Shiga toxin-producing and other diarrhoeagenic *Escherichia coli* in Finland: pheno- and genotypic epidemiology

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Academic dissertation

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This thesis is based on the following publications, which are referred to in the text by their Roman numerals. In addition, some unpublished results are presented.

I Keskimäki M, Ikäheimo R, Kärkkäinen P, Scheutz F, Ratiner Y, Puohiniemi RL and A Siitonen. 1997. Shiga Toxin-Producing *Escherichia coli* Serotype OX3:H21 Causing Hemolytic Uremic Syndrome. Clin. Infect. Dis. 24:1278-1279.

II Keskimäki M, Saari M, Heiskanen T and A Siitonen. 1998. Shiga toxin-producing *Escherichia coli* in Finland from 1990 through 1997: Prevalence and characteristics of the isolates. J. Clin. Microbiol. 36:3641-3646.

III Keskimäki M, Ratiner Y, Oinonen S, Leijala E, Nurminen M, Saari M and A Siitonen. 1999. Haemolytic-uraemic syndrome caused by verotoxin-producing *Escherichia coli* serotype Rough:K⁻:H49. Scand. J. Infect. Dis. 31:141-144.

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ABBREVIATIONS

AA	Aggregative adhesion
A/E	Attaching-and-effacing
ATCC	American type culture collection
ASM	American Society for Microbiology
BFP	Bundle-forming pilus
CF	Colonisation factors
CFA	Colonisation factor antigen
CLED	Cystine-, Lactose-, Electrolyte-Deficient agar
CT	Cholera enterotoxin
DAEC	Diffusely adherent *E. coli*
EAEC	Enteroaggregative *E. coli*
EAF	EPEC adherence factor
EAggEC	Enteroaggregative *E. coli*
EAST1	EAEC heat-stable enterotoxin
EHEC	Enterohaemorrhagic *E. coli*
Ehly	Enterohaemolysin
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
HC	Haemorrhagic colitis
HNT	H-nontypeable
HUS	Haemolytic Uraemic Syndrome
LEE	Locus of enterocyte effacement
LEP	Laboratory of Enteric Pathogens
LT	Heat-labile enterotoxin of ETEC
ONT	O-nontypeable
PCF	Putative colonisation antigen
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
SDS-PAGE	Sodiumdodecylsulphate-polyacrylamide gel

SMAC	Sorbitol MacConkey agar
ST	Heat-stable enterotoxin of ETEC
Stx	Shiga toxin of STEC
SLTEC	Shiga-like toxin-producing *E. coli*
STEC	Shiga toxin-producing *E. coli*
TD	Tourist diarrhoea or traveller's diarrhoea
Tir	Translocated intimin reseptor
TTP	Thrombotic thrombocytopenic purpura
VTEC	Vero toxin-producing *E. coli*
WHO	World Health Organiztion

CONTENTS

1. INTRODUCTION

Escherichia coli is the predominant facultative anaerobic bacterium in the human colonic flora. It usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even *E. coli* strains of normal flora can cause infection. Three general clinical syndromes result from infection with pathogenic *E. coli* strains: (i) urinary tract infection; (ii) sepsis/meningitis; and (iii) enteric/diarrhoeal disease (Nataro and Kaper 1998). This thesis concentrates on the diarrhoeagenic *E. coli* strains, which include several emerging pathogens of worldwide public health importance.

Contaminated food and water are important vehicles for transmission of various enteric pathogens. The food industry, restaurants and private homes occasionally fail to meet adequate cooling, storage, preparation and other hygiene standards, and food may be contaminated with faecal flora due to improper practices of those who prepared the food. This makes possible the distribution of enteric pathogens and the spread of diseases caused by them. Among enteric pathogens, diarrhoeagenic *E. coli* belong to the most common bacteria causing intestinal infections in both developing and industrialised countries.

Diarrhoeagenic *E. coli* have been classified into different categories according to their special characteristics. Enteropathogenic *E. coli* (EPEC) has been linked to infant diarrhoea in developing countries. They were previously commonly found also in industrialised countries and, were known as "dyspepsiekoli." Enterotoxigenic *E. coli* (ETEC) has been regarded as the most common pathogen causing tourist diarrhoea (TD), irrespective of destination. Enteroaggregative *E. coli* (EAEC) and Shiga toxin-producing *E. coli* (STEC) are new *E. coli* pathogroups. The former have been implicated as one of the aetiological agents of diarrhoea both in developing countries and in outbreaks of gastro-enteritis in industrialised countries. STEC strains are well-known causes of bloody diarrhoea and haemorrhagic colitis (HC) in humans.

Diarrhoeagenic *E. coli* were among the first pathogens for which molecular diagnostic methods were developed. Molecular methods, especially polymerase chain reaction (PCR), are nowadays considered the most reliable techniques for differentiating diarrhoeagenic *E. coli* strains from

nonpathogenic members of the stool flora and for distinguishing one *E. coli* pathogroup from another (Nataro and Kaper 1998). This has made it possible to re-evaluate the role of various diarrhoeagenic *E. coli* groups in diarrhoea in humans.

2. REVIEW OF THE LITERATURE ON DIARRHOEAGENIC *E. COLI*

2.1 Microbial diarrhoea

Diarrhoea associated with bacterial, viral or parasitic infections is the most common infectious illness experienced by millions of children in developing countries, and also the major cause of disease in international travellers. According to the World Health Organization (WHO 1993), 16 million deaths were due to infectious diseases in 1990. Of these deaths, diarrhoea accounted for over 3 million. The main examples of diarrhoeal disease associated with bacteria are cholera and diarrhoea due to ETEC. Cholera remains an important cause of illness in many developing countries and has been estimated to result in more than 120 000 deaths each year. In children in developing countries, ETEC is responsible for more than 650 million diarrhoeal episodes and 800 000 deaths annually (Cravioto et al. 1998). However, diarrhoea as a syndrome is not limited to developing countries.

The most readily recognised bacterial agents of gastroenteritis in industrialised countries are *Salmonellae* and *Campylobacter* (Murray et al. 1995). Moreover, acute diarrhoea may be associated with a large number of other bacteria, such as *Yersinia enterocolitica, Staphylococcus aureus* and *Bacillus cereus*. The various *Shigellae* can cause diarrhoea or the syndrome known as bacillary dysentery. In particular, incompletely prepared or incompletely heated food has been reported as the source of different diarrhoeal pathogens.

Additionally, other organisms such as parasites and viruses are common causes of diarrhoea around the world. *Entamoeba histolytica* and *Giardia lamblia* are usually acquired by ingestion of food or water contaminated with faeces (Murray et al. 1995). Usually no symptoms result from the presence of these parasites in the colon, but some people experience abdominal pain and diarrhoea. Rotaviruses are a common cause of diarrhoeal disease in the whole world (Murray et al. 1995). Recently, caliciviruses have caused large diarrhoea outbreaks in industrialised countries. In Finland, caliciviruses have spread via drinking water and frozen berries (Kukkula 1998).

2.2 Isolation, identification and common themes in virulence of *E. coli*

E. coli is the type species of genus *Escherichia* which contains mostly motile gram-negative bacilli within the family *Enterobacteriaceae* and the tribe *Escherichia*. The most highly conserved feature of diarrhoeagenic *E. coli* strains is their ability to colonise the intestinal mucosal surface despite peristalsis and competition for nutrients (Nataro and Kaper 1998). The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains. However, diarrhoeagenic *E. coli* strains possess specific fimbrial antigens that enhance their intestinal colonising ability and allow adherence to the small bowel mucosa, a site that is not normally colonised (Levine et al. 1984). Three general paradigms have been described by which *E. coli* may cause diarrhoea; each is described in more detail in the appropriate section below: (i) enterotoxin production (ETEC and EAEC); (ii) invasion (Enteroinvasive *E. coli* (EIEC)); and (iii) intimate adherence with membrane signalling (EPEC and STEC). However, the interaction of the organisms with the intestinal mucosa is specific for each category (Nataro and Kaper 1998).

The versatility of the *E. coli* genome is conferred mainly by two genetic configurations: virulence-related plasmids and chromosomal pathogenicity islands. STEC, EPEC, EAEC and EIEC strains typically harbour highly conserved plasmid families, each encoding multiple virulence factors (Nataro and Kaper 1998). Chromosomal virulence genes of STEC and EPEC are organised as a cluster referred to as a pathogenicity island (McDaniel et al. 1995).

2.2.1 Diagnostics

E. coli can be recovered from clinical specimens on general or selective media at 37ºC under aerobic conditions. *E. coli* in stools are most often recovered on MacConkey- or Eosin methylene-blue agar, which selectively grow members of the *Enterobacteriaceae* and permit differentiation of enteric organisms on the basis of their morphology (Murray et al. 1995).

Identification of diarrhoeagenic *E. coli* strains requires that these organisms can be differentiated from non-pathogenic members of the normal flora. Substantial progress has been made in the development of nucleic acid-based technologies. The use of DNA probes for detection of heat-

labile (LT) and heat-stable (ST) enterotoxins in ETEC revolutionised the study of these organisms by replacing the awkward and costly animal models of toxin detection (Mosley et al. 1982). Since then, gene probes have been introduced for all diarrhoeagenic *E. coli* categories. Another widely used method is Polymerace Chain Reaction (PCR). It has been a major advance in molecular diagnostics of pathogenic microorganisms, including *E. coli*. In PCR, a pair of primers (20-40 bases) is used for selective amplification and detection of a certain DNA sequence in a target organism. PCR primers have successfully been developed for all categories of diarrhoeagenic *E. coli*. PCR can be used in both diagnosing and typing *E. coli* strains. Advantages of PCR include high sensitivity, spesificity and appropriate rapidity in the detection of target DNA templates. However, substances within stools have been shown to interfere with PCR (Stacy-Phipps et al. 1995). However, PCR has been found beneficial in diagnosing diarrhoeagenic *E. coli*. Recently, it was concluded that PCR detects significantly more ETEC infections than does the standard probe-based hydridisation method (Caeiro et al. 1999). In diagnostics, PCR is commonly used for detecting different virulence associated genes of *E. coli*, such as toxin and adherence associated genes (Table 1). PCR is also widely used in subtyping by doing virulence gene profiles for different diarrhoeagenic *E. coli* strains.

Other methods specifically capable of detecting certain types of *E. coli* have been developed, especially for STEC. The latex agglutination test (Verotox-F assay, Denka Seyken, Tokio, Japan) for detection of toxins produced by STEC has been found 100% sensitive and 100% specific in comparison with the classical Vero cell assay (Karmali et al. 1999). Immunomagnetic separation with magnetic beads coated with antibody against *E. coli* O157 have been found more sensitive than direct culture of these strains (Chapman and Siddons 1996).

2.2.2 Serotyping

Prior to the identification of specific virulence factors in diarrhoeagenic *E. coli* strains, serotypic analysis was the predominant means by which pathogenic strains were differentiated. In 1947, Kauffmann proposed a scheme for the serologic classification of *E. coli* (Kauffmann 1947), which is still used in a modified form today. According to the modified Kauffmann scheme, *E. coli* are now serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigens

(Ørskov and Ørskov 1984). However, generally only extraintestinal *E. coli* are encapsulated (Jann and Jann 1992). Thus, among diarrhoeagenic *E. coli*, usually a specific combination of O and H antigens defines the serotype of the strain. *E. coli* of specific serogroups can be associated with certain clinical syndromes (Table 1), but it is not in general the serologic antigens themselves that confer the virulence (Ørskov and Ørskov 1984).

2.2.3 Genotyping

In recent years, the use of molecular "fingerprinting" methods has become standard practice in microbiology for evaluating the epidemiology of infectious diseases, investigating suspected outbreaks of bacterial infections, and typing bacteria (Mickelsen 1997). Pulsed-field gel electrophoresis (PFGE) allows the generation of simplified chromosomal restriction fragment patterns without having to resort to probe hydridisation methods. In this method, restriction enzymes that infrequently cut DNA are used for generating large fragments of chromosomal DNA, which are then separated by special electrophoresis (Swaminatham and Matar 1993). PFGE has been applied to subtyping of several gram-positive and gram-negative bacteria. It is now widely used in epidemiological surveillance and common interpretation schemes have been published (Tenover et al. 1997). In 1995, the Centers for Disease Control and Prevention (CDC) in the USA initiated PulseNet, a national computer network of public health laboratories that employs standard methods to subtype STEC O157:H7 strains. Laboratories within the network can transmit PFGE patterns electronically to a databank at the CDC, where they are automatically compared with patterns of other isolates. If the patterns submitted by laboratories in different locations during a defined time period are found to match, the CDC computer will alert PulseNet participants of a possible multistate outbreak (Centers for Disease Control 2001).

Ribotyping is a method based on DNA probes that recognize conserved RNA operon genes. Ribotyping is essentially a Southern blot analysis in which strains are characterised for restriction fragment length polymorphism of their individual ribosomal genes. Within a species, and particularly within a strain, the DNA sequences and the restriction digest patterns of genes encoding rRNA are highly conserved and thus serve as a molecular fingerprint for that organism. All organisms have ribosomal genes. This technique is universally applicable and has also found acceptance as a

clinical tool for typing *E. coli* (Tarkka et al. 1994). All these new molecular typing methods have allowed highly discriminant genotyping, and are useful tools for demonstrating that isolates from different sources are identical, closely related or not related at all (Mickelsen 1997).

Table 1. Characteristics of the diarrhoeagenic *E. coli*

Category	**Characteristic serogroups** [1)]	**Virulence determinants** [2)]		**Main targets in diagnostics** [2)]	**Published molecular detection methods**
	Characteristic O serotypes	**Toxins**	**Other**		
STEC	O5, O22, O26, O55, O91, O103, O111, O113, O117, O118, O128, O145, O157	Stx1, Stx2, EAST1, Ehly	A/E, LEE	*stx_1*, *stx_2*	Olsvik and Strockbine 1993 (PCR) Fratamico et al. 1995 (PCR) Paton and Paton 1999 (PCR) Cocolin et al. 2000 (PCR)
EPEC	O26, O55, O86, O111, O114, O119, O125, O126, O128, O142, O158	EAST1	A/E, EAF, BFP, LEE	*eae,* EAF-plasmid, *bfpA*,	Jerse et al. 1990 (probe) Gannon et al. 1993 (PCR) Heuvelink et al. 1995 (PCR) Batchelor et al. 1999 (PCR) Cocolin et al. 2000 (PCR)
EAEC	O3, O15, O44, O77, O86, O111, O127	EAST1	AA	EA-plasmid	Baudry et al. 1990 (probe) Schmidt et al. 1995 (PCR)
ETEC	O6, O8, O25, O78, O126, O148, O153, O169	LT, ST, EAST1	CFAs	*elt, etx*	Murray et al. 1987 (probe) Frankel et al. 1989 (PCR) Olsvik and Strockbine 1993 (PCR) Schultz 1994 (PCR and probe) Yavzori et al. 1998 (PCR)

1) The information presented is derived from several publications (Robins-Browne 1987, Wolf 1997, Nataro and Kaper 1998, Law and Chart 1998, Bettelheim 2000). 2) See the text for abbreviations and discussion

2.3 Shiga toxin-producing *E. coli* (STEC)

The recognition of STEC as a distinct class of pathogenic *E. coli* resulted from two epidemiological observations. The first was the report of Riley et al. in 1983, who investigated two outbreaks characterised by severe abdominal pain and watery diarrhoea followed by bloody diarrhoea. This illness, designated as HC, was associated with the ingestion of undercooked hamburgers at a fast food restaurant chain. Stool cultures from these patients yielded a previously rare *E. coli* serotype O157:H7. The second essential observation, also in 1983, was by Karmali et al. (1983) who reported the association of sporadic cases of Haemolytic Uraemic Syndrome (HUS) with faecal cytotoxin and cytotoxin producing *E. coli* in stools. Thus, the two clinical microbiological observations, one based on a rare *E. coli* serotype and the other on the production of a specific cytotoxin, led to the recognition of a novel and increasingly important class of enteric pathogens causing intestinal and renal disease.

