Escherichia coli and persistent diarrhea
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Chapter 1

Diarrheagenic *Escherichia coli*, an overview

*Enterotoxigenic E. coli* (ETEC) colonizes the small intestine of humans and animals, causing diarrhea. Symptoms include nausea, vomiting, and cramping. Morning sickness in pregnant women is believed to be caused by a toxin produced by *E. coli*.

*Rossellia enterotoxigenica E. coli* (ETEC) colonizes the large intestine of humans and animals, causing diarrhea. Symptoms include abdominal pain, fever, and tenesmus (inability to pass stools).

*Enteropathogenic E. coli* (EPEC) colonizes the small intestine of humans and animals, causing diarrhea. Symptoms include fever, vomiting, and abdominal pain.

*Enterohemorrhagic E. coli* (EHEC) colonizes the small intestine of humans and animals, causing diarrhea. Symptoms include fever, vomiting, and abdominal pain.

*Enteroaggregative E. coli* (EAEC) colonizes the small intestine of humans and animals, causing diarrhea. Symptoms include fever, vomiting, and abdominal pain.

*Enteroadherent E. coli* (EAAEC) colonizes the small intestine of humans and animals, causing diarrhea. Symptoms include fever, vomiting, and abdominal pain.

*Enteroaggregative-reaggregative E. coli* (EAREC) colonizes the small intestine of humans and animals, causing diarrhea. Symptoms include fever, vomiting, and abdominal pain.

These categories are based on the ability of the *E. coli* strains to adhere to the intestinal epithelial cells. The ability to adhere to the intestinal epithelial cells is correlated with the ability of the *E. coli* strains to produce toxins and virulence factors.

Individuals travelling from developed to developing countries are at risk of infection with ETEC. Children and adults living in developing countries generally develop (innocuous) symptoms after exposure to ETEC but may shed ETEC in large numbers in the stool. Individuals travelling from developed to developing countries are at risk of infection with ETEC. Children and adults living in developing countries generally develop (innocuous) symptoms after exposure to ETEC but may shed ETEC in large numbers in the stool. Individuals travelling from developed to developing countries are at risk of infection with ETEC. Children and adults living in developing countries generally develop (innocuous) symptoms after exposure to ETEC but may shed ETEC in large numbers in the stool. Individuals travelling from developed to developing countries are at risk of infection with ETEC. Children and adults living in developing countries generally develop (innocuous) symptoms after exposure to ETEC but may shed ETEC in large numbers in the stool.
Introduction

*Escherichia coli* was first described by Theodore Escherich in 1885 and was initially regarded as a non-pathogenic commensal present in colon flora. The bacterial flora in the large intestine consists mainly of anaerobic bacteria but *E. coli* is considered the most important facultative-anaerobic inhabitant of the colon. *E. coli* is a Gram-negative rod-shaped bacterium. On the basis of antigenic differences in its outer membrane lipopolysaccharides (O-antigen), flagella (H-antigen) and capsule (K-antigen), more than 700 serogroups can be distinguished. Besides commensal strains, which can become potentially harmful in the immunocompromised host or when gastro-intestinal barriers are violated, certain clones of *E. coli* possess virulence properties and cause disease in humans. The main clinical syndromes associated with these pathogenic *E. coli* are urinary tract infections, neonatal meningitis, with or without bacteremia, and diarrhea.

This chapter summarizes the current knowledge about diarrheagenic *E. coli* in humans, with emphasis on the pathogenesis of disease. For additional details of the available data, the reader is referred to the excellent review of the literature by Nataro and Kaper (1). Six major categories of diarrheagenic *E. coli* can be distinguished. These are called enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), entero-aggregative adherent *E. coli* (EAggEC), diffuse adherent *E. coli* (DAEC) and entero-invasive *E. coli* (EIEC). Each category will be addressed in this chapter.

