included, 133 children with diarrhea and 117 matched controls without diarrhea. Children aged less than one year were represented by about one third (36%) of the total children examined, but accounted for 43.6% of the total diarrhea cases, while the majority of diarrhea cases (69.2%) were found in children aged less than three years old. This fact along with the common clinical findings of fever and vomiting as well as the presence of watery diarrhea more than bloody diarrhea in our examined children were similar to many studies described from the developed countries (Guerrant *et al.*, 1990, Nataro and Kaper, 1998).

The development of a simple, rapid, and efficient protocol for PCR analysis was key to the sensitive and specific detection of diarrheagenic *E. coli* during the course of infection (Stacy and Phipps, 1995). All diarrheal stool samples and controls were screened for diarrheagenic *E. coli* strains; ETEC, EIEC, EAggEC, and EHEC.

Most of diarrheagenic *E. coli* types were found in both diarrheal and control stools without any significant difference, but EIEC strains (2,9%) were isolated only from patients with diarrhea. In addition, the high percentage of ETEC isolates (9.8%) in this study was statistically not significant ($p>0.05$) in association with diarrheal children as compared with the high number of isolates in control children (11.1%). This finding is similar to that found in Bangkok, and Djibouti among diarrheal children (Mayatepek *et al.*, 1993, Mikhail *et al.*, 1990), but less than that reported from Bangladesh (17%) (Albert *et al.*, 1999) and Egypt (17%) (Mikhail *et al.*, 1989). A recent study from Nigeria found ETEC in 2.4% of children with diarrhea (Okeke *et al.*, 2000). Also, ETEC is a common cause of diarrhea in children in certain underprivileged population in the United States (Sack *et al.*, 1995). It is well established that ETEC is a common causative agent of traveller's diarrhea among persons traveling from developed to developing countries (Keskimaki *et al.*, 2000). Children in the developing countries are exposed to ETEC normally early in their lives and acquire immunity to its toxins. The percentage of diarrheal episodes caused by ETEC in these children , however, have varied from study to study, nation to nation, and from season to season (Keskimaki *et al.*, 2000, Albert *et al.*, 1999, Nataro and Kaper, 1998, Biswas *et al.*, 1996 ,Mathewson, 1987).

The rate of occurrence of EAggEC in this study is 9% in children with diarrhea and 6% in controls without diarrhea. A second study carried out in the North of Jordan revealed that the prevalence of the EAggEC in children without diarrhea was nearly twice that detected in children with diarrhea (Malkawi and Youssef, 1998). On the other site a third study which was carried out in the Jordan Valley by Meqdam *et al.*, (1997) reported no isolation of EaggEC from diarrheal children. However, this study and that of Malkawi and Youssef in 1998 emphasized that there is no significant correlation between diarrhea and EaggEC, in contrast to the other studies carried out in Nigeria, India and Mexico which considered EAggEC as an important pathogen in their pediatric population (Okeke *et al.*, 2000, Bhan *et al.*, 1989 and Mathewson *et al.*, 1987).

The present study has not detected EHEC strains in any of the patients nor controls. EHEC is an important pathogen in Europe, Japan, Canada and North America, and its isolation rates exceed in some countries other diarrheagenic *E. coli* strains (Nataro and Kaper, 1998, Griffin, 1995, Karmali, 1989). The prevalence of EHEC in these developed countries was associated with the consumption of poorly cocked foods such as hamburgers (Riley, 1987), fast-food restaurants (Grimm, 1995) or milk and other dairy products (Griffin, 1995). Numerous studies have suggested that cows and pigs are important

animal reservoirs of this human pathogen (Griffin, 1995). In developing countries such as Jordan, the role of EHEC strains in diarrheal disease is very limited, because the majority of the population depends mostly on well cooked food, and well pasteurized milk, which are the main sources of infection. However, the recent wide spread of fast – food restaurants in Jordan could be associated with the emergence of EHEC as important causative agent of diarrhea in the near future. Therefore, it was expected to find a few isolates of EHEC in this study, because the social habits of the population in Amman is closer to those of developed countries. Despite the fact that there was no isolation of EHEC strains in this study or other studies carried in Jordan, EHEC may have caused a few cases of HUS in Jordanian children (personal communication, R.Hamad, JUH, 2001).

The incidence of EIEC in this study was 2.9% in children with diarrhea, and this result is significant compared with no isolation of the EIEC from control children. This finding was similar to that of another study carried out in the Jordan Valley in which EIEC was the most frequent isolate from patients with diarrhea (4 out 17 cases) (Meqdam *et al*, 1997). The incidence of EIEC infection in other developing countries was reported to be 1.2% in Nigeria (Okeke *et al.*, 2000) or slightly higher and was considered an important cause of pediatric diarrhea in China 6.8 % (Kain, *et al.*, 1991) and in Thailand 4 – 9 % (Tailor *et al.*, 1988).