2.3.1 Nomenclature

The discovery of this pathogen along distinct paths of investigation resulted in a parallel nomenclature, a situation that still exists. The term Verotoxigenic *E. coli* or Vero cytotoxin-producing *E. coli* (VTEC) was derived from the observation that these strains produced a toxin that was cytotoxigenic for Vero cells (Konowalchuk et al. 1977). The term VTEC is still widely used in the United Kingdom and in many European scientific publications. The term enterohaemorrhagic *E. coli* (EHEC) was originally coined to denote strains that cause HC and HUS (Nataro and Kaper 1998). According to the latest nomenclature, these strains are called Shiga toxin-producing *E. coli* (STEC) (formerly Shiga like toxin-producing *E. coli* (SLTEC), a term that reflects the cytotoxin produced by these strains (Calderwood et al. 1997). The term STEC is used especially in American scientific papers.

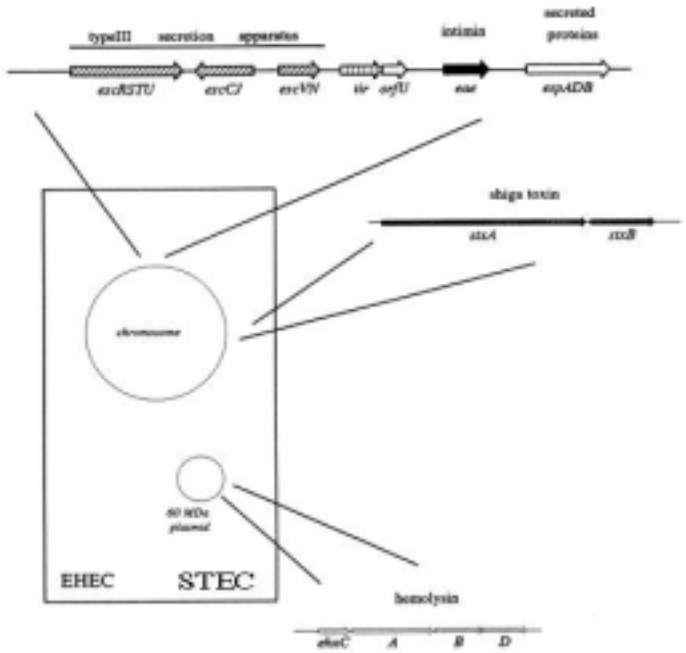
STEC and VTEC are equivalent terms, and both refer to *E. coli* strains that produce one or more cytotoxins. However, it is not clear that mere possession of toxin genes confers pathogenicity in the absense of other virulense factors (see below). Strains carrying toxin genes are commonly found

from many healthy domestic animals. However, these strains often lack some, or all, other virulence determinants often connected with STEC (Beutin et al. 1995). Thus, all STEC are not believed to be pathogens, whereas all EHEC are (Nataro and Kaper 1998). However, other types of diarrhoeagenic *E. coli* can also cause bloody diarrhoea. Usage within the scientific world will determine the final name(s) of these strains, and whether one or all of the names will be used.

2.3.2 Shiga toxins (Stx) and other factors affecting the pathogenic properties of STEC

STEC strains produce Stx toxins, also known as Vero toxins (VT) or Shiga-like toxins (Slt). Stx toxin is essentially identical at the genetic and protein levels to the Stx-toxin produced by *Shigella dysenteriae* 1. The Stx family contains two major, immunologically non-cross-reactive groups called Stx1 and Stx2, encoded by the stx_1 and stx_2 genes. Both toxins are composed of five B subunits (encoded by *stxB*) and a single A subunit (encoded by *stxA*) and both, *stxA* and *stxB*, are located on a temperate bacteriophage inserted into the STEC chromosome. Genes involved in STEC pathogenesis are presented in Figure 1 (Nataro and Kaper 1998). A single STEC strain may express Stx1 only, Stx2 only, or both toxins, or even multiple forms of Stx2. The prototypical Stx1 and Stx2 toxins, respectively, have 55% and 57% sequence identity in A and B subunits, (Jackson et al. 1987). While Stx1 is highly conserved, sequence variation exists in Stx2. Three types of Stx2 have been identified: Stx2, Stx2c and Stx2e (Pierard et al. 1998). The subtype Stx2e is classically associated with pig oedema disease rather than human disease, but occasional strains that express only this variant are isolated from patients with HUS (Thomas et al. 1994). The various subtypes are wholly interchangeable between the Stx and VT nomenclatures (i.e. Stx1=VT1=Slt1, Stx2e=VT2e=Slt2e etc.) (Caldervood et al. 1997). Most molecular diagnostic methods for STEC are aimed at the detection of genes encoding Stx (Olsvik and Strockbine 1993, Cocolin et al. 2000). Improved diagnostics have increased the variety of STEC types found in patients with HC or some other STEC associated disease, and new clones continue to emerge, for example Stx2-producing STEC O26:H11 found in Germany (Zhang et al. 2000).

Figure 1. Genes involved in STEC (i.e. EHEC, see 2.3.1) pathogenesis. Genes involved in STEC pathogenesis are similar to those implicated for EPEC, excecpt for the presence of the Stx-encoding phage on the STEC chromosome and the presence of the characteristic STEC 60-MDa plasmid instead of the EAF plasmid of EPEC (adapted from Nataro and Kaper 1998).



Other virulence associated factors of STEC are the production of enterohaemolysin (Ehly) and, possibly, heat-stable enterotoxin (EAST1). Genes encoding Ehly are located in the 60-MDa plasmid found in nearly all O157:H7 strains and also widely in non-O157 STEC strains. In Germany, approximately 90% of all STEC strains isolated from patients possessed genes encoding Ehly (Beutin et al. 1994). However, the significance of Ehly production as a true virulence factor is hard to judge, since in a study by the same author, the Stx-negative and Ehly-positive *E. coli* were in vitro incapable of causing visible damage to Vero, Hep-2 or HeLa cells (Beutin et al. 1989). EAST1, first described in EAEC, is also found in many STEC strains. The significance of EAST1 in the pathogenesis of STEC is unknown, but it migth account for some of the non-bloody diarrhoea frequently seen in persons infected with STEC (Nataro and Kaper 1998).

The only potential STEC adherence factor that has been demonstrated as playing a role in intestinal colonisation is the 94-97 kDa outer membrane protein intimin. It is encoded by the *eae* gene and produces extensive attaching-and-effacing (A/E) lesions in the large intestine, featuring intimate adherence of the bacteria to the epithelial cells (Donnerberg et al. 1993b). This *eae* gene is also found in EPEC. The *eae* is only one of many genes located on the 35 kb pathogenicity island called the locus of enterocyte effacement (LEE), which confers the A/E phenotype for EPEC. The STEC LEE contains genes encoding intimin, translocated intimin reseptor (Tir), the secreted proteins EspA and EspB, and a type III secretion pathway. Formation of A/E lesions depends upon interaction between bacterial outer membrane protein (intimin) and bacterially encoded reseptor protein Tir, which is exported from the bacterium and translocated into the host cell membrane (Paton et al. 1998). The true roles of EspA and EspB are unknown, but type III secretion systems are responsible for secretion and translocation of critical virulence determinants found in a variety of gram-negative human, animal, and plant pathogens (Jarvis and Kaper 1996). However, all STEC do not carry *eae*. Thus, it has also been suggested that intimate bacterial attachment and induction of extensive A/E lesions have only slight effect on the uptake of Stx2 producing STEC to cause the disease (Tzipori et al. 1995).

Most STEC outbreaks have been caused by O157:H7 strains, suggesting that this serotype is in some way more virulent or more transmissible than other serotypes. The unique biochemical marker for this group is that most STEC O157:H7 strains cannot ferment sorbitol or produce β-glucuronidase. Thus, in many countries the STEC diagnostics are based on the detection of sorbitol-negative STEC O157:H7 strains only. However, most *E. coli* strains of normal flora, including pathogenic non-O157 STEC strains, can ferment sorbitol and produce β-glucuronidase. Moreover, *E. coli* strains belonging to over 200 serotypes can express Stx, but within most serotypes, both Stx-positive and Stx-negative strains can be found (Johnson et al. 1996). More than 100 of these serotypes have been associated with HC or HUS in humans. The most common non-O157 serotypes associated with human disease include O26:H11, O103:H2, O111:HNM and O113:H21 (WHO 1994).

Non-bloody diarrhoea, HC and HUS or TTP (thrombotic thrombocytopenic purpura) are the most common symptoms STEC can cause, although the infection may also be lethal. Additionally,

a large number of other complications may arise and they include cholecystis, colonic perforation, pancreatitis, posthemolytic biliary lithiasis, postinfectious colonic stricture, rectal prolapse, appendicitis, hepatitis, haemorrhagic cystitis, pulmonary oedema, myocardial dysfunction, and neurological abnormalities (Griffin 1995, Tarr 1995). The frequencies of the various syndromes include about 10% non-bloody diarrhoea, 90% HC, and 10% HUS/TTP. Additionally, < 5% are associated with different intestinal and extraintestinal complications (Tarr 1995).

2.3.3 Sources of STEC infections

STEC can be found in the faecal flora of a variety of animals including cattle, sheep, goats, pigs, cats and dogs (Beutin et al. 1993, Beutin et al. 1995, Chapman et al. 1997), horses (Chalmers et al. 1997) and even seagulls (Makino et al. 2000). The most important animal species in terms of human infection is cattle, but the prevalence of STEC O157:H7 in cattle varies significantly from country to country. Surveys of bovine cattle have found STEC O157:H7 in 1.3% of animals in Finland (Lahti et al. 2001), 10.8% in Germany (Montenegro et al. 1990), and 15.7% in the United Kingdom (Chapman et al. 1997). STEC strains are usually isolated from healthy animals, but may be associated with an initial episode of diarrhoea in young animals followed by asymptomatic colonisation. The STEC O157:H7 isolation rates from animals are much lower than those of non-O157 serotypes. The main route of STEC into the food chain is through contamination of meat by intestinal contents and faeces in the abattoir (Butler 1996).

Usually STEC are transmitted to humans by food, water, and from person to person. Most cases are caused by ingestion of contaminated foods, particularly foods of animal origin, beef being a major vehicle of infection. In the United States, ingestion of undercooked hamburgers, prepared in a restaurant or at home, has been a particularly significant cause of outbreaks (Griffin 1995). In Japan, the largest reported STEC outbreak, where over 6000 people were infected and 13 died, was associated with centrally distributed school food (National Institute of Health... 1996). In Scotland, over 400 people were infected with, and 18 elderly patients died of, infections associated with a butcher's shop and contaminated meat products (Christie 1997). However, the spectrum of vehicles implicated in disease due to STEC is expanding beyond the initial hamburger-associated outbreaks. STEC outbreaks have been linked to consumption of different foods, such

as mayonnaise (Griffin 1995) unpasteurised apple juice (McCarthy 1996), alfalfa sprouts (Centers for Disease Control 1995a) and fermented hard salami (Centers for Disease Control 1995b). These vehicles illustrate the notable ability of STEC to grow in foods under conditions where other pathogens would not survive. Molecular subtyping methods, especially PFGE, have been extensively used to distinguish between outbreak-associated and sporadic or unrelated O157:H7 infections (Bender et al. 1997). However, while the large STEC outbreaks involving hundreds of individuals have garnered the most attention, sporadic infections comprise the major burden of this pathogen (Lansbury and Ludlam 1997).

2.4 Enteropathogenic *E. coli* (EPEC)

During the 1920s in Germany, Adam showed by serologic typing that strains of "dyspepsiekoli" could be implicated in outbreaks of paediatric diarrhoea (Adam 1923). In 1955, the term enteropathogenic *E. coli* was first coined by Neter et al. (1955) to indicate those strains of *E. coli* that had been epidemiologically linked with childhood diarrhoea. It was defined for decades solely on the basis of O and H serotypes found typically in diarrhoeal children younger than two years (Robins-Browne 1987). However, this definition has changed drastically in recent years as knowledge of this organism has increased. EPEC is still an important category of diarrhoeagenic *E. coli*, which then was linked to infant diarrhoea in industrialised countries and now in the developing world. Citing recent pathogenic data, the Second International Symposium on EPEC in 1995 reached a consensus on the basic characteristics of EPEC, and now EPEC is defined on the basis of molecular pathogenic characteristics (Kaper 1995).

2.4.1 Attaching-and-effacing (A/E) adherence of EPEC strains

According to a recent resolution, a diarrhoeagenic *E. coli* that produces a characteristic histopathology known as attaching-and-effacing (A/E) and does not produce Shiga toxins can be called EPEC (Kaper 1995). Genes involved in EPEC pathogenesis are similar to those implicated for STEC (Table 1), except for the Stx-encoding phage on STEC chromosome and the presence

of some other proposed virulence determinants found in STEC (Nataro and Kaper 1998). The A/E lesion is characterised by intimate adherence between bacteria and epithelial cells and effacement of intestinal microvilli. A/E can be observed in intestinal biopsy specimens from patients or infected animals (Knutton et al. 1987). The gene necessary for mediating A/E has been identified and termed *eae.* The *eae* gene codes for a protein called intimin, which is required for full virulence of EPEC (Donnerberg et al. 1995a). Similar A/E lesions are seen in animal and culture models of many STEC strains and *Hafnia alvei* isolated from children with diarrhoea (Albert et al. 1992). The overall pattern for these *eae* sequences from different bacteria shows high conservation in the N-terminal region and variability in the C-terminal region (Nataro and Kaper 1998). The role of intimin in human disease was demonstrated by studies on volunteers, who ingested an isogenic *eae* negative mutant of EPEC E2348/69 (O127:H6). Diarrhoea was seen in all volunteers who ingested the wild-type E2348/69 compared with four of 11 volunteers who ingested the isogenic mutant. (Donneberg et al. 1995a).

A secreted enterotoxin that would explain the mechanism of diarrhoea due to EPEC has been unsuccesfully sought for over many years (Robins-Browne et al. 1982, Nataro and Kaper 1998). In the absence of recognised enterotoxins, attempts to develop improved diagnostic tests for EPEC have centred on their adherence properties. In one EPEC strain (E2348/69), production of a plasmid called EPEC adherence factor (EAF) was found necessary for adherence in a localized pattern, and a 1-kb fragment from this region was developed as a diagnostic DNA probe (Baldini et al. 1983). However, the possessing of EAF plasmids seems to occur only in some EPEC serovars (class 1 EPEC) (Nataro et al. 1985, Tamura et al. 1996). PCR primers have been developed to detect the *eae* gene of EPEC (Gannon et al. 1993, Heuvelink et al. 1995, Batchelor et al. 1999). Subsequent studies have revealed that genes involved in adherence are required for expression and assembly of bundle-forming pilus (BFP). BFP are fimbriae, encoded by *bfpA* in EAF plasmid, and they also have a role in EPEC adherence (Donnerberg et al. 1992).

2.4.2 Epidemiology of EPEC infections

As with other diarrhoeagenic *E. coli*, transmission of EPEC is fecal-oral, with contaminated hands, contaminated food, or contaminated fomites serving as vehicles. In adult outbreaks, waterborne

and foodborne transmission has been reported, but no particular type of food has been implicated as more likely to serve as a source of infection (Levine and Edelman 1984). The most notable feature of the epidemiology of disease due to EPEC is the striking age distribution seen in persons infected with this pathogen. EPEC infection is primarily a disease of infants younger than 2 years. Illness caused by EPEC is often clinically acute, severe diarrhoea. The reason(s) for the relative resistance of adults and older children is not known, but loss of specific receptors with age is one possibility. However, EPEC can cause diarrhoea in an adult if the bacterial inoculum is high enough. The infectious dose in naturally transmitted infection in infants is not known, but it is presumed to be much lower than with adults. (Nataro and Kaper 1998).

Although several outbreaks of diarrhoea due to EPEC have been reported in healthy adults in industrialised countries (Costin et al. 1964, Schroeder et al. 1968, Viljanen et al. 1990, Hedberg et al. 1999), very little is known about the current status of EPEC as a diarrhoeagenic agent, since it has no longer been routinely assayed from stool samples. In a recent study in the USA, EPEC was detected with a DNA probe in 3.6% of children's diarrhoeal samples and exceeded the rates of *Salmonella* or *Campylobacter* (Bokete et al. 1997).