**Enterotoxigenic *Escherichia coli***

Enterotoxigenic *E. coli* (ETEC) causes watery diarrhea, nausea and abdominal cramping. Weaning infants in developing countries and traveler’s to these areas, are particularly prone to infection with ETEC (1). Children and adults living in developing countries generally develop (mucosal) immunity after exposure to ETEC but may shed ETEC in large numbers in the stool (1). Individuals traveling from developed to developing countries are considered immunologically naive and 20-40% of them experience diarrhea due to ETEC in the first week of their stay (2). Occasionally, outbreaks with ETEC also occur in developed countries (3,4). Fecal-oral transmission is the most important route of infection with ETEC and diarrhea generally occurs through ingestion of contaminated food and water.
ETEC strains colonize the small intestine of humans and animals, through production of fimbrial adhesins, named colonization factor antigens (CFA's) (5). A large number of such fimbriae have been characterized, with differences in morphology and variability in structural and accessory proteins, conferring antigenic heterogeneity to ETEC. The antigenic heterogeneity of ETEC is expressed in the differences in susceptibility of certain animals, such as pigs and calves, to various ETEC strains. Epidemiologic studies suggest that the majority of human ETEC strains express CFA/I, CFA/II or CFA/IV (5). The genes encoding CFA's are located on plasmids.

After adherence to the small intestine, ETEC produces either one or both of two distinct classes of enterotoxins, a heat-labile toxin (LT) and a heat-stable toxin (STI)(6). The genes encoding LT and STI are located on the plasmids which also carry the genes encoding CFA's. Certain CFA's are often associated with a certain combination of toxins, suggesting that the combined set of genes arrived on transposons(5). ETEC expressing STI or STI/LT are more often linked to diarrhea than ETEC which express LT only (5).

LT consists of an A subunit and 5 identical B subunits. The B subunits confer binding capacity of the toxin to the intestinal epithelial cell by binding to ganglioside GM1, located in the epithelial cell membrane. After binding, the toxin is endocytosed and the A subunit is delivered to adenylate cyclase at the basolateral membrane of the cell. Activation of adenylate cyclase increases intracellular cyclic AMP levels, which in turn leads to activation of protein kinase A. Activation of protein kinase A results in phosphorylation of chloride channels, in particular the cystic fibrosis transmembrane regulator (CFTR), which results in increased chloride secretion from the crypt cells and inhibition of NaCl absorption by the villus cells. The increased luminal ion content promotes osmotic diarrhea through passive paracellular secretion of water (6).

ETEC LT shows high homology with cholera toxin (CT) (80% identity in protein sequence)(7-9). The effects of LT on the intestinal epithelial cell are also very similar to CT. There is considerable evidence, that additional responses to the ones described above, add to the secretory effect of CT, such as activation of arachidonic acid metabolites such as prostaglandins, stimulation of the enteric nervous system mediated by serotonin and vasoactive intestinal peptide, and stimulation of IL-6 production. Whether all of these responses also occur with LT remains to be determined (6).

There are two variants of the human ETEC heat-stable toxin, STIa and STIb (STII is mainly found in ETEC from pigs). These variants show high nucleotide sequence homology
STI is a small protein which uses guanylate cyclase C (GC-C) as receptor for binding to the epithelial cell. GC-C is located in the apical membrane and has an extracellular binding domain and an intracellular domain which shows enzymatic activity. The hormone guanylin, which is involved in normal gut homeostasis, is considered to be the physiological agonist of GC-C. Binding to GC-C leads to increased intracellular cyclic GMP, which ultimately leads to activation of the CFTR, similar to LT. However, STI acts much faster than LT since it does not require endocytosis and translocation to the basolateral membrane for activation of cellular proteins. A role for serotonin has been suggested as an additional mediator of STI activated secretion (6).

Watery diarrhea of short duration is the hallmark of ETEC infection. This clinical syndrome can entirely be explained by the pathogenic mechanisms described above. The immunity observed after infection with ETEC has led to the development of oral vaccines, including vaccines which make use of the similarities between LT and CT (11,12). However, the development of a vaccine with broad protection is complicated by the antigenic variability of ETEC, in particular of the CFA’s.