5.2 Prevalence of diarrheagenic *E. coli* in clinical cases according to different variables:

In relation to the presence of diarrheagenic *E. coli* and other variables, our results show that about 70% of diarrheagenic *E. coli* isolates were recovered from children aged less than three years with and without diarrhea, and this is a statistically significant correlation between the prevalence of diarrheagenic *E. coli* isolates and age of children (p=0.001).

Other recently published studies from developing countries also reported that children aged between 0 – 3 years are at a greater risk of contracting diarrhea due to a variety of diarrheagenic *E. coli* (Okeke *et al.*, 2000, Gascon *et al.*, 2000, Albert *et al.*, 1999, Milaat and Elassouli, 1995). A study carried out in Mogadishu observed a decrease in the ETEC isolation rate with increasing age (Mikhail *et al.*, 1990) which is very close to the results of this study. In addition, the present study shows that all of the EIEC strains were isolated from children aged more than 3 years, which is similar to the study of Meqdam *et al.*, 1996 in the Jordan Valley. The present study and all other studies showed that there was no significant correlation between sex and prevalence of diarrheagenic *E. coli* in children with diarrhea (Nataro and Kaper, 1998).

Other characteristics of diarrheal children such as vomiting, temperature, frequency of diarrhea, and results of macroscopic examination of stool samples were not significant with isolation of diarrheagenic *E. coli*. The presence of fecal leukocytes was also not significant according to the presence of diarrheagenic *E. coli in* this study, therefore most positive stool specimens would have been missed if fecal leukocytes had been used as a screening test (Fan *et al.*, 1993).

In general, there was no statistical significant correlation between bloody stool and diarrheagenic *E. coli* infection ($p>0.05$), but for EIEC most stool samples contained abundant RBC's and was associated with diarrhea (75%) This result was confirmed by the fact that this organism have been shown to invade the colonic epithelium and emphasize the significant correlation between bloody diarrhea and presence of EIEC in stool (Nataro and Kaper, 1998).

There were no deaths associated with diarrheal illness during this study. Diarrheal deaths had decreased markedly in the Jordanian children during the last decade and are now extremely rare (Khuri – Bolus, 1999).

5.3 Antimicrobial susceptibility patterns

Previous studies have shown a high prevalence of antibiotic resistance among certain gram-negative bacterial isolates in the Jordan University hospital during the recent years, particularly *E.coli* isolates from different clinical specimens other than stools proved to be highly resistant to ampicillin (Shehabi, 1996, Shehabi *et al.*, 2000). Also, this study has revealed that diarrheagenic *E. coli* isolates were highly resistance to co-trimoxazole (60%) and ampicillin (88.7%) which are commonly used antibiotics in Jordan. These findings are in agreement with the results of several studies from Jordan and other developing countries (Vila *et al.*,1999, Malkawi and Youssef, 1998, Barttoloni *et al.*, 1998, Mumun *et al.*, 1993, Mayatepek *et al.*, 1993). However, antimicrobial resistance to nitofurantoin, gentamicin, norfloxacin , nalidixic acid and cefuroxime were low and ranged between 5.7% to 17% among diarrheagenic *E. coli* isolates. Recent studies also showed that nalidixic acid and ciprofloxacin have very good activity against enterotoxigenic *E.coli* isolates causing diarrhea in travelers to India (Vila et al., 1999) and against diarrheagenic *E. coli* isolates in Tanzania (Vila et al., 1999) and Kenya (Sang, et al., 1997).

Diarrhea caused by multidrug-resistant bacteria is an important public health problem among children in developing countries (Bern, *et al.*, 1992), in addition bacterial resistance to antimicrobial agents is an

increasing problem in many areas of the world, especially in countries where there is little information available to determine the patterns of resistance in different pathogens (Shears, 2000). Therefore, we suggest that guidelines to monitor prevalence of antimicrobial resistance and to improve rational and effective drug use in developing countries are needed.

6. Summary and Conclusion

The majority of diarrheagenic *E.coli* isolates (69.2%) were detected in children aged less three years old.

- Diarrheagenic *E. coli* isolates were mostly found in both diarrheal and control patients without any significant difference, but EIEC strains (2.9%) were isolated only from children with diarrhea.
- The rate of ETEC isolates in diarrheal children (9.8%) was statistically not significant as compared with the rate of isolates in control children (11.1%).
- Most diarrheagenic *E. coli* isolates were highly resistance to co-trimoxazole (60%) and ampicillin (88.7%), whereas antimicrobial resistance to nitofurantoin, gentamicin, norfloxacin , nalidixic acid and cefuroxime were low and ranged between 5.7% to 17% .
- A rapid and sensitive PCR methods will be useful for detecting diarrheagenic *E. coli* strains and to investigate outbreaks of diarrhea in community.
- The knowledge of antimicrobial resistance patterns among diarrheagenic *E. coli* isolates in Jordan can improve rational and effective drug use in diarrhea cases.