In contrast to the limited importance of EPEC in industrialised countries, EPEC is a major cause of infant diarrhoea in developing countries (Nataro and Kaper 1998). Studies in Brazil (Gomes et al. 1989), Mexico (Cravioto et al. 1988), and South Africa (Robins-Browne et al. 1982) have shown that 30-40% of infant diarrhoea can be attributed to EPEC. EPEC strains are an important cause of disease in all settings of nosocomial outbreaks, outpatient clinics, patients admitted to hospitals, community-based longitudinal studies, and urban and rural settings (Nataro and Kaper 1998).

2.5 Enteroaggregative *E. coli* (EAEC)

EAEC, often also EaggEC, has been epidemiologically implicated as one of the etiological agents of diarrhoea in industrialised and developing countries. The group is heterogenous, comprising a diverse range of serotypes that possess a variety of putative virulence factors (Law and Chart 1998).

2.5.1 Aggregative adhesion (AA) and other proposed virulence characteristics

EAEC strains are currently defined as *E. coli* strains that do not secrete enterotoxins LT or ST and that adhere to HEp-2 cells in an aggregative adhesion (AA) pattern (Baydry et al. 1990). The pathogenesis and the site of EAEC infection in the human intestine are not well understood. However, a characteristic histopathologic lesion and several candidate virulence factors have been described. Virtually all EAEC strains carry a 60 Mda plasmid that contains a gene that confers AA and a gene that encodes EAEC heat-stable enterotoxin-1 (EAST1). However, the role of EAST1 in diarrhoea has not yet been determined, although EAST1 clones yield net increases in short-circuit current in the rabbit mucosa when the chamber model is used (Savarino et al. 1991). Other virulence associated factors have been proposed. These include putative haemolysins and toxins, and various types of fimbriae and outer membrane proteins that may be involved in the adhesion process (Law and Chart 1998). The role of these factors and mechanisms by which EAEC adhere to eukaryotic cells have not been identified, but carriage of plasmid-encoded gene encoding AA is essential (Baydry et al. 1990).

Diagnosis of EAEC infection is problematic; serotyping cannot identify strains of *E. coli* expressing the EAEC phenotype. Currently, EAEC are identified by either cell adhesion tests or DNA-based tests involving gene probes or PCR for detecting the plasmidial genes encoding cell adhesion, but these methods cannot be used in a routine clinical laboratory (Law and Chart 1998).

2.5.2 Diarrhoea caused by EAEC

A growing number of studies have supported the association of EAEC with diarrhoea, most prominently with persistent diarrhoea (>14 days). In most studies conducted in recent years, EAEC have been detected either with PCR (Schmidt et al. 1995) or with a DNA probe (Baydry et al. 1990). Although most reports have implicated EAEC in sporadic endemic diarrhoea, an increasing number of reports have described EAEC also in outbreaks. In Brazil, EAEC has been detected in up to 68% of persistent diarrhoea cases (Fang et al. 1995). In India, EAEC have been associated with persistent diarrhoea (Bhan et al. 1989a) and cases of sporadic diarrhoea detected

in household surveillance (Bhan et al. 1989b). Interestingly, in Brazil, the isolation of EAEC from the stools of infants was associated with a significantly lower height and/or weight of the EAEC carriers than the mean of the populations, irrespective of the presence of diarrhoael symptoms (Steiner et al. 1998).

In industrialised countries, very little is known about the epidemiology of EAEC. However, it has been reported that in Germany 2% of children with diarrhoea have EAEC in their stools (Hubberz et al. 1997). In Austria, EAEC was the most common *E. coli* group found in patients with diarrhea (Presterl et al. 1999). In Japan in 1993, EAEC ONT:H10 caused a large outbreak where 2 697 schoolchildren developed severe diarrhoea (Itoh et al. 1997). In the United Kingdom, four outbreaks of diarrhoea have been reported as being due to EAEC strains representing several different serotypes (O19, O62, O73, O86, O113, O116, O125, O134). Each of the outbreaks was associated with consumption of a restaurant meal, but no single source could be implicated (Smith et al. 1997). In another British study, EAEC were the most common diarrhoeagenic *E. coli* group detected in diarrhoeal patients (Tompkins et al. 1999). In the United States and Switzerland, EAEC have been linked with diarrhoea in human immunodeficiency virus-infected patients (Mayer and Wanke 1995, Durrer et al. 2000).

2.6 Enterotoxigenic *E. coli* (ETEC)

ETEC strains are an important world-wide cause of diarrhoeal disease in humans, mainly affecting children in developing countries and travellers going from industrialised countries to less developed countries. It was DuPont et al. (1971) who first showed that ETEC strains were capable of causing diarrhoea in adult volunteers. In general, ETEC strains are considered to represent a pathogenic prototype: the organism colonises the surface of the small bowel mucosa and elaborates their enterotoxins, giving rise to secretion of electrolytes and water (Nataro and Kaper 1998).

2.6.1 Heat-stable (ST) and heat-labile (LT) enterotoxin production and other virulence associated factors

ETEC is defined as *E. coli* strains that produce at least one of the two defined groups of enterotoxins: ST and LT enterotoxins (Levine 1987). Thus, detection of ETEC has long relied on detection of the enterotoxins. The use of DNA probes for detection of LT and ST enterotoxins in ETEC revolutionised the study of all diarrhoeal organisms by replacing the awkward and costly animal models of toxin detection (Mosley et al. 1982).

LTs are large oligomeric toxins that are closely related in structure and function to the cholera toxin (CT) expressed by *Vibrio cholerae*. The two immunotypes of LT, LT-I and LT-II, share the same ganglioside receptor and mode of action, but are antigenically distinct (Sixma et al. 1993). The genes encoding LT (*elt* or *etx*) reside on plasmids that may also contain genes encoding ST and/or colonisation factor antigens (CFAs) (Nataro and Kaper 1998). In contrast to LTs, the STs are small, monomeric toxins that contain multiple cysteine residues, whose disulphide bonds account for the heat stability of these toxins (Nataro and Kaper 1998). ST is not a single toxin but a family of small toxins that fall into two subgroups (methanol soluble) STa (or STI) and (methanol insoluble) STb (or STII), which differ in nucleotide and amino acid sequences (Salyers and Whitt 1994). Besides the production of LT and/or ST, some ETEC strains may also express EAST1 (Savarino et al. 1996), which was first found in EAEC.

ETEC strains have two major virulence determinants, enterotoxins and CFAs. To cause diarrhoea, ETEC strains must first adhere to small bowel enterocytes, an event mediated by surface fimbriae. ETEC adhere to epithelial surfaces by means of CFAs and putative colonisation antigens, jointly referred as CFs. There exist three major morphologic varieties of CFs: rigid rods; bundle-forming flexible rods; and thin flexible wiry structures. At least 20 different CFs in ETEC pathogenic to humans have been described, but they are almost always encoded by plasmids also encoding ST and/or LT enterotoxins (Gaastra and Svennerholm 1996). ETEC strains cause diarrhoea through the action of these enterotoxins. Diarrhoea caused by ETEC has much in common with cholera; both result from ingestion of rather large inocula of bacteria that will colonise in the small intestine and produce toxins that cause net fluid secretion into the intestinal lumen. Typically, the diarrhoea is

watery, usually without blood, but not severe, and it resolves without treatment in about a week. In some cases, ETEC infection may result in severe purging similar to that seen in cholera (Arduino and DuPont 1993).

2.6.2 Infant diarrhoea and tourist diarrhoea

Food and water are the most common vehicles for ETEC infection. Thus, faecal contamination is the principal reason for the high incidence of ETEC infection throughout the developing world. ETEC infections in areas of endemic infections tend to be clustered in warm, wet months, when multiplication of ETEC in food and water is most efficient (Levine 1987). Sampling of both food and water sources from areas of endemic ETEC have demonstrated strikingly high rates of ETEC contamination (Ryder et al. 1976). Asymptomatic ETEC excretion is also commonly found (Abu-Elyazeed et al. 1999). Thus, it is not surprising that, in developing countries, children under 2 years of age typically have two to three episodes of diarrhoea per year, with ETEC infections representing more than 25% of all these diarrhoeal cases, and contribute significantly to the mortality of this group. Oral rehydration therapy is often lifesaving in infants and children with ETEC diarrhoea (Black et al. 1981).

Although ETEC infection occurs most frequently in infants, immunologically naive adults are also susceptible. Thus, ETEC has been regarded as the most common pathogen in TD, irrespective of the tourist destination (Mattila 1995). The percentages of ETEC, however, have varied from study to study, from nation to nation, and from season to season. Studies suggest that 20-60% of travellers visiting areas where ETEC infection is endemic, experience diarrhoea; typically, 20-40% of cases are believed to be due to ETEC (Arduino and DuPont 1993, Nataro and Kaper 1998). Many studies have investigated the effect of dietary self-restrictions on the risk of TD. However, the dietary self-restrictions have had no or only limited benefit (Loewenstein et al. 1973, Mattila 1995, Mattila et al. 1995). An oral cholera vaccine containing killed *V. cholerae* and purified cholera toxin B subunit has been reported to provide some protection against TD due to ETEC (Clemens et al. 1988, Peltola et al. 1991). This protection is presumably due to the structural and antigenic similarity between LT and CT.

2.7 Other groups of diarrhoeagenic *E. coli*

2.7.1 Enteroinvasive *E. coli* (EIEC)

EIEC strains are often biochemically atypical and difficult to identify. Antigenically, biochemically and pathogenically these strains resemble *Shigella* so much that the illness caused by EIEC has often been mistaken for shigellosis. Clinical features include fever, abdominal cramps, malaise, toxaemia and watery diarrhoea or typical dysentery with blood, mucus and many faecal leukocytes. Both *Shigella* spp. and EIEC have been shown to invade the colonic epithelium, a phenotype mediated by both plasmid and chromosomal loci (Sansonetti 1992). In addition, both have one or more secretory enterotoxins that may play roles in diarrhoeal pathogenesis (Nataro and Kaper 1998). True prevalence of EIEC diarrhoea is not known. Endemic sporadic disease occurs in some areas, generally where *Shigella* spp. are also prevalent, but the epidemiologic features may be different from those of *Shigella* spp (Taylor et al. 1988). EIEC has also been associated with diarrhoea occurring in travellers and with outbreaks of food poisoning due to ingestion of contaminated food (Pushker 1994).

2.7.2 Diffusely adherent *E. coli* (DAEC)

The term diffusely adherent *E. coli* (DAEC) was initially used to refer to any Hep-2-adherent *E. coli* strain that did not form EPEC-like microcolonies. With the discovery of EAEC, most authors now recognise DAEC as an independent category of potentially diarrhoeagenic *E. coli* (Nataro and Kaper 1998). Little is known about the pathogenic features of DAEC induced diarrhoea and no PCR assay has been described as identifying DAEC. Levine et al. (1993) showed that in Santiago, Chile the relative risk of DAEC in association with diarrhoea increased with age from 1 to 4-5 years. The reason for such an age-related phenomenon is yet unknown. Jallat et al. (1993) have shown that DAEC strains account for a large proportion of diarrhoea cases among hospitalised patients in France who have no other identified enteropathogen. Other epidemiological features and the true role of DAEC remain to be determined.

3. AIMS OF THE PRESENT STUDY

The aims of this study were to:

-set up and use new molecular detection and typing methods for diagnosing and genotyping diarrhoeagenic *E. coli* (EAEC, EPEC, ETEC and STEC);

-study the prevalence and (molecular) epidemiology of diarrhoagenic *E. coli* in diarrhoea in Finland, and in diarrhoea associated with travel abroad. With diarrhoeas in Finland, special emphasis was placed on STEC;

-compare the prevalence of diarrhoaegenic *E. coli* with that of conventional enteric pathogens.

This study was carried out at the Laboratory of Enteric Pathogens (LEP) of the National Public Health Institute (Mannerheimintie 166, FIN-00300 Helsinki, Finland).

4. MATERIALS AND METHODS

4.1 Subjects and specimens (I, II, III, IV, V)

The number of subjects and faecal specimens (samples or cultures) investigated in the different parts of the study are presented in Table 2.

Table 2. Number of subjects with or without diarrhoea, and the faecal cultures available for investigation

Study	Subjects	Faecal samples/cultures	
		Diarrhoeal	Non-diarrhoeal
I	1	1	0
II	481	481	0
III	1	1	0
IV	204	65	219
V	603	603	92

4.2 Study design (II, IV, V)

4.2.1 Collection of samples and strains (II, V)

The diarrhoeal samples and STEC strains used in this study were collected at LEP in four phases.

January 1990-January 1996. The diagnosis of STEC infections in Finnish clinical microbiology laboratories (in total 29) was based on rather occasional culturing of stools on Sorbitol

MacConkey agar (SMAC) and isolation of sorbitol negative colonies. The pure cultures of these colonies were sent to LEP on a voluntary basis. From 1994 on, these laboratories were obliged to send all STEC O157:H7 isolates to LEP on the basis of regulations issued by the Ministry of Social Affairs and Health. The strains were stored in skimmed milk at -70°C until use.

February 1996-January 1997. The clinical microbiology laboratories of 20 acute-care hospitals culturing stool samples were approached by a letter providing diagnostic services free of charge for potential STEC or other diarrhoeagenic *E. coli* infections in all patients with bloody diarrhoea. For this purpose, these laboratories were asked to send the primary SMAC or Cystine-Lactose Electrolyte-Deficient (CLED) agar plates cultured from stool samples of these patients by mail to LEP.

Additionally, 122 stool cultures from consecutive diarrhoeal patients without macroscopically visible blood in their stools were investigated. These samples were cultured in a hospital in the central part of Finland (samples taken 7 January, 1997 – 21 February, 1997) and were investigated at LEP for diarrhoeagenic *E. coli*.

February 1997-December 1997. After the enhanced surveillance period, the service to examine *stx* genes from stool cultures was offered to all 29 clinical microbiological laboratories in Finland in cases where the patient was hospitalised for bloody diarrhoea or a suspected STEC infection. The methods used at LEP were the same as used previously.

4.2.2 Setting for TD study (IV)

The Ethical Committee of the Finnish National Public Health Institute (KTL) approved the study protocol. A charter-flight on an aircraft with 320 seats had been booked for a whole journey round the world. An introductory letter was sent via the travel agent to all the tour participants. The study was carried out among those 204 travellers who agreed to participate. They travelled round the world (Helsinki, Finland [April 19] - Shanghai, China [April 20-22] - Kuching, Malaysia [April 22-24] - Sydney, Australia [April 24-27] - Fiji [April 27-30]- Santiago de Chile, Chile [April 30-May 2]- Recife, Brazil [May 2-4]- Helsinki, Finland [May 5]) within 16 days in spring 1996. Each

volunteer received two doses of the ETEC/recombinant B-subunit oral vaccine (SBL Vaccine Ab, Stockholm, Sweden) (Jertborn et al. 1992, Sanchez et al. 1993) or a placebo containing heat-killed *E. coli* K-12 suspended in phosphate buffered saline. The first dose was to be ingested about 21 days before the trip, the second dose two weeks after the first dose. All participants gave at least two fresh faecal samples: one immediately before and one after the trip. In addition, if diarrhoea occurred during the journey, a faecal sample was collected as soon as possible and another sample on the following day**.** Also, if diarrhoea occurred within a week after the return home, a sample was taken. Additionally, a sample for parasitology was taken four weeks after the return.

4.2.3 Controls (IV, V)

Stool samples taken from 92 Finns without diarrhoea before they left for a round-the-world trip (cultured at LEP on April 19, 1996) formed the comparison group (Table 2).

4.3 Culturing and identification of diarrhoeal bacteria (I, II, III, IV, V)

All stool samples were cultured for *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia, Aeromonas* and *Plesiomonas* species by standard methods (Murray et al. 1995) in hospital laboratories (samples from diarrhoeal patients) and at LEP (samples from healthy controls, subjects on return and with TD). For detecting *E. coli*, nonselective SMAC or CLED plates were used.

4.4 Polymerase Chain Reaction (PCR) (I, II, III, IV, V)

Investigation of diarrhoeagenic *E. coli* was done with PCR. Genes detected, oligonucleotide primers and optimiced reaction conditions used for PCR are presented in Table 3. For PCR, a loopful of gram-negative bacterial growth, taken from the first streaking area of the primary faecal culture plate, was suspended in 0.5 ml of sterile distilled water and boiled for 20 minutes. Selected primer sequences matched completely with only the corresponding genes of corresponding *E. coli*

pathoroups in GenBank/EMBL database libraries. The oligonucleotides used as primers were purchased from Pharmacia Biotech (Uppsala, Sweden). In PCR amplification, all protocols used (Table 3) included denaturation steps at 95°C, amplification steps with 30 amplification cycles and final elongation steps at 72°C. The positive controls are presented in Table 3, and the negative controls used were sterile distilled water and *E. coli* strain ATCC 25922.