Both LT and CT show strong activity as a mucosal adjuvant which has resulted in the use of mutant toxins, which retained their adjuvant activity, for mucosal delivery of various mucosal vaccines, such as tetanustoxoid (1,13).

ETEC used to be detected by demonstration of LT and/or STI activity in animal models or in cell culture (14,15). Assays have become available for detection of LT and ST in clinical isolates, using antibodies against the toxin (1). In addition, several methods have now been described for detection of the genes encoding LT and STI, including polynucleotide probes, oligonucleotide probes and PCR (16-18). The detection of CFA’s, for example with immunological assays, is impractical due to their great number and heterogeneity.

**Enteropathogenic Escherichia coli**

Enteropathogenic *Escherichia coli* (EPEC) is an important cause of childhood diarrhea in developing countries. EPEC primarily causes disease in infants younger than 2 years. In older children and adults in developing countries, EPEC can be isolated from both healthy and ill individuals. Whether the low incidence of EPEC diarrhea in older children and adults is due to acquired immunity or decreased inherent susceptibility is not known. Recent studies in volunteers suggest that after infection, only immunity to homologous O-serotypes of EPEC...
strains develops (19). Despite the high incidence of EPEC infection in countries where ETEC infections are also common, EPEC has never been implicated as a cause of traveler’s diarrhea (20,21). EPEC infection in developed countries is generally related to outbreaks among children in hospitals or day-care centres. Outbreaks of diarrhea in healthy adults due to EPEC have also been reported but appear to be rare (1). EPEC causes acute watery diarrhea, often associated with vomiting and low-grade fever, but cases of persistent diarrhea due to EPEC have also been described.

The main characteristic of infections due to EPEC is the development of histological abnormalities, called attaching-effacing (A/E) lesions, which can be observed in small intestinal biopsies and can be reproduced in cell culture (22,23). A/E lesions are characterized by intimate adherence of bacteria to the epithelial cell membrane and effacement of microvilli. Below the area of intimate adherence, cytoskeletal changes occur in the epithelial cell to which the bacteria adhere. These changes include accumulation of polymerized actin and formation of pedestal structures extending from the epithelial cell, on which the bacteria appear to reside. The area of the epithelial cell containing polymerized filamentous actin can be detected using fluorescein isothiocyanate (FITC)-labeled phalloidin, a mushroom toxin, which binds specifically to polymerized filamentous actin (24).

In the last decade a vast amount of research on EPEC has resulted in remarkable insight in the pathogenesis of EPEC diarrhea, reviewed in reference (1). This has led to the development of a three stage model of pathogenesis which is summarized below. These three stages include localized adherence, which is followed by signal transduction, leading to the final stage, intimate adherence. 

Localized adherence is considered to be the first step in EPEC pathogenesis. Localized adherence can be observed when EPEC strains are co-incubated with cells from the continuous cell line Hep-2 and with cultured small intestinal cells. The bacteria adhere to the epithelial cells forming three-dimensional micro-colonies (figure). Localized adherence to Hep-2 cells is most likely mediated by fimbriae which tend to aggregate and form bundles, called the “bundle-forming pilus” (BFP). A cluster of 13 genes, in addition to the structural gene bfpA, located on a 60 MDa plasmid, is required for the expression and assembly of BFP (25). This plasmid is called the EPEC adherence factor (EAF) plasmid. Regulation of expression and assembly of BFP requires the regulatory element Per (plasmid encoded regulator) which is also located on the EAF plasmid. However, a recent study suggests that the BFP is not required for localized adherence to cultured intestinal tissue from children, in contrast to
Adherence patterns of diarrheagenic *Escherichia coli* to HEp-2 cells

A: Localized adherence

B: Diffuse adherence

C: Aggregative adherence

D: No adherence
adherence to the Hep-2 cells. In this study, the BFP was shown to enhance the three-dimensional micro-colony formation after intimate attachment and A/E-lesion formation were achieved (26). In addition, EPEC has been isolated which showed localized adherence to HEp-2 cells but which did not contain the EAF plasmid. It is suggested that the initial non-intimate adherence to the host cell surface is mediated by another, as yet unknown adhesin(s) or by intimin (see below). Recent studies in volunteers however, showed that the BFP is required for full virulence of EPEC (27).