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Appendix I

RESEARCH PROJECT DIARRHEA

1- Patient's details:

- Serial No. .....
- Patient's hospital No. .....
- Name .....
- Sex .....
- Age .....
- Duration of hospitalization .....

2- Clinical features of diarrhea cases:

- frequency .....
  - Macroscopic examination of specimen.
    a. watery b. mucoid c. bloody
- other signs and symptoms:
  - vomiting
    a. yes b. no
  - temperature .....
  - fits .....

3- Microscopic stool examination:

WBCs ..... RBCs .....
Parasites or ova .....
Others .....

4- Antibiotic treatment:

a. yes (type) ..... b. no
- other treatment
a. antiemtic b. antispasm c. other .....

5- Type of diarrheagenic <u>E.</u> <u>coli</u> after investigation:

Appendix II

RESEARCH PROJECT CONTROL

1- Patient's details:

- Serial details .....
- Patient's hospital NO. .....
- Name .....
- Sex .....
- Age .....
- Duration of hospitalization .....

2- The main cause of administration to the hospital

3- Type of Diarrheagenic E. coli in the stool sample

الملخص

دراسة مدى انتشار بكتيريا عصيات القولون المسببة للاسهال عند الاطفال في عمان باستخدام تفاعل البوليمريز المتسلسل

اعداد

كمال غازي حجاج

المشرف

الاستاذ الدكتور عاصم الشهابي

المشرف المشارك

الاستاذة الدكتورة نجوى بولوص خوري

ما تزال الاسهالات الحادة من اهم اسباب الوفيات في العالم الثالث وخاصة عند الرضع والاطفال. تضم العصيات القولونية المسببة للاسهال ذراري متنوعة من البكتيريا المسؤولة عن الاسهالات في الانسان. تعتبر تقنيات البيولوجيا الجزيئية من اكثر الطرق المعتمدة في تصنيف هذه البكتيريا وتمييزها عن بعضها البعض وتحديد صفاتها الامراضية. وبالتالي قمنا بدراسة مدى انتشار هذه البكتيريا عند الاطفال في عمان – الاردن ، باستخدام هذه التقنيات الحديثة، حيث اجريت الدراسة من شهر كانون الثاني ٢٠٠٠م ولغاية كانون الاول ٢٠٠٠م.

في هذه الدراسة قمنا بدراسة ٢٥٠ عينة براز من الاطفال المدخلين الى مستشفى الجامعة الاردنية والمستشفى الاسلامي. حيث شملت الدراسة ١٣٣ طفل يعاني من الاسهال و ١١٧ طفل يعانون من امراض اخرى عدا الاسهال واعتبروا مجموعة ضابطة. تراوحت اعمار الاطفال الذين شملتهم الدراسة من عمر يوم واحد وحتى عمر ١٣ عاما بمتوسط ٣٠ شهر لمجموعة الاطفال المصابين بالاسهال و٣٣ شهر لمجموعة الاطفال الضابطة. تم فحص جميع عينات ابراز باستخدام تقنية (PCR) الاحادية (Single) والمتعددة (multiplex PCR) للكشف عن وجود الذراري الايشريشا القولونية المسببة للاسهال.

تم الكشف عن وجود ذراري (ETEC) بنسبة ٩,٨% و ذراري (EIEC) بنسبة ٢,٩ % و ذراري (EAggEC) بنسبة ٩,٠% و ذراري ايشريشا قولونية متنوعة بنسبة ١,٥%

عند الاطفال المصابين بالاسهال. كما تم الكشف عن وجود ذراري (ETEC) بنسبة ١١,١ % و ذراري (EAggEC) بنسبة ٦,٠% و ذراري ايشريشا قولونية متنوعة بنسبة ١,٧% عند الاطفال غير المصابين بالاسهال. بينت الدراسة وجود فرق ذا دلالة احصائية ايجابية بين نسبة عزل بكتيريا (EIEC) من عينات الاطفال المصابين بالاسهال بالمقارنة مع عينات الاطفال غير المصابين بالاسهال.

كشفت الدراسة عن عدم وجود ذراري (EHEC) في اي من عينات الاطفال المصابين بالاسهال أو الاطفال غير المصابين بالاسهال. كما تبين ان معظم العزلات القولونية كانت مقاومة للمضاد الحيوي كوتريموكسازول (cotrimoxazole) بنسبة ٦٠% والمضاد الحيوي امبسيلين(ampicillin) بنسبة ٨٨,٧% مع العلم ان هذه المضادات الحيوية من اكثر المضادات الحيوية المستخدمة في الاردن.