Table 3. Primers, amplification protocols and positive controls used for PCR

Target	Primer sequence 5' 3'	Amplicon size (bp)	Reference of primers	Reaction conditions (°C)	Controls (Reference)
STEC /*stx*$_1$	CAGTTAATGTGGTGGCGAAG CTGCTAATAGTTCTGCGCATC	894	Olsvik and Strockbine 1993	95°, 95°, 60°, 72°, 72° 10', 1' 1' 1', 5'	ATCC 43894, RH 3536
STEC /*stx*$_2$	CTTCGGTATCCTATTCCCGG CGATGCATCTCTGGTCATTG	478	Olsvik and Strockbine 1993	95°, 95°, 60°, 72°, 72° 4.5', 1' 1' 1', 5'	ATCC 43894, RH 3536
EPEC /*eae*	TGCGGCACAACAGGCGGCGA CGGTCGCCGCACCAGGATTC	629	Heuvelink et al. 1995	95°, 95°, 72°, 72° 5', 1' 1', 5'	RH 4283 (=EPEC E2348/69) (Baldini et al. 1990)
EAEC	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630	Schmidt et al. 1995	95°, 95°, 62°, 72°, 72° 5', 1' 1' 1', 10'	RH 4260 (=*E. coli* 17-2) (Baydry et al. 1990)
ETEC /LT	TCTCTATATGCACACGGAGC CCATACTGATTGCCGCAAT TCTCTATGTGCATACGGAGC	321	Olsvik and Strockbine 1993	95°, 95°, 62°, 72°, 72° 5', 1' 1' 1', 10'	ATCC 35401, ATCC 43886
ETEC /ST	TCTGTATTGTCTTTTTCACC TTAATAGCACCCGGTACAAGC	186	Frankel et al. 1989	95°, 95°, 62°, 72°, 72° 5', 1' 1' 1', 10'	ATCC 35401

4.5 Isolation and identification of *E. coli* (I, II, III, IV, V)

From the PCR-positive primary faecal culture, distinct *E. coli*-like and other gram-negative colonies were isolated and tested for the presence of the sequence which had initially given a positive result. As many colonies as required (however, no more than 100) for finding the isolate carrying these particular genes were assayed. The isolates were subsequently characterised biochemically by Api 20 E (bioMerieux sa, Marcy l'Etoile, France). Their ability to ferment sorbitol was also tested on SMAC. The PGUA test was executed according to the manufacturer's instructions (AS Rosco, Taastrup, Denmark).

4.6 Serotyping of the isolates (I, II, III, IV, V)

O grouping was carried out by bacterial agglutination (Ørskov and Ørskov 1984) with antisera against "traditional EPEC" (O26, O44, O55, O86, O111, O112, O114, O119, O124, O125, O126, O127, O128 and O142) and other O groups (O1, O2, O4, O6, O7, O8, O9, O11, O15, O16, O18, O22, O25, O50, O75, O77, O83, O85, 086, O100 and O157) (Siitonen 1992). A strain giving clumping with 4% saline was defined as rough. Flagellar H antigens were identified by agglutination of motile strains with seven anti-H serum pools (Ratiner 1989), capable of identifying single *E. coli* flagellar antigens (anti-O and anti–H sera were kindly provided by Dr. Yuli Ratiner from Mechnikov Research Institute for Vaccines and Sera, Moscow, Russia). A strain which did not grow through semi-solid agar within a week was defined as nonmotile (HNM), and a strain which was nontypable after six passages through semi-solid agar tubes was defined as nontypable (HNT). In Work II, the presence of capsular polysaccharide was determined by a rocket immunoelectrophoresis modification (Kuusi 1990) of precipitation with cetrimide (Cetavlon®, Sigma Chemical Co, St Louis, USA) after gel electrophoresis of K antigen extracts (Ørskov 1976). A strain not reacting with cetrimide was defined as noncapsulated (K^-). STEC strains which were ONT (O-nontypeable) with any of the antisera in use at LEP were sent for typing to the International *Escherichia* and *Klebsiella* Centre of the WHO at the Statens Seruminstitut (Copenhagen, Denmark) (II).

4.7 Detection of enterohaemolysin (Ehly) (I, II, III)

The production of Ehly was detected on a tryptose agar plate (Difco Laboratories, Detroit, USA) supplemented with 10 mM $CaCl_2$ and defibrinated sheep blood washed three times in phosphate-buffered saline, pH 7.2 (Beutin et al. 1989). Blood agar with unwashed sheep blood was used as a comparison plate. The plates were grown overnight in ambient air at 37°C. A halo around the colonies on the plate with washed blood cells only was interpreted as Ehly positive.

4.8 Shigatoxin production (I, II, III)

The ability of an isolate to produce Stx1 and/or Stx2 toxin was determined by the reversed passive latex agglutination test (Verotox F, Denka Seiken, Tokyo, Japan) according to the manufacturer's instructions; the test allowed toxin titers to a dilution of 1:128.

4.9 Antimicrobial susceptibility (II)

Antimicrobial susceptibility of the isolates was studied by the disk diffusion technique on Iso-Sensitest medium, using the zone size criteria recommended by the disk manufacturer and based on breakpoints established by the Swedish reference group for antibiotics (1990). The following antimicrobial agents (AS Rosco, Taastrup, Denmark) were used: ampicillin; ceftriaxone; chloramphenicol; ciprofloxacin; imipenem; mecillinam; nalidixic acid; neomycin; streptomycin; sulphonamide; tetracycline; and trimethoprim.

4.10 Pulsed-field gel electrophoresis (PFGE) (II, IV)

Bacterial cells were grown on Luria-agar overnight in ambient air at 37°C. They were suspended in 5 ml of TEN-buffer and were partially embedded in low-melting-temperature agarose (SeaPlaque agarose, FMC BioProducts, Rockland, USA), and were digested overnight with Proteinase-K (Boehringer Mannheim, Mannheim, Germany) at 55°C. The plugs were first washed

for 30 minutes with TE buffer, then for 60 minutes with TE plus 200 µl of 100 mM phenyl methanolsulphonyl fluoride and finally with TE three times for 30 minutes. Restriction endonuclease digestion was done with 10 U of *Xba*I (Boehringer Mannheim, Mannheim, Germany) overnight at 37°C. PFGE was performed using Bio-Rad GenePath™ System in 1% agarose gel in 0.5x TBE buffer at 14°C, a linear ramp of 2.20 to 54.20 seconds over a period of 22 hours, a 120-degree switch angle, and a gradient of 6.0 V per cm. After PFGE, the gels were stained with ethidium bromide and were photographed under UV transillumination.

In Work II, an isolate with at least two differences in the banding pattern among bands larger than 100 kb, was regarded as belonging to another subtype. Each subtype was given a number within a serotype: serotype O157:H7 isolates were numbered 1a, 1b, etc., isolates of serotype O26:H11 were 2a, 2b etc. In Work V, a different letter was assigned (A, B, C...) to each different PFGE-type (Tenover et al. 1995), to indicate its dissimilarity to other isolates.

4.11 Characterisation of LPS with SDS-PAGE (III)

The technique described previously was used for the deproteinisation of bacterial whole cell samples (Hitchcock and Brown 1983) Two *E. coli* O157:H7 strains (ATCC 43894, RH 1438) were used as controls. The samples were electrophoresed through 12 % sodiumdodecylsulphate-polyacrylamide gel (SDS-PAGE) as described by Laemmli (1970).

4.12 Determination of antibodies (III)

Agglutinins in the serum of the patient against boiled O157 antigen were titrated in wells of a microtiter plate (Titertek®, Flow Laboratories, UK) using the Widal reaction method (Farmer 1995). The known positive, taken in the acute phase of O157 infection, and negative human sera (tested previously at the Public Health Laboratory Service, Colindale Avenue, London, UK) were used as controls.

4.13 Virology and parasitology (IV)

The investigation for rotaviral and adenoviral antigens in samples from diarrhoeal patients was carried out during the round-the-world trip by latex agglutination (Diarlex Rota-Adeno Kit, Orion Diagnostica, Espoo, Finland) according to the manufacturer's instructions. The specimens for parasitology were collected before the trip and four weeks after the return, and were analysed at the Unit of Parasitology of Helsinki University Central Hospital, Helsinki. The standard formalin-ether concentration method was used (Murray et al. 1995).

4.14 Statistical methods (IV, V)

Differences between the cases and controls were compared using Fisher's exact test (Epi-Info 6.04 software; WHO, Geneva, Switzerland and CDC, Atlanta, USA). P-values lower than 0.05 were considered significant.

5. RESULTS

Publications I - V contain the results in detail.

5.1 Demographic characteristics of the subjects (I - V)

The ages of the 668 diarrhoeal patients (I - V) ranged from 0 to 98 years, and the ages of subjects without diarrhoea from 15 to 76 years (IV, V) (Table 4). The gender distribution was similar in all groups of subjects. Information about possible stays abroad before (maximum 2 weeks) the onset of symptoms was available for 266 of the 603 diarrhoeal patients (V).

Table 4. Demographic characteristics of the subjects

Number of subjects	Symptoms[2)]	Male/Female	Age(years)			Publication
			range	mean	median	
481	Bd	252/223[3)]	0 - 98	37	37	I, II, III,V
122	D	59/63	0 - 88	42	42	V
204[1)]		99/105	17-86	64	61	IV
65[1)]	D	34/31	45-86	63	62	IV
92[1)]	Nd	47/45	29 - 85	61	62	IV, V

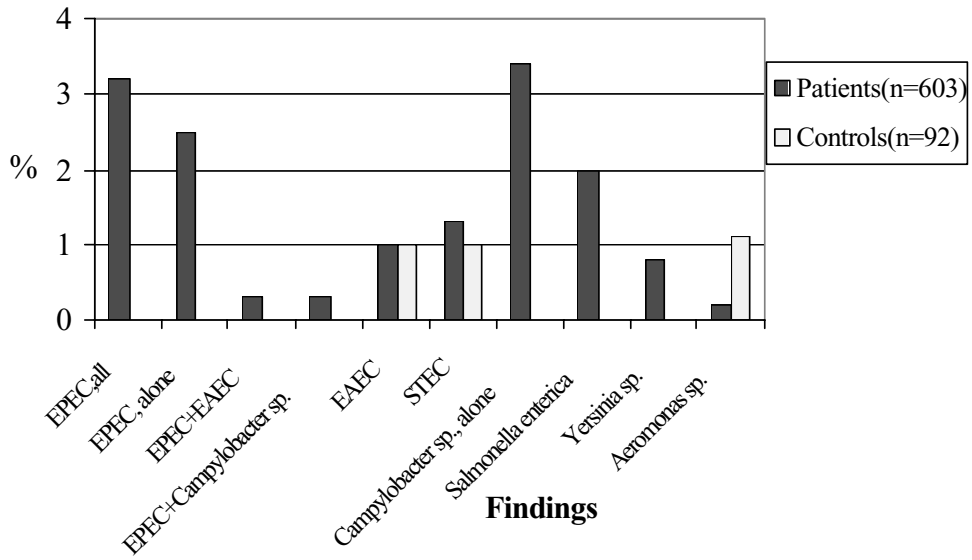
1) 65 and 92 are subsets of the 204 subjects, 2) Bd=bloody diarrhoea, D=diarrhoea, Nd=no diarrhoea, 3) No gender information was available for six subjects

5.2 Diarrhoaegenic *E. coli* and other bacterial findings in faeces of Finns with diarrhoea (I, II, III, V)

5.2.1 Findings of EPEC and EAEC in diarrhoea and characteristics of the isolates (V)

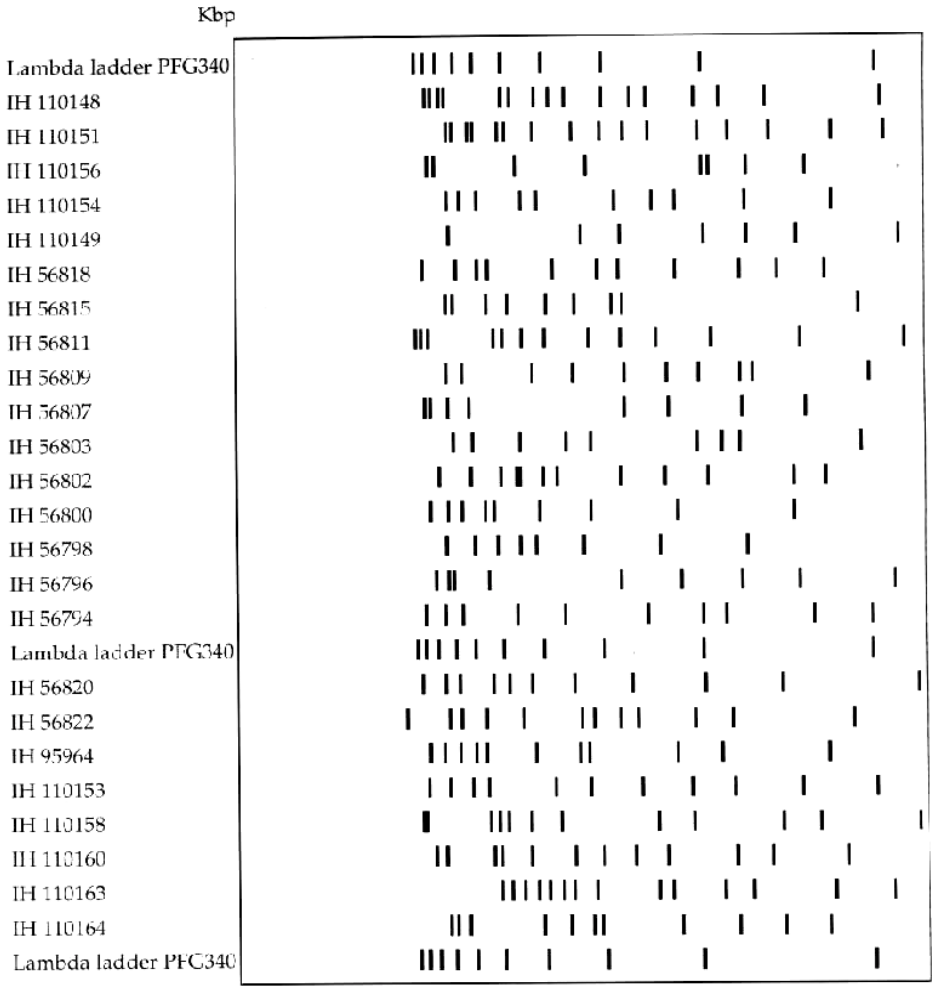
EPEC infection was detected by PCR in 19 (3.2%) and EAEC infection in six (1.0%) of the 603 diarrhoeal patients (Figure 2). Four EPEC infections were associated with trips abroad (Egypt (two cases), Ghana and Peru). Four EAEC patients had visited in the Dominican Republic, Egypt, Ghana and Kenya.

Figure 2. Findings of diarrhoeagenic *E. coli* in faecal samples from 603 subjects and findings of other enteric bacterial pathogens in samples from 506 subjects with diarrhoea. The findings in samples from 92 subjects formed the control group.



Seventeen of the 19 EPEC (89.5%) and all seven EAEC (100%) isolares were available for subtyping; only two of them could be characterised for their O groups (O15 and O55). In H typing, most of the EPEC or EAEC isolates had a distinct H antigen. In PFGE, digestion of chromosomal DNA with *Xba*I yielded 7 to 12 bands for EPEC and 10 to 12 bands for EAEC (Figure 3). All EPEC and EAEC isolates showed different PFGE patterns when compared visually.