Signal transduction is considered the second step in EPEC pathogenesis. A 35-kb pathogenicity island, called the locus of enterocyte effacement (LEE), located on the chromosome, encodes the genes which are responsible for signal transduction activity. At least four proteins are secreted extracellularly by EPEC. Three of these, called EPEC secreted protein (Esp) A, EspB and EspD are essential for formation of A/E lesions and are all located on the LEE (28). The function of the fourth protein, EspC is not clear (29). The EspA, EspB and EspD proteins are secreted by a type III protein secretion system, for which the genes are also located on the LEE. Type III protein secretion systems can be found in various Gram-negative micro-organisms and are responsible for the transport of proteins from the cytoplasm, across the periplasmic space, to the external environment (30).

The effects of signal transduction induced by the secreted proteins result in a number of changes in the epithelial cells to which EPEC adheres. These changes have been studied in various cell culture systems, including HEp-2 cells, HeLa cells, T84 cells and Caco-2 cells and in human intestinal tissue culture. It has long been assumed that increases in intracellular calcium levels are important for intestinal secretory responses to EPEC, resulting in inhibition of Na⁺ and Cl⁻ absorption and enhanced Cl⁻ secretion. However, a recent study using sensitive methods for measuring intracellular calcium levels, failed to show any change in intracellular calcium levels after infection of HEp-2 cells (31). EPEC genes encoding EspA, EspB and EspD are essential for modulation of Caco-2 cell electrolyte transport, including stimulation of chloride secretion, which is observed after infection with EPEC (32). In addition to secretion of proteins, adherence of EPEC results in the phosphorylation of several epithelial proteins, possibly resulting in changes in electrolyt secretion and in changes in permeability of tight junctions. Furthermore, a decrease in the transepithelial resistance of cell culture monolayers and migration of polymorphonuclear leukocytes (PMNs) across intestinal epithelial cell monolayers have been described (33). Finally, binding of EPEC activates the eukaryotic
transcription factor NF-κB, which upregulates expression of IL-8, a potent chemo-attractant of PMNs (34).

Addition of purified preparations of EspA, EspB or EspD to epithelial cells in culture, has no effect on signal transduction. Delivery of the proteins by an attached EPEC appears to be required for signal transduction activity.

**Intimate adherence** is the third step in EPEC pathogenesis and is mediated by a 94- to 97-kDa outer membrane protein called intimin. The gene encoding intimin (eae) is located on the LEE and is also under regulatory control of the Per locus. The eae gene is also found in enterohemorrhagic *E. coli*, *E. coli* strain RDEC-1, which is pathogenic to rabbits, *Hafnia alvei* and *Citrobacter rodentium*. The N-terminal region of the gene is conserved while the C-terminal region shows variability among EPEC strains and the other bacteria which contain the eae gene (35). Intimin from EPEC binds to a 90k-Da protein, called Hp90, which only functions as a receptor after tyrosin-phosphorylation. Recently, it has been shown that Hp90 is of bacterial origin (36). EPEC secretes Hp90 in its non-phosphorylated form, after which it is inserted into the epithelial cell membrane. After tyrosine-phosphorylation, it serves as a receptor for intimin, thereby mediating intimate adherence of the bacteria. Hp90 has therefore been renamed Tir (Translocated intimin receptor) (36). The delivery of Tir to the host cell is dependent on the presence of the type III secretion system and EspA and EspB.

The considerable insight gained into EPEC pathogenesis, suggests several possible mechanisms by which EPEC infection may result in diarrhea. These include active chloride secretion, which may account for the rapid onset of diarrhea observed in volunteers after ingestion of EPEC, malabsorption due to loss of microvilli, local inflammation due to the influx of PMNs, and increased intestinal permeability. A combination of these mechanisms is most likely to be involved in EPEC diarrhea.