Figure 3. Schematic view of PFGE patterns of EPEC (IH 110148 – IH 56794) and EAEC (IH 56820 – IH 110164) isolated from Finns with or without diarrhoea

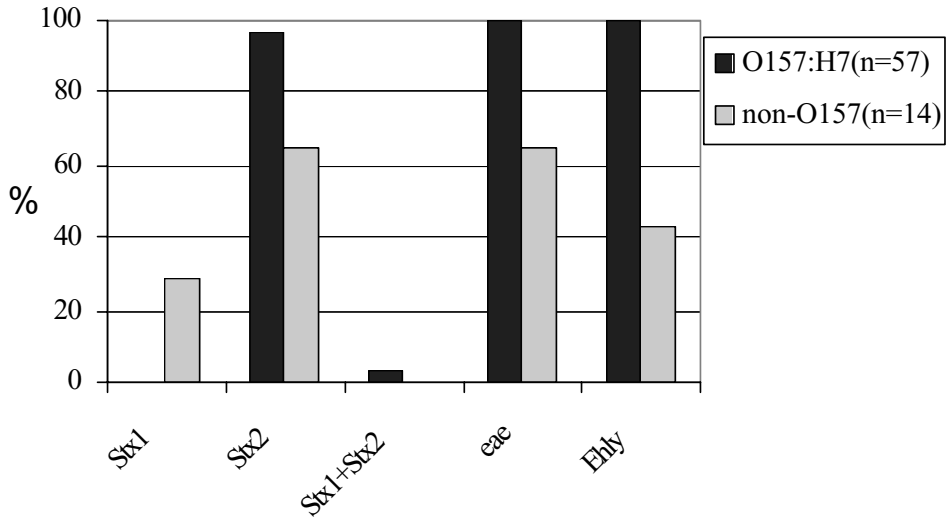


5.2.2 Findings of STEC isolated in Finland 1990-1997 (I, II, III)

Before the enhanced surveillance was started in 1996, only four sporadic infections caused by STEC O157:H7 were diagnosed in Finland in the 1990s. During the enhanced surveillance 1996-1997, eight infections were discovered, three caused by O157:H7 and five by non-O157 strains. Of these, two represented unique STEC serotypes (OX3:H21 and Rough:H49) and caused HUS. After the enhanced surveillance period, 59 STEC infections were detected. Of these, two STEC O157:H7 infections resulted in deaths of the patients.

Of all 71 STEC isolates detected in Finland during 1990-1997, 57 were sorbitol and PGUA negative and belonged to the O157:H7 serotype. One additional O157 strain was HNM, sorbitol and PGUA positive. Of the 14 non-O157:H7 strains, STEC serotype O26:H11 was the most common and was found in four patients. Of the 71 STEC strains, 63 carried the stx_2 gene only, five isolates (all O26) carried the stx_1 gene only, and three isolates (all O157) carried both genes (Figure 4). The *eae* gene was detected in all other isolates except five isolates of serotypes O2:H29, O91:H121, O107:H27, OX3:H21 and Rough:H49. Production of Ehly was detected in all but eight STEC isolates, all representing non-O157:H7 serotypes. Isolates found 1996 and before were all of different genotypes. Four distinct genomic subtypes were found among STEC O157:H7 strains isolated in 1997.

Figure 4. Comparison of STEC O157:H7 and non-O157:H7 isolates



All isolates belonging to the serotype with more than one isolate (57 O157:H7 and 4 isolates of O26:H11) were subtyped with PFGE. Digestion of chromosomal DNA with *Xba*I yielded 12 to 17 bands. Seven distinct genomic profiles could be seen among the 57 O157:H7 isolates and three distinct profiles among the four O26:H11 isolates. Type 1a was the most common genotype found among 36 (65%) of the O157:H7 strains. All 15 strains isolated in July 1997 in western Finland had this 1a type PFGE pattern. Other common O157:H7 subtypes were 1b (n=7), 1c (n=6) and 1d (n=4). These subtypes were associated with sporadic cases of infection or with cases of infection occurring within a family.

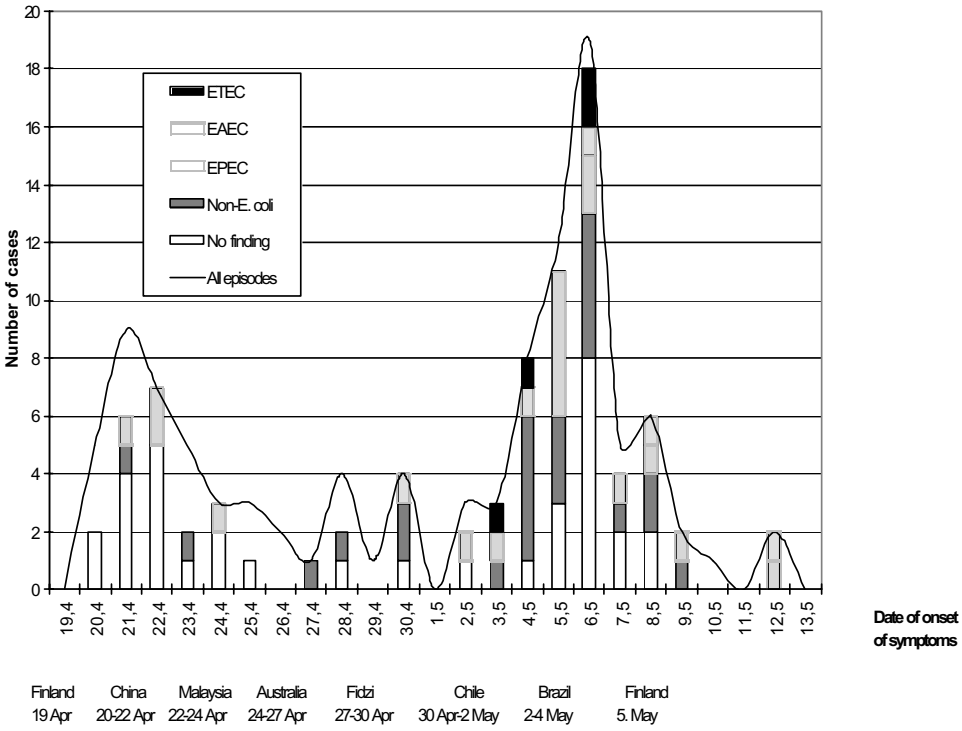
Table 4. Characteristics of human STEC isolates in Finland, 1990 to 1997

Phenotype	**Virulence determinants**	**PFGE-genotypes (number of isolates)**	**Number of strains**
O157:H7, sor-	*stx*$_2$, *eae*, Ehly	1a (36), 1b (7), 1c (5) 1d(4), 1e (1), 1g (1)	55
O157:H7, sor-	*stx*$_1$ + *stx*$_2$, *eae*, Ehly	1c (1), 1f (1)	2
O157:HNM, sor+	*stx*$_2$, Ehly	-	1
O26:H11, sor+	*stx*$_1$, *eae*, Ehly	2a (2), 2b (1), 2c (1)	4
O26:HNM, sor+	*stx*$_1$, *eae*	-	1
Rough:H49, sor+	*stx*$_2$	-	1
O2:H29, sor+	*stx*$_2$	-	1
O91:H21, sor+	*stx*$_2$, *eae*	-	1
O91:H40, sor+	*stx*$_2$, *eae*	-	1
O101:HNM, sor+	*stx*$_2$, *eae*	-	1
O107:H27, sor+	*stx*$_2$	-	1
O165:H25, sor+	*stx*$_2$, *eae*, Ehly	-	1
OX3:H21, sor+	*stx*$_2$	-	1

5.3 Findings associated with a round-the-world trip of Finns (IV)

During the trip in April 1996, diarrhoeal episodes took place in two peaks; the first occurred two and the second 18 days after the departure (Figure 4). This second peak appeared one day after the return to Finland. A stool sample was available for culture from 65 episodes of diarrhoea (60 subjects; 80% of all 75 patients with diarrhoea and 72% of all 90 diarrhoeal episodes). The causes of the diarrhoeal episodes in China and Malaysia remained mostly unknown. The episodes in the latter part of and after the trip probably originated in South America.

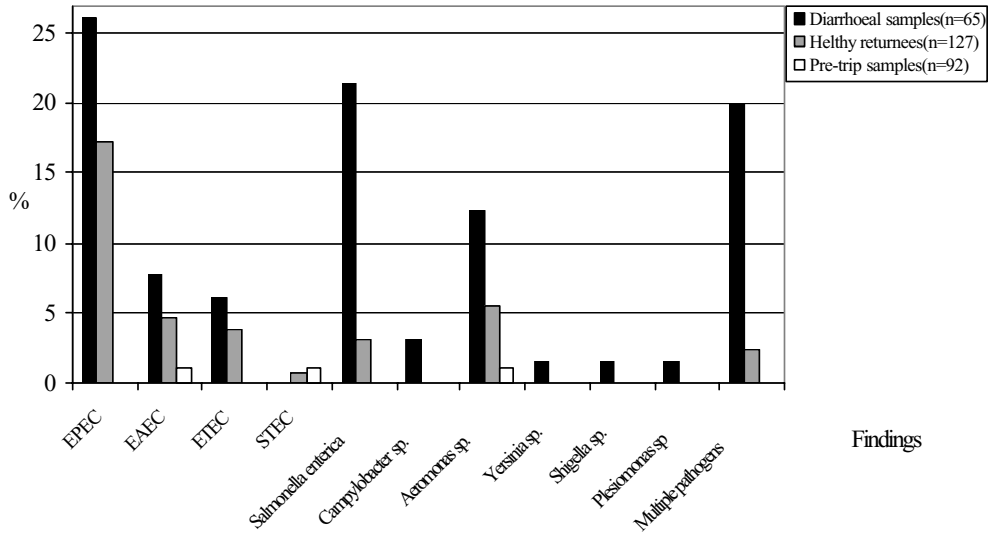
Figure 4. Onset and etiology of diarrhoeal episodes during and after a round the world trip



Three of the 92 stool samples taken from non-diarrheal subjects before the trip were positive for potential enteric pathogenic bacteria (EAEC, STEC, *Aeromonas* sp.). Of these samples, compared with the findings from 65 diarrhoeal samples, significant differences were found in EPEC

(0/92 vs. 13/65; $P < 0.001$) and *S. enterica* (0/92 vs. 8/65; $P < 0.001$). In 65 diarrhoeal episodes, enteropathogenic bacteria were found in 62%. The corresponding figure for the 127 subjects without diarrhoea, healthy returnees, was 33% ($P < 0.001$). Of the 65 diarrhoeal episodes, various pathogenic *E. coli* were found in 35% (23/65) and of the samples of the healthy returnees in 26% (33/127) (*P*=NS). In 13 diarrhoeal episodes, they were a single pathogen and in 10, they were found as a mixed culture with some other bacterial pathogen. The numbers of single *E. coli* findings from diarrhoeal episodes and from healthy returnees did not differ statistically.

Figure 5. Bacterial findings in stool samples from diarrhoeal episodes of subjects (n=65) and from healthy returnees (n=127). Findings from diarrhoeal episodes with several bacterial pathogens are included in each column.



Non-ETEC strains were found in 29% (n=19) of the episodes: the *eaeA*-positive EPEC was found in 26% (n=17) accompanied by EAEC in 8% (n=5) (Figure 5). The ST-toxin encoding gene of ETEC was found in three and LT in one of the diarrhoeal episodes. All of the four episodes (6%) caused by ETEC were episodes that started during or after a stay in South America. Because the number of ETEC strains identified was low, the efficacy of the vaccine could not be evaluated.

Several bacterial pathogens were detected in 20% (n=13) of the diarrhoeal episodes (Figure 5), whereas in specimens of 127 healthy subjects several pathogens were found on return in 2% (n=3, *P*= <0.001). Of these three latter findings, all were EPEC in combination with either *Aeromonas* sp., *Salmonella* or STEC. Of all bacterial findings, EPEC strains were the most common, independent of the clinical picture of the subjects, detected in a total of 39 samples, whereas *Salmonella enterica* was more commonly found as a single pathogen in diarrhoeal episodes than in samples from healthy returnees (12% vs. 2%; *P*=0.005). The numbers of single non-*E. coli* findings in diarrhoeal episodes and in healthy returnees differed significantly (22% vs. 7%; *P*=.003).

5.4 Conventional enteric pathogens in patients with diarrhoea and in subjects travelling around-the-world (IV, V)

In patients with diarrhoea (V), the results of the stool cultures for non-*E. coli* enteric pathogens were available for 506 of the 603 patients with diarrhoea (83.9%). Other enteric bacterial pathogens were found in 34 (6.7%) of these 506 patients. *Campylobacter jejuni/coli* was the most common finding, isolated from 19 patients (3.8%) (16 *C. jejuni,* 3 *C. coli* infections) (Figure 5). In two of these cases, both EPEC and *C. jejuni* were detected in the same patient. *Salmonella enterica* was found in ten (2.0%) patients. *Yersinia enterocolitica* was found in four patients and *Aeromonas* sp. in one. Of these 34 patients, travel histories were known for 28, of whom 16 (57.1%) had travelled abroad before becoming ill.

In patients during or after the round-the-world trip (IV), strains of various *Salmonella* serotypes were the most common non-*E. coli* pathogens (Figure 5). They were isolated either alone or with another bacterial pathogen in 24% (n=14) of the diarrhoeal episodes and in 3% (n=4) of the healthy returnees (*P*=0.003). No rotaviral or adenoviral antigens were detected in diarrhoeal patients.

6. DISCUSSION

6.1 General aspects

Infectious diarrhoeal diseases contribute a considerable problem globally and are responsible for considerable morbidity and mortality, especially in the developing countries (WHO 1993). Diarrhoea also remains an important problem in the industrialised countries, but the course of the disease is generally mild, and the mortality has decreased drastically over time (Cravioto et al. 1998). However, economic losses due to the cost of medical care and the absence of patients from work or school may be considerable. Moreover, the numbers of diarrhoeal outbreaks have increased during recent years in the industrialised countries (Armstrong et al. 1996). Recently it was concluded that 20% of Britons had infectious intestinal disease each year (Wheeler et al. 1999). In Finland, the numbers of outbreaks associated with food and water increased during the whole 1990s (Kukkula 1998).

Rapid and reliable detection and typing methods are required for successful microbiological surveillance and investigation of infectious diseases. Traditionally, pathogenic bacteria from stools have been analysed using conventional culturing methods and various animal models, which are time-consuming and laborious but still widely in use today. However, DNA based detection methods have become more and more common in clinical microbiology. They enable the analysis to be done within 24 hours, or even within one working day, and provide more specific detection of the desired organism (Nataro and Kaper 1998).

In this study, PCR was used to for detecting diarrhoaegenic *E. coli*. PCR, without enrichment steps, will recognise culturable, viable but nonculturable, and even nonviable cells. Thus, the inability of PCR discriminate between living and dead cells has been considered a negative aspect. However, the detection of diarrhoaegenic *E. coli* in this study was done from stool cultures i.e. all *E. coli* findings made were actually alive, and therefore potentially capable of causing symptoms. In this study, before a round-the-world trip, few subjects carried any potential enteric pathogens, whereas, on return of the subjects from the trip, a high number of subjects carrying various

pathogenic *E. coli* non-symptomatically was found. This is not surprising, since in many developing countries this kind of carriage of EPEC, EAEC or ETEC is common (Nataro and Kaper 1998). Also, the high sensitivity of the PCR assay and the high infectious dose of these bacteria may explain the nonsymptomacy of the subjects to whose normal microbial flora these bacteria do not belong. Molecular methods, especially PCR, are nowadays considered the most reliable and sensitive techniques for differentiating diarrhoeagenic *E. coli* strains from nonpathogenic members of the stool flora, and for distinguishing one *E. coli* pathogroup from another (Nataro and Kaper 1998, Caeiro et al. 1999). In practice, PCR was a rapid and constant method for investigating diarrhoeal samples. This was confirmed with *stx* PCR, as practically all strains isolated were also Stx-producing when tested by another method (Verotox-F, Denka Seiken, Tokyo, Japan). There is no doubt that in the near future, PCR based detection methods will play a larger role in clinical microbiology laboratories.

PFGE was used for genotyping *E. coli* isolates in this study. PFGE was capable of discriminating between the strains within a single serotype and, therefore, confirmed whether isolates were related or not. PFGE has become a standard tool in molecular epidemiology (Mickelsen 1997). It has been stated that routine surveillance by this technique can identify outbreaks that are not detected by traditional phenotypic methods and can ascertain whether a sudden increase in reported cases is due to sporadic isolated cases or an outbreak (Nataro and Kaper 1998). In the USA, in the PulseNet, PFGE patterns of STEC O157:H7 can be electronically transmitted to a databank at the CDC, where they are automatically compared with patterns of other isolates. If the patterns submitted by laboratories at different locations during a defined time period are found to match, the CDC computer will alert of a possible outbreak (Centers for Disease Control 2001). PFGE is a valuable tool in epidemiological research. However, the disadvantage of PFGE is that it is a slow, labour- and equipment-intensive method. If shorter protocols are not developed, it is hard to expect wider use of PFGE outside the research and reference laboratories.

6.2 Prevalence of diarrhoeagenic *E. coli* in diarrhoea in Finland

Stool samples of 603 patients with sporadic diarrhoea were investigated by PCR for diarrhoeagenic *E. coli*. Diarrhoeagenic *E. coli* were detected in 5.5% of the 603 patients with

diarrhoea. EPEC were the most common diarrhoaegenic *E. coli*, found in 3.2% of the patients. In four of these cases, EPEC was found together with other pathogens. EAEC and STEC infections were both discovered in eight patients (1.3%). In practice, specific *E. coli* strains were isolated from all PCR positive stool cultures; this indicates their significant prevalence in *E. coli* flora in diarrhoeal stools. In a large recent Swedish study (Svenungsson et al. 2000), 5% of diarrhoeas in adult patients were caused by STEC, EPEC or EAEC. The Swedish results are in accordance with the present results. Also, in the present study, the mean age of the patients was about 40 years, indicating that diarrhoeagenic *E. coli* causes diarrhoea in adults in Finland, as well as in Scandinavia, and probably also in other industrialiced countries.

Among the 506 of 603 patients regarding whom all conventional bacterial findings were available, a non-*E.coli* pathogen was identified in 6.7%. *Campylobacter* sp. and *Salmonella* sp. were the most common non-*E. coli* pathogens, found in 3.8% and 2.0% of the patients, respectively. *Salmonella* sp. and *Campylobacter* sp. were also the most common non-*E. coli* pathogens found in a recent study in Sweden (Svenungsson et al. 2000). However, in that study, *Salmonella* sp. and *Campylobacter* sp. were found in total of 20% of the patients, a figure which is notably higher than in our study. This difference might be due to different sampling areas. In the Swedish study, all samples were gained from one hospital serving people in southern and western parts of Stockholm, whereas in our study, the samples were collected from 16 hospitals all around Finland.

By the conventional serotyping, only one (O55) of the 17 EPEC isolates could be classified under a traditional scheme of EPEC serotypes, suggesting that this old serotypic classification of "dyspepsiekoli" is evidently not valid. This was also concluded in a recent Japanese report (Sunabe and Honma 1998) investigating the relation between O serogroup and pathogenic factor genes in *E. coli*, and in another report (Ansaruzzaman et al. 2000) on the clonal groups of EPEC associated with diarrhoae in Bangladesh. Moreover, Vidotto et al. (2000) studied EPEC associated with infant diarrhoea in Brazil. They concluded that virulence characteristics of EPEC may be found in strains not belonging to the classical EPEC serotypes, and that molecular identification is required for studying the epidemiology of diarrhoea suspected to be caused by *E. coli*.

6.3 STEC infections in Finland

6.3.1 Overall situation

The number of identified STEC infections in Finland was very low at the beginning of the 1990s. The four sporadic O157:H7 infections were the only STEC detected during a 6-year period (1990 to 1995). After the first Scandinavian epidemic in Sweden in 1995 (Andersson and de Young 1996), LEP started an enhanced microbiological surveillance study of patients with bloody diarrhoea to evaluate the actual prevalence of STEC infections in Finland. During this study, three O157:H7 and five non-O157 infections were diagnosed during that 12-month period. After this surveillance, the incidence of STEC infections changed dramatically. In all, the rise in the number of STEC infections was over 10-fold from 1996 to 1997. In July 1997, the first STEC outbreak in Finland caused by STEC serotype O157:H7 started (Paunio et al. 1998). A total of 15 microbiologically confirmed STEC cases were found to be associated with this outbreak. These cases still accounted for only 25% of all 61 STEC infections in Finland in 1997. Two infections were fatal. In most cases, the sources of the infections remained unknown. The outbreak was associated with swimming water (Paunio et al. 1998) and, in one case, the patient had drunk unpasteurised milk produced on a neighbouring farm (Tast et al. 1998). During the whole 8-year period, only 10 (14%) of all 71 STEC infections were associated with travel outside Finland.

6.3.2 Pheno- and genotypes

In total, there were seven different STEC O157:H7 genotypes found during 1990-1997. All isolates known to be associated with the outreak in western Finland (Paunio et al. 1998) showed identical PFGE patterns (genotype 1a). Several sporadic isolates from different parts of Finland, isolated before, during, and after the outbreak, represented the same PFGE pattern, which together represented 65% of the O157:H7 strains isolated. Yet, no connection or common source has been found for these isolates. However, when Finnish human and bovine STEC O157:H7 isolates were compared by different subtyping methods, PFGE and phagetyping, the same

subtypes were found, suggesting that these infections could be of animal origin (Keskimäki et al. 2000, Lahti et al. 2001).

All 57 O157:H7 isolates found during the study were negative by sorbitol fermentation. A distinctive characteristic of the Finnish O157:H7 strains was that they had the *eae* gene and produced Stx2 and Ehly but not Stx1. Subtyping of Stx2 produced by these strains could provide useful information for epidemiological research. Similar Stx2-producing STEC O157:H7 strains have been described previously in other European countries. Of the human STEC O157:H7 isolates, 89% (Heuvelink et al. 1995) and 100% (Pierard et al. 1999) were solely Stx2 producing in the Netherlands and Belgium, respectively. In North America, STEC isolates typically produce both Stx1 and Stx2 toxins (Advisory comm... 1995).

Of the 14 non-O157 isolates found, serotype O26:H11 was the most common type, with four isolates; one further O26 strain was non-motile. All O26 isolates produced Stx1 and were *eae*-, sorbitol- and Ehly-positive. STEC O26:H11 has also been the most common non-O157 serotype found in some other European countries, for example, in Germany and Denmark (WHO 1997). The production of only Stx1 appears to be typical of STEC O26:H11. In a British study, 70% of STEC O26:H11 produced Stx1 only (Scotland et al. 1990). Moreover, in Germany and the Czech Republic, Stx1-producing STEC O26:H11 were the exclusive type identified until 1994, but after 1997, 71% of STEC O26:H11 isolates have been Stx2-producing (Zhang et al. 2000). Most other STEC strains produce Stx1 very rarely (Advisory comm… 1995, Zhang et al. 2000); in the present study, except for the O26 strains, no such strain was found. The other non-O157 STEC strains detected in this study, were Stx2-producing and sorbitol-positive, but their possession of the *eae* gene varied.

Two of the non-O157:H7 strains represented unpublished STEC serotypes (OX3:H21 and Rough:H49) associated with HUS. These strains were Stx2-producing and *eae*-negative. In Sweden, Rough-type STEC (STEC without O antigen) has been associated with HUS and bloody diarrhoea (Welinder-Olsson et al. 2000). Since it is generally believed that loss of the O antigen of bacteria results in loss of virulence (Nikaido 1996), these findings emphasise the need for further research in STEC pathogenesis. Additionally, following our publications, STEC strains

representing O-type OX3, many also Stx2-producing and *eae*-negative, have been commonly found in France, from cattle and associated with HUS (Bonnet et al. 1998, Pradel et al. 2000), and in Argentina, from cattle and ground beef (Parma et al. 2000). Nowadays, this serotype OX3 is called O174 (Scheutz 2000). All these STEC infections were sporadic and not associated with travel abroad, suggesting their endemic prevalence in these countries, possibly in cattle.

6.4 Diarrhoeagenic *E. coli* associated with TD

Causes of TD are numerous, but bacteria, and especially ETEC, are believed to be its major cause (Ericsson and DuPont 1993, Mattila 1995). In our prospective study, a group of 204 Finns travelled round the world in 16 days. The incidence of diarrhoea was registered, and a stool sample of each patient was taken and cultured immediately for *E. coli* during the course of the journey. Rotaviruses and adenoviruses were also investigated immediately by a nurse or laboratory technician who travelled with the group. Pathogenic *E. coli* was found in 35% of the 65 episodes of diarrhoea but also often (26%) in the subjects without diarrhoea. Conventional enteric pathogens (*S. enterica*, *Campylobacter*, etc.) were found in lesser amounts from diarrhoeal episodes (22%) than diarrhoaegenic *E. coli*, as a group, and parasites or rota- and adenoviruses were not found to be associated with diarrhoea.

ETEC has been regarded as the most common pathogen in TD, irrespective of the tourist destination (Ericsson and DuPont 1993, Mattila 1995). However, during recent years, very few studies have been made about the current aetiology of TD or diarrhoea in different countries. Recently, ETEC was associated with three waterborne outbreaks on cruise ships (Daniels et al. 2000), and in a Dutch study, ETEC was found in 11% of returned travellers with diarrhoea (Schultsz et al. 2000). In our study, ETEC strains were found in only four (6%) of all the 65 diarrhoeal episodes. ETEC detected in these subjects were from episodes that started after a stay of 2-3 days in South American countries (Chile and Brazil). Thus, all ETEC detected in the subjects probably originated in South America. Furthermore, ETEC has been reported to be a common diarrhoeal pathogen in Brazil (Torniepoth et al. 1995). In the present study, although the number of TD episodes was higher after a visit to South America than after visits to other places previously, EPEC rather than ETEC was the major pathogroup of *E. coli* found in these diarrhoeal

episodes. The same type of aetiology of diarrhoea, where EPEC, not ETEC, is the most prevalent enteric pathogen, has been found also in other developing countries, e. g. Nigeria (Akinyemi et al. 1998).

In our study, EPEC strains were the most common finding both in diarrhoeal samples and in samples taken from healthy subjects on return. EPEC was not found in pre-trip samples, and the difference between the pre-trip samples and the diarrhoeal samples was significant. In a recent Swedish study (Svenungsson et al. 2000), EPEC was detected in same numbers in both returning diarrhoeatic travellers and healthy subjects. In developing countries, while nonsymptomatic carriage of EPEC is common, it is still an important cause of infant diarrhoea. However, it has not been traditionally implicated as a cause of TD (Nataro and Kaper 1998). In Brazil, one of the countries visited during the present trip, EPEC has been the most prevalent enteropathogen in diarrhoeal children (Rosa et al. 1998). In Nigeria, EPEC was also the most common enteropathogen found in 27% of patients with acute diarrhoea (Akinyemi et al. 1998). In the present study, the finding that EPEC was the most common *E. coli* pathogroup in stools of diarrhoeal patients suggests that EPEC alone (15%), or together with another pathogen (9%), is capable of causing TD, although nonsymptomatic carriage was also high (15%).

7. SUMMARY AND CONCLUSIONS

Diarrhoea associated with bacterial infections is the most common illness experienced by millions of people in developing countries, as well as the major cause of disease in international travellers. The development of new molecular diagnostic methods has made it possible to re-evaluate the prevalence of different diarrhoeagenic *E. coli* in diarrhoea. In this study, the aetiology of diarrhoea was investigated among diarrhoeal patients in Finland during 1996-1997 and among adult Finnish tourists participating in a round-the-world trip in April 1996. Special emphasis was placed on the prevalence and characteristics of various diarrhoaegenic *E. coli*: STEC, EPEC, EAEC and ETEC.

In diarrhoeal patients in Finland, diarrhoeagenic *E. coli*, as a group, proved to be a more common potential cause of diarrhoea than *Salmonella* sp. or *Campylobacter* sp. or any other enteric bacterial pathogen commonly studied in cases of diarrhoea. Also, nonsymptomatic carriage of diarrhoeagenic *E. coli* was found. This may reflect a need for further study to evaluate the extension of this phenomenom and, moreover, to evaluate the significance of *E. coli* findings from diarrhoeal patients. Nevertheless, although the new diarrhoeagenic *E. coli* groups, EAEC and STEC, as well as the old group EPEC, were found at low frequencies in diarrhoeal stool samples from patients of different ages, they were more prevalent and more often of domestic origin than was expected. The annual incidence of detected STEC infections increased 10-fold during the period of the study and the first STEC outbreak in Finland took place. Two children died of serious illness caused by STEC. All these findings suggest that the contribution of diarrhoeagenic *E. coli* is greater than is currently appreciated in Finland and, probably, in the industrialised world, and therefore their study with appropriate methods is recommended.

In the genotyping of diarrhoeal *E. coli* isolates, all EPEC and EAEC strains isolated represented different PFGE genotypes, suggesting that all cases were sporadic. These PFGE results indicate that there are a large number of different EPEC and EAEC types that may cause diarrhoea in humans. By the conventional O serotyping, only one of all EPEC isolates could be classified under a traditional EPEC serotype (O55), suggesting that this serotypic classification of "dyspepsiekoli" no longer seems to be valid. The genotyping of STEC O157:H7 isolates showed that one type was far more common than the others, but several other types were also found. The number of non-

O157 infections was consistent during the years when they were investigated. Moreover, unique non-O157:H7 STEC associated with HUS were found, indicating the importance of detection all STEC strains, not just sorbitol-negative O157:H7 strains.

The new molecular diagnostic methods made it possible to re-evaluate the causes of TD and also made it possible to extend the analysis to new *E. coli* pathogroups. In this study, a new group, EAEC, and an old group, EPEC, proved to be the major *E. coli* groups associated with TD, whereas STEC and ETEC seemed to have only limited roles as causative agents.

The numbers of diarrhoeagenic *E. coli* and other enteric pathogens, found in diarrhoeal patients in Finland and in subjects participating in a trip round the world, were very much alike. Diarrhoeagenic *E. coli,* especially EPEC, was the most common pathogen found in both groups. *S. enterica* and *Campylobacter* sp. were the most common non-*E. coli* pathogens found. The only difference between the subject groups was the common asymptomatic carriage of diarrhoeagenic *E. coli* among subjects returning from the round-the-world trip. All these results could mean that, although the enteric bacterial pathogens found in patients with diarrhoea in Finland and in the rest of the world seem to be similar in types and proportions, the incidence of these bacteria still remains lower in Finland.

8. ACKNOWLEDGEMENTS

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9. REFERENCES

Abu-Elyazeed R, T F Wierzba, A S Mourad, L F Peruski, B A Kay, M Rao, A M Churillo, A L Borbeois, A K Mortagy, S M Kamal, S J Savarino, J R Campbell, J R Murphy, A Naficy, and J D Clemens. 1999. Epidemiology of enterotoxigenic *Escherichia coli* diarrhoea in pediatric cohort in a periurban area of Lower Egypt. J. Infect. Dis. 179:382-389.

Adam A. 1923. Über die Biologie der dyspepsiekoli und ihre Beziehungen zur Pathogenese der Dyspepsie und Intoxication. Jahrbuch für kinderheilkunde und physiche Erziehung 101:295-314.

Advisory Committee on the Microbiological Safety of Food. 1995. Report on Verocytotoxin-producing *Escherichia coli*. HMSO, London, United Kingdom.

Akinyemi K O, A O Oyefolu, B Opere, V A Otunba-Payne, and A O Owuru. 1998. *Escherichia coli* in patients with acute gastroenteritis in Lagos, Nigeria. East Afr. Med. J. 75:512-515.

Albert M J, S M Faruque, M Ansaruzzaman, M M Islam, K Haider, K Alam, I Kabir, and R Robins-Browne. 1992. Sharing of virulence associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*. J. Med. Microbiol. 37:310-314.

Andersson Y, and B de Young. The first Swedish outbreak of VTEC, abstract. In: Proceedings and Programme of Food Associated Pathogens-conference, May 6-8, 1996, Uppsala, Sweden. 1996;177.

Ansaruzzaman M, M J Albert, S Nahar, R Byun, M Katouli, I Kuhn, and R Mölldy. 2000. Clonal groups of enteropathogenic *Escherichia coli* isolated in case-control studies of diarrhoea in Bangladesh. J. Med. Microbiol. 49:177-185.

Arduino R C, and H L DuPont. 1993. Traveler's diarrhoea. Baillieres Clin. Gastroenterol. 7:365-385.

Armstrong G L, J Hollingsworth, and J G. Morris Jr. 1996. Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. Epidem. rev. 18:29-51.

Baldini M M, J B Kaper, M M Levine, C D Candy, and H W Moon. 1983. Plasmid-mediated adhesion in enteropathogenic *Escherichia coli.* J. Pediatr. Gastroenterol. Nutr. 2:534-538.

Batchelor M, S Knutton, A Caprioli, V Huter, M Zanial, G Dougan, and G Frankel. 1999. Development of a universal intimin antiserum and PCR primers. J. Clin. Microbiol. 37:3822-3827.

Baudry B, S J Savarino, P Vial, J B Kaper, and M M Levine. 1990. A sensitive and specific DNA probe to identify enteroaggregative *Escherichia coli*, a recently discovered diarrhoeal pathogen. J. Infect. Dis. 161:1249-1251.

Bender J B, C W Hedberg, J M Besser, D J Boxrud, K L MacDonald, and M T Osterholm. 1997. Surveillance for *Escherichia coli* O157:H7 infections in Minnesota by molecular subtyping. N. Engl. J. med. 337:388-394.

Bettelheim K A. 2000. Role of non-O157 VTEC. J. Appl. Microbiol. Symp. Supl. 88:38S-50S.

Beutin L, M A Montenegro, I Ørskov, F Ørskov, J Prada, S Zimmermann, and R. Stephan. 1989. Close association of verotoxin (shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli.* J. Clin. Microbiol. 27:2559-2564.

Beutin L, D Geier, S Steinruck, S Zimmerman, and F Scheutz, F**.** 1993. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Esherichia coli* in seven different species of healthy domestic animals. J. Clin. Microbiol. 31:2483-2488.

Beutin L, S Aleksic, S Zimmermann, and F Scheutz. 1994. Virulence factors and phenotypic traits of verotoxigenic *Escherichia coli* isolated from human patients in Germany. Med. Microbiol. Immunol. (Berlin) 183:13-21.

Beutin L, D Geier, S Zimmerman, and H Karch. 1995. Virulence markers of Shiga-like toxin-producing *Esherichia coli* strains originating from healthy domestic animals of different species. J. Clin. Microbiol. 33:631-635.

Bhan M K, V Khoshoo, V H Sommerfelt, P Raj, S Sazawal and, R Srivastava. 1989a. Enteroaggregative *Esherichia coli* and *Salmonella* associated with nondysenteric persistent diarrhea. Pediatr. Infect. Dis. J. 8:499-502.

Bhan M K, P Raj, M M Levine, J P Kaper, N Bhandari, R Srivastava, R Kumar, and S Sazawal. 1989b. Enteroaggregative *Esherichia coli* associated with persistent diarrhea in a cohort of rural children in India. J. Infect. Dis. 159:1061-1064.

Black R E, H M Merson, I Huq, A R M Aleim, and N Yunus. 1981. Incidence and severity of rotavirus and *Esherichia coli* in rural Banglades. Lancet I:141-143.

Bokete T N, T S Whittam, R AWilson, C R Clausen, C M O'Callahan, S L Moseley, T R Fritsche, and P I Tarr. 1997. Genetic and phenotypic analysis of *Escherichia coli* with enteropathogenic characteristics isolated from Seattle children. J. Infect. Dis. 175:1382-1389.

Bonnet R, B Souweine, G Gauthier, C Rich, V Livrelli, J Sirot, et al. 1998. Non-O157:H7 Stx2-producing *Escherichia coli* strains associated with sporadic cases of Hemolytic-uremic syndrome in adults. J. Clin. Microbiol. 36:1777-1780.

Butler D. 1996. Novel pathogens beat food safety checks. Nature 384:397.

Caeiro J P, J J Mathewson, M A Smith, Z D Jiang, M A Kaplan, and H I DuPont. 1999. Etiology of outpatient pediatric nondysenteric diarrhoea: a multicenter study in the United States. Pediatr. Infect. Dis. J. 18:94-97.

Caeiro J P, M T Estrada-Garcia, Z D Jiang, J J Mathewson, J A Adachi, R Steffen, and HL DuPont. 1999. Improved detection of enterotoxigenic *Escherichia coli* among patients with travelers' diarrhea, by use of the polymerase chain reaction technique. J. Infect. Dis. 180:2053-2055

Calderwood S B, D W K Acheson, G T Keuch, T J Barrett, P M Griffin, N A Strockbine et al. 1997. Proposed new nomenclature for SLT(VT) family. ASM News 1997:118-119.

Centers for Disease Control. 1995. *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami-Washington and California, 1994. Morbid. Mortal. Weekly Rep. 44: 157-160.

Centers for Disease Control. 1997. Outbreks of *Escherichia coli* O157:H7 infection associated with eating alfalfa sprouts-Michigan and Virginia, June-July 1997. Morbid. Mortal. Weekly Rep. 46: 741-744.

Centers for Disease Control. 2001. Diagnosis and Management of Foodborne Illnesses: A Primer for Physicians. Morbid. Mortal. Weekly Rep. 50(RR02):1-69.

Chalmers, R M, R L Salmon, G A Willshaw, T Cheasty, N Looker, I Davies, and C Wray. Vero-cytotoxin-producing *Escherichia coli* O157 in a farmer handling horses. Lancet 349:1816.

Chapman P A, and C A Siddons. 1997. A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from cases of bloody diarrhoea, non-bloody diarrhoea and asymptomatic contacts. J. Med. Microbiol. 44:267-271.

Chapman P A, C A Siddons, A T Cerdan Malo, and M A Harkin. 1997. A 1-year study of *Escherichia coli* in cattle, sheep, pigs and poultry. Epidemiol. Infect. 119:245-250.

Christie B. 1996. *E. coli* O157 kills five people in Scotland. BMJ 313:1424.

Clemens J D, D A Sack, J R Harris, J Chackraborty, P K Neogy, B Stanton, et al. 1988. Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli*: results of a large-scale field trial. J. Infect. Dis. 158:372-377.

Cocolin L, M Manzano, C Cantoni, and G Comi. A multiplex-PCR method to detect enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* in artificially contaminated foods. Int. J. Hyg. Environ. Health 203:159-164.

Costin, I., D Voiculescu, and V. Gorcea. 1964. An outbreak of food poisoning in adults associated with *Escherichia coli* serotype 86:B7:H34. Pathol. Microbiol: 27:68-78.

Cravioito A R, E Reyes, and R Ortega. 1988. Prospective study of diarrhoeal disease in a cohort of rural Mexican children: incidence and isolated pathogens during the first two years of life. Epidemiol. Rev. 101:123.

Cravioto A, C Eslava, Y Lopez-Vidal, and R Cabrera. 1998. Strategies for control of common infectious diseases prevalent in developing countries. p. 577-586. In P Williams, J Ketley and G Salmond (edt). Methods in microbiology, vol 27, Bacterial Pathogenesis. 620 p. Academic Press, London, UK.

Daniels NA, J Neimann, A Karpati, U D Parashar, K D Greene, J G Wells, et al. 2000. Traveler's diarrhea at sea: three outbreaks of waterborne enterotoxigenic *Escherichia coli* on cruise ships. J Infect Dis 181:1491-1495.

Donnerberg M S, J A Giron, J P Nataro, and J B Kaper. 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Esherichia coli* associated with localized adherence. Mol. Microbiol. 6:3427-3437.

Donnerberg M S, C O Tacket, S P James, G Losonsky, J P Nataro, S S Wasserman, J B Kaper and M M Levine. 1993a. Role of *eae* gene in experimental enteropathogenic *Escherichia coli* infection. J. Clin. Invest. 92: 1412-1417.

Donnerberg M S, S Tzipori, M L McKee, A D O'Brien, J Alroy and J B Kaper. 1993b. The role of *eae* gene of enterohemorrhagic *Escherichia coli* in intimate attachement in vitro and in porcine model. J Clin. Invest. 92:1418-1424.

DuPont H L, S B Formal, R B Hornick, M J Snyder, J P Libonati, D G Sheahan, E E LaBrec and J P Kalas. 1971. Pathogenesis of *Esherichia coli* diarrhea. N. Engl. J. Med. 285:1-9.

Durrer P, R Zbinden, F Fleisch, M Altwegg, B Lederberger, H Karch, and R Weber. 2000. Intestinal infection due to enteroaggregative *Escherichia coli* among human immunodeficiency virus-infected persons. J. Infect. Dis. 182:1540-1504.

Ericsson C D, and H L DuPont. 1993. Travelers' diarrhea: Approaches to prevention and treatment. Clin. Inf. Dis. 16:616-26.

Fang, G.D, A A M Lima, C V Martins, J P Nataro and R L Guerrant. 1995. Etiology and epidemiology of persistant diarrhoea in northeastern Brazil: a hospital-based, prospective, case-control study. J. Pediatr. Gastroenterol. Nutr. 21:137-144.

Farmer J J. 1995. *Enterobacteriaceae*: Introduction and identification, p. 438-449. In Murray, P.,R., Baron, E., J., Phaller, M., A., Tenover, F., C and Yolken R., H. Manual of Clinical Microbiology, Sixth edition. American society for microbiology, Washington, DC.

Frankel G, J A Giron, J Valmassol, and G K Schoolnik. 1989. Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool. Molecular Microbiology 3:1729-34.

Fratamico P M, S K Sackitey, M Wiedmann, and M Y Deng. 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR. J. Clin. Microbiol. 33:2188-2191.

Gaastra W, and A-M Svennerholm. 1996. Colonization factors of human enterotoxigenic *Esherichia coli* (ETEC). Trends Microbiol. 4:444-452.

Gannon V P J, M Rashed, R K King, and E. J. Golsteyn Thomas. 1993. Detection and characterization of the *eae* gene of shiga-like toxin-producing *Escherichiacoli* using polymerase chain reaction. J. Clin. Microbiol. 31:1268-1274.

Gascon J, J Vila, M Valls, E Ruiz, L Vidal, J Corachan, et al. 1993. Etiology of traveller's diarrhoea in Spanish travellers to developing countries. Eur. J. Epidemiol 92:217-223.

Gomes T A, P A Blake and L R Trabulsi. 1989. Prevalence of *Escherichia coli*strains with localized, diffuse, and aggregative adherance to HeLa cells in infants with diarrhoea and matched controls. J. Clin. Microbiol. 27:266-269.

Griffin P M. 1995. *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli.* p. 739-761. In M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greeberg, and R. L. Guerrant (ed.). Infections of the gastrointestinal track. Raven Press, New York.

Hedberg CV, S J Savarino, J M Besser, C J Paulus, V M Thelen, L J Myers, et al. 1997. An outbreal of foodborne illness caused by *Esherichia coli* O39:NM: an agent that that does not fit into the existing scheme for classifying diarrheagenic *E. coli*. J. Infect. Dis. 176:1625-1628.

Heuvelink A E, N C A J van de Kar, J F G M Meis, L A H Monnens, and W J G Melchers. 1995. Characterization of verocytotoxin-producing *Esherichia coli* O157 isolates from patients with haemolytic uremic syndrome in Western Europe. Epidemiol. Infect.115:1-14.

Hitchcok P J, and T M Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. 154:269-277

Holzman D. 1998. Foodborne disease:New means for quantifying the burden. ASM News. 64:319

Huppertz H I, S Rutkowski, S Aleksic, and H Karch. 1997. Acute and chronic diarrhoea and abdominal colic associated with enteroaggregative *Escherichia coli* in young children living in western Europe. Lancet 349:1660-1662.

Itoh Y, I Nagano, M Kunishima, and T Ezaki. 1997. Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable:H10 associated with massive outbreak of gastrointestinal illness. J. Clin. Microbiol. 35:2546-2650.

Jackson M P, R J Neill, A D O'Brien, R K Holmes, and J W Newland. 1987. Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Esherichia coli* 933. FEMS Microbiol. Lett. 44:109-114.

Jallat C, V Livrelli, A Darfeuille-Michaud, C Rich, and B Joly. 1993. *Esherichia coli* strains involved in diarrhea in France: high prevalence and heterogenity of diffusely adhering strains. J. Clin. Microbiol. 31:2031-2037.

Jann K, and B Jann. 1992. Capsules of *Escherichia coli*, expression and biological significance. Can. J. Microbiol. 38:705-10.
Jarvis K G, and J B Kaper. 1996. Secretion of extracellular proteins by enterohemorrhagic *Escherichia coli* via a putative type III secration system. Infect. Immun. 64:4826-4829.

Jertborn M, A M Svennerholm, and J Holgren. 1992. Safety and immunogenicity of an oral recombinant cholera B subunit-whole cell vaccine in Swedish volunteers. Vaccine 10:130-132.

Jerse A E, W C Martin, J E Galen, and J B Kaper. 1990. Oligonucleotide probe for detection of the enteropathogenic *Escherichia coli* (EPEC) adherence factor of localized adherent EPEC. J. Clin. Microbiol. 28:2842-2844.

Johnson R P, R C Clarke, J B Wilson, S C Read, K Rahn, S A Renwick, et al. 1996. Growing conserns and recent outbreaks involving non-O157:H7 serypes of verotoxigenic *Escherichia coli*. J. Food. Prot. 59: 1112-1122.

Karmali M A, B T Steele, M Petric, and C Lim. 1983. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cyto toxin and cytotoxin-producing *Escherichia coli* in stool. Lancet i:619-620.

Karmali M A, M Petric, and M Bielaszewska. 1999. Evaluation of microplate latex agglutination method (Verotex-F assay) for detecting and characterizing verotoxins (Shiga toxins) in *Escherichia coli*. J. Clin. Microbiol. 37:396-399.

Kauffmann F. 1947. The serology of *coli* group. J. Immunol. 57:71-100.

Keskimäki M, M Saari, T Honkanen-Buzalski, and A Siitonen. 2000. Typing of enterohaemorrhagic *Escherichia coli* isolates from human and cattle samples in Finland (In Finnish). National Food Administration/Research Notes 8/2000, 11 p. + 2 app.

Knutton S, M M Baldini, J B Kaper, and A S McNeish. 1987. Role of plasmid-encoded adherence factors in adhesion of enteropathogenic *Escherichia coli* to Hep-2 cells. Infect Immun 55:78-85.

Konowalchuk J, J I Speirs, and S Stavric. 1977. Vero responce to a cytotoxin of *Esherichia coli*. Inf. Imm. 18(3):775-779.

Kukkula M. 1998. Increased effectiveness in the surveillance of foodborne outbreaks in Finland. p. 237-242. In: 4th World Congress, Foodborne Infections and Intoxications, Proceedings. Berlin, Germany July, 7-12, 1998. Federal institute for health protection of consumers and veterinary medicine (FAO/WHO collobrating centre for research and training in food hygiene and zoonoses.

Kuusi N. 1979. A technical improvement for crossed immunoelectrophoresis. J. Immunol. Methods. 31:229-237.

Laemmli U K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-5.

Lahti E, M Keskimäki, L Rantala, A Siitonen, P Hyvönen, and T Honkanen-Buzalski. 2001. Occurrence of *Escherichia coli* O157 in Finnish cattle. Vet. Microbiol. 2:239-251.

Lansbury L E ja H Ludlam. 1997. *Escherichia coli* O157: Lessons from the past 15 years. J. Inf. 34:189-198.

Law D, and H Chart. 1998. Enteroaggregative *Escherichia coli*. J. Appl. Microbiol. 685-697.

Levine M M, and R Edelman. 1984. Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: epidemiology and pathogenesis. Epidemiol. Rev. 6:31-51.

Levine M M, P Ristaino, G Marley, S Knutton, E Boeker, R Black, et al. 1984. Coli surfase antigens 1 and 3 of colonization factor antigen II-positive enterotoxigenic *Escherichia coli*: morphology, purification, and immuno responses in humans. Infect. Immun. 44:409-420.

Levine M M. 1987. *Esherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemoorhagic, and enteroadherant. J. Infect. Dis. 155:377-389.

Levine M M, C Ferreccio, V Prado, M Cayazzo, P Aprego, J Martinez, et al. 1993. Epidemiological studies of *Esherichia coli* diarrheal infections in a low socioeconomic lever peri-urban community in Santiago, Chile. Am. J. Epidemiol. 30:849-869.

Loewenstein M S, A Balows, and E J Gangarosa. Turista at an international congress in Mexico, Lancet 1:529-531.

Makino S, H Kobori, H Asakura, M Watarai, T Shirahata, T Ikeda, et al. 2000. Detection and characterization of Shiga toxin-producing *Escherichia coli* from seagulls. Epidemiol. Infect. 125:55-61.

Mattila L. 1995. Travelers' diarrhea among Finnish tourists. Publications of The National Public Health Institute, KTL A6/1995. 97 p.

Mattila L, A Siitonen, H Kyrönseppä, I Simula and H Peltola. 1995. Risk behaviour for travelers' diarrhea among Finnish travelers. T. Travel Med. 2:77-84.

Mayer H B and C A Wanke. 1995. Enteroaggregative *Escherichia coli* as a possible cause of diarrhoea in an HIV-infected patient. N. Engl. J. Med. 332:273-274.

McCarthy M. 1996. *E. coli* O157:H7 outbreak in USA traced to apple juice. Lancet 348:1299.

McDaniel, T K, K G Jarvis, M S Donnerberg, and J B Kaper. 1995. A genetic locus of the enterocyte effecement conserved among diverse enterobacterial pathogens. Proc. Natl. Acad. Sci. 92:1664-1668.

Mickelsen P A. 1997. The use of molecular strain typing has become a standard practise. Clin. Microbiol. Newsletter. 19:137-144.

Montenegro M, M Bülte, T Trumpf, S Aleksic, G Reuter, E Bulling and R Helmuth. 1990. Detection and characterization of fecal verotoxin-producing *Escherichia coli* from helthy cattle. J. Clin. Microbiol. 28:1417-1421.

Moseley S L, P Echeverria, J Seriwatana, C Tirapat, W Chaicumpa, T Sakuldaipeara, and S Falkow. 1982. Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. J. Infect. Dis. 146:863-869.

Murray B E, J J Mathewson, H L DuPont, and W E Hill. 1987. Utility of oligodeoxyribonucleotide probes for detecting enterotoxigenic *Escherichia coli*. J. Infect. Dis. 155:809-811.

Murray P R, E J Baron, M A Phaller, F C Tenover, and R H YolkeN. 1995. Manual of Clinical Microbiology, Sixth edition. American society for microbiology, Washington, DC. 1482 p.

Nataro J P, M M Baldini, J B Kaper, R E Black, N Bravo, and M M Levine. 1985. Detection of an adherance factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. 152560-565.

Nataro J P, and J B Kaper 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rew. 11:142-201.

National institute of health and infectious diseases control division, Ministry of health and welfare of Japan. 1996. Outbreaks of enterohaemorrhagic *Escherichia coli* O157:H7 infection. 1996. Japan. Infectious Ag. Surveillance Report. 17:180-181.

Neter E, O Westphal, O Lüderitz, R M Gimo, and E A Gorzynski. 1955. Demonstration of antibodies against enteropathogenic *Escherichia coli* in sera of children of various ages. Pediatrics 16:801-808.

Nikaido H. 1996. Outer membrane. In: FC Neidhart et al. (Edt) *Escherichia coli* and *Salmonella*- cellular and molecular biology. vol 1, second edition, p. 29-43. AMS, Washington, D.C. USA.

Olsvik, Ø. and N A Strockbine. 1993. PCR detection of heat-stable, heat-labile, and shiga-like toxin genes in *Escherichia coli*. p. 271-276. In: Persing, D., H., Smith, T., H., Tenover, F. ja White, T.J. (Edt) Diagnostic molecular microbiology-Principles and applications. AMS, Washington DC. USA. 641 p.

Ørskov F. 1976. Agarose electrophoresis combined with second dimensional Cetavlon precipitation. A new method for demonstration of acidic polysaccharide K antigens. Acta Pathol. Microbiol. Scand (B) 84:319-20.

Ørskov F, and I Ørskov. 1984. Serotyping of *Esherichia coli*. In T Bergan (Edt), Methods in Microbiology, vol. 14. Academic Press Inc., Ltd. London, p. 43-112.

Parma A E, M E Sanz, J E Blanco, J Blanco, M R Vinas, M Blanco, et al. 2000. Virulence genotypes and serotypes of verotoxigenic *Esherichia coli* isolated from cattle and foods in Argentina. Eur. J. Epidem. 16:757-762.

Paton A W, P A Manning, M C Woodrow, and J C Paton. 1998. Translocated intimin reseptors (Tir) of Shiga-toxigenic *Escherichia coli* isolates belonging to serogroups O26, O111, and O157 react with sera from patients with Hemolytic-uremic syndrome and exhibit marked sequence heterogeneity. Infect. Immun. 66:5580-5586.

Paton A W, and J C Paton. 1999. Direct detection of Shiga toxigenic Escherichia coli strains belonging to serogroups O111, O157, and O113 by multiplex PCR. J. Clin. Microbiol. 37:3362-3365.

Paunio M, R Peapody, M Keskimäki, M Kokki, P Ruutu, S Oinonen, et al. 1999. Swimming associated outbreak of *Escherichia coli* O157:H7. Infect. Epidem. 122:1-5.

Peltola H, A Siitonen, H Kyrönseppä, I Simula, L Mattila, P Oksanen, et al. 1991. Prevention of travellers' diarrhoae by oral B-subunit/whole-cell cholera vaccine. Lancet 338:1285-1289.

Pierard D, G Muyldermans, L Moriau, D Stevens and S Lauwers. 1998. Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Esherichia coli* isolates. J. Clin. Microbiol. 36:3317-3322.

Pierard D, G Corny, W Proesmans, A Dediste, F Jacobs, J Van de Walle, et al. 1999. Hemolytic uremic syndrome in Belgium: incidence and association with verocytotoxin-producing *Escherichia coli* infection. Clin. Microbial. Infect. 5:16-22.

Pradel N, V Livrelli, C De Champs, J-B Palcoux, A Reynaud, F Scheutz, et al. 2000. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France. J. Clin. Microbiol. 38:1023-1031.

Presterl E, R Nadrchal, D Wolf, M Rotter, and A M Hirschl. 1999. Enteroaggregative and enterotoxigenic *Escherichia coli* among isolates from patients with diarrhea in Austria. Eur. J. Clin. Microbiol. 18:209-212.

Pushker R. 1994. Pathogenesis and laboratory diagnosis of *Esherichia coli*-associated enteritis. Clin. Microbiol. Newsletter 15:89-93.

Ratiner Y. 1989. Serotyping of *Escherichia coli* flagellar antigens. In: G Stain, R Fünfstück, hrsg. Harnwegsinfektion. Aktuelle Gesichtspunkte zur Pathogenese, Diagnostic und Therapie. II. Wissenschaftliches Symposium, Jena, 30. Aug. bis 1. Sept.1989.

Riley L, W Remis, and R S Helgersson. 1983. Hemorrhagic colitis associated with rare *Esherichia coli* Serotype. New Engl. J. Med. 308:681-685.

Robins-Browne R M, M M Levine, B Rowe, and E M Gabriel. 1982. Failure to detect conventional enterotoxins in classical enteropathogenic (serotyped) *Escherichia coli* strains of proven pathogenicity. Infect. Immun. 38: 798-801.

Robins-Browne R M. 1987. Traditional enteropathogenic *Escherichia coli* of infantile diarrhoea. Rew. Infect. Dis. 9:28-53.

Rosa A C P, A T Mariano, A M S Pereira, A Tibana, T A T Gomes, and J R C Andrade. 1998. Enteropathogenicity markers in *Escherichia coli* isolated from infants with acute diarrhoea and healthy controls in Rio de Janeiro, Brazil. J. Med. Microbiol. 47:781-790.

Ryder R W, D A Sack, A Z Kapikian, J C McLaughin, J Chakraborty, A S Mizanur Rahman, et al. 1976. Enterotoxigenic *Escherichia coli* and revirus-like agent in rural Bangladesh. Lancet i:659-663.

Salyers A A and D D Whitt. 1994. *Escherichia coli* gastrointestinal infections (p. 190-204). In Salyers A A and D D Whitt (Ed.) Bacterial pathogenesis: a molecular approach. AMS, Washington DC. USA.

Sanchez J L, A F Trofa, D N Taylor, R A Kuschner, R F DeFraites, S C Craig, et al. 1993. Safety and immunogenicity of the oral, whole cell/recombinant B subunit cholera vaccine in North American volunteers. 167:1446-1449.

Sansonetti P J. 1992. *Escherichia coli, Shigella*, antibiotic-associated diarrhea, and prevention and treatment of gastroenteritis. Curr. Opin. Infect. Dis. 5:66-73.

Savarino S J, A Fasano, D C Robertson, and M M Levine. 1991. Enteroaggregative *Escherichia coli* eleborate a heat-stable enterotoxin demonstrable in an in vitro rabbit intestinal model. J. Clin. Invest. 87:1450-1455.

Savarino S J, A McVeigh, J Watson, A Craviato, J Molina, P Echeverria, et al. 1996. Enteroaggregative *Escherichia coli* heat-stable enterotoxin is not restricted to enteroaggregative *E. coli*. J. Infect. Dis. 173:1019-1022.

Scheutz F. 2000. International *Escherichia* and *Klebsiella* Centre (WHO), Statens Seruminstitut. Copenhagen, Denmark. Personal communication.

Schmidt H, C Knop, S Franke, S Aleksic, J Heeseman, and H Karch. 1995. Development of PCR for screening of enteroaggregative *Escherichia coli*. J. Clin. Microbiol. 33:701-705.

Schroeder S A, J R Caldwell, T Vernon, P C White, S I Granger, and J V Bennett. 1968. A waterborne outbreak of gastoenteritis in adults associated with *Esherichia coli*. Lancet i:737-740.

Schultsz C. 1994. Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. 32:2393-2397.

Schultsz C, J van den Ende, F Cobelens, T Vervoort, A van Gompel, J C F M Westneyn, and J Dankert. 2000. Diarrheagenic *Escherichia coli* and acute and persistent diarrhea in returned travelers. J. Clin. Microbiol. 38:3550-3554.

Siitonen A. 1992. *Escherichia coli* in fecal flora of healthy adults: serotypes, P and type 1C fimbria, Non-P Mannose-resistant adhesins and hemolytic activity. J. Infect. Dis. 166:1058-65.

Sixma T K, K H Kalk, B A van Janten, Z Dauter, J Kingma, B Witholt, and W G Hol. 1993. Refined structure of *Esherichia coli* heat-labile enterotoxin, a close relative of cholera toxin. J. Mol. Biol. 230:890-918.

Smith H R, T Cheasty, and B Rowe. 1997. Enteroaggregative *Escherichia coli* and outbreaks of gastroenteritis in UK. The Lancet 350:814-815.

Stacy-Phipps S J, J Mecca, and J B Weiss. 1995. Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during the course of infection. J. Clin. Microbiol. 33:1054-1059.

Steiner T S, A A M Lima, J P Nataro, and R L Guerrant. 1998. Enteroaggregative *Escherichia coli* produce intestinal inflammation and growth impairment and cause interleukin-8 release from intestinal epithelial cells. J. Infect. Dis. 177:88-96.

Sunabe T, and Y Honma. 1998. Relationship between O-serogroup and presence of pathogenic factor genes in *Escherichia coli.* Microbiol. Immonol. 42:845-849.

Swaminatham B, and G M Matar. 1993. Molecular typing methods. pp. 26-48. In: Persing, D., H., Smith, T., H., Tenover, F. ja White, T.J. (Edt) Diagnostic molecular microbiology-Principles and applications. AMS, Washington DC. USA. 641 p.

Swedish reference group for antibiotics. 1990. Antimicrobial susceptibility testing of bacteria-a reference and methodology manual. The Swedish Medical Society and Statens Bakteriologiska Laboratorium, Stockholm, Sweden.

Svenungsson B, Å Lagergren, E Ekwall, B Evengård, K O Hedlund, A Kärnell, et al. 2000. Enteropathogens in adult patients with diarrhea and healthy control subjects: A 1-year prospective study in a Swedish clinic for infectious diseases. Clin. Inf. Dis. 30:770-778.

Tamura K, R Sakazaki, M Murase, and Y Kosako. 1996. Serotyping and categorisation of *Escherichia coli* strains isolated between 1958 and 1992 from diarrhoeal diseases in Asia. J. Med. Microbiol. 45:353-358.

Tarkka E, H Åhman, and A Siitonen. 1994. Ribotyping as an epidemiologic tool for *Escherichia coli*. Epidemiol. Infect. 112:263-274.

Tarr P I. 1995. *Escherichia coli* o157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin. Infect. Dis. 20:1-10.

Tast E, M Keskimäki, V Myllys, P Suomala, M Saari, A Siitonen and T Honkanen-Buzalski. Vero cytotoxin producing *Escherichia coli* O157:H7 infection in a 6-year-old child associated with consumption of unpasteurised milk. In: Proceedings of World congress on food hygiene. August 24-29, 1997. Hague, Alankomaat. 1997:202.

Taylor D N, P Echeverria, O Sethabutr, C Pitarangi, U Leksomboon, N R Blacklow, et al. 1988. Clinical and microbiologic features of *Shigella* and enteroinvasive *Escherichia coli* infections detected by DNA hybridization. J. Clin. Microbiol. 26:1362-1366.

Tenover F C, R D Arbeit, and R V Goering. 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Molecular Typing Working Group of the Society for Healthcare Epidemiology of America. Infect. Control Hosp. Epidemiol. 18:426-39.

Thomas A, T Cheasty, H Chart, and B Rowe. 1994. Isolation of vero cytotoxin-producing *Escherichia coli* serotypes O9ab:H- and O101:H- carrying VT2 variant gene sequences from a patient with haemolytic uraemic syndrome. Eur. J. Clin. Microbiol. Infect. Dis. 13:1074-1076.

Tornieporth N G, J John, K Salgado, P de Jesus, E Latham, M Clotildes de Melo, et al. 1995. Differentatiation of pathogenic *Escherichia coli* strains in Brazilian children by PCR. J. Clin. Microbiol. 33:1371- 1374.

Tzipori S, F Gunzer, M S Donnenberg, L de Montigny, and J B Kaper. 1995. The role of the *eaeA* gene in diarrhea and neurological complications in gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. Inf. Immun. 63:3621-7.

Vidotto M C, R K T Kobayashi, and A M G Dias. 2000. Unidentified serogroups of enteropathogenic *Escherichia coli* (EPEC) associated with diarrhoea in infants in Londrina, Parana, Brazil. J. Med. Microbiol. 49:823-826.

Viljanen M K, T Peltola, S Y T Junnila, L Olkkonen, H Järvinen, M Kuistila, and P Huovinen. 1990. Outbreak of diarrhoea due to *Esherichia coli* O11:B4 in schoolchildren and adults: association of Vi antigen reactivity. Lancet 336:831-834.

Welinder-Olsson C, E Kjellin, M Badenfors, and B Kaijser. 2000. Improved microbiological techniques using the polymerase chain reaction and the pulsed-field gel electrophoresis for diagnosis and follow-up of enterohaemorrhagic *Escherichia coli* infection. Eur. J. Microbiol. Infect. Dis. 19:843-851.

Wheeler J G, D Sethi, J M Cowden et al. 1999. Study of infectious intestinal disease in England: rates in community, presenting to general practise, and reported to national surveillance. BMJ 318:1046-1050.

WHO (World Health Organization). 1993. Global Health Situation III. WER 6,33.

WHO. 1994. Report of WHO working group meeting on Shiga-like toxin producing *Esherichia coli* (SLTEC), with emphasis on zoonotic aspects. WHO/CDC/VPH/94. 136:1-16. Geneve, Switzerland.

WHO. 1997. Consultation on the prevention and control of enterohaemorrhagic *Escherichia coli.* 28 April-1 May, 1997. Geneve, Switzerland.

Wolf M K. 1997. Occurrence, distribution, and associations of O and H serogroups, colonizatin factor antigens, and toxins of enterotoxigenic *Escherichia coli*. Clin. Microbiol. Rev. 10:569:584.

Yavzori M, N Porath, O Ochana, R Dagan, R Orni-Wasserlauf, and Cohen D. 1998. Detection of enterotoxigenic *Escherichia coli* in stool specimens by polymerase chain reaction. Diagn. Microbiol. Infect. Dis. 31:503-509.

Zhang W L, M Bielaszewska, A Liesegang, H Tschape, H Schmidt, M Bitzan M, and H Karch. 2000. Molecular characteristics and epidemiological significance of Shiga toxin-producing *Escherichia coli* O26 strains. J. Clin. Microbiol. 38:2134-2140.