In early days, EPEC was detected by O- and H-serotyping, since *E. coli* of specific serotypes were associated with diarrhea. Serotyping is laborious and can only be performed in reference laboratories. In addition, it has become clear that different categories of diarrheagenic *E. coli* may occur within a certain serotype. Nowadays, detection of EPEC in clinical samples is therefore performed using tests for detection of EPEC virulence factors. For detection of virulence associated localized adherence to HEp-2 cells, associated with the presence of the EAF plasmid, cell culture adherence assays are used (37). The presence of the EAF plasmid itself can be detected using a 1-kb fragment probe, obtained from a region of the plasmid of which the function is unknown (38). Oligonucleotide probes and PCR primers have
also been developed for detection of the EAF plasmid. In addition, a fragment probe, oligonucleotide probes and PCR have been used for detection of \textit{bfpA}, the structural gene which encodes the BFP and which is located on the EAF plasmid (39, 40).

Detection of A/E lesion formation can be done using phenotypic or genotypic tests. The A/E phenotype can be identified using the previously mentioned fluorescein isothiocyanate (FITC)-labeled phalloidin which detects polymerized filamentous actin inside the epithelial cell (FAS-test) (24). The presence of the \textit{eae} gene, which encodes intimin, can be detected using a 1-kb fragment DNA-probe which has shown high sensitivity and specificity and an excellent correlation with the FAS-test (41). Primers have been developed, which are used in PCR.

Typical EPEC are considered to show the localized adherence and the A/E phenotypes and to contain both the EAF plasmid and the LEE pathogenicity island which contains the \textit{eae} gene. However, EPEC which did not contain the EAF plasmid have been associated with outbreaks and sporadic cases of diarrhea (42) and it is possible that EPEC loses the EAF plasmid spontaneously. Therefore strains lacking the EAF plasmid which are \textit{eae}-positive are called atypical EPEC and may be pathogenic.

\textbf{Enterohemorrhagic \textit{Escherichia coli}}

Enterohemorrhagic \textit{E. coli} (EHEC) was first recognized as a distinct class of pathogenic \textit{E. coli} in 1983 after an outbreak of hemorrhagic colitis associated with the rare \textit{E. coli} serotype O157:H7. Subsequently, hemorrhagic colitis due to \textit{E. coli} O157:H7 was shown to be associated with the development of hemolytic uremic syndrome (HUS), possibly due to the bacterial production of a cytotoxin, called Verocytotoxin. EHEC is now an important pathogen in the USA, Canada, Europe and Japan and has been shown to comprise additional serotypes besides O157:H7 (43). Surprisingly, in developing countries EHEC is much less frequently isolated than other diarrheagenic \textit{E. coli}, such as EPEC or ETEC. In contrast to the other types of diarrheagenic \textit{E. coli}, EHEC is mainly transmitted through ingestion of contaminated undercooked food, particularly of bovine origin. Large outbreaks of EHEC have been described, often related to the ingestion of undercooked hamburgers obtained in fast-food chains. However, outbreaks associated with foods which are contaminated through water have also been described (44). Cattle is considered the main reservoir of EHEC (45). Person to person transmission is also observed, possibly due to the very low infectious dose required for disease (45). Infection occurs in all age groups, and can result in a spectrum of disease ranging
from asymptomatic carriage to severe hemorrhagic colitis. The incidence of HUS is particularly high in children and the elderly. In contrast to ETEC and EPEC, EHEC primarily colonizes the large intestine.

The major virulence factor of EHEC is the production of a cytotoxin, previously called Verocytotoxin, but presently named Shiga toxin (Stx). Two distinct groups of Stxs can be distinguished, which are immunologically not cross-reactive. Stx1 is identical to Shiga toxin from *Shigella dysenteriae* type 1. Stx2 shows sequence variation, which has resulted in the description of different variants, e.g. Stx2c, Stx2vhb or Stx2e. One EHEC strain may express Stx1 only, Stx2 only, or both, or different forms of Stx2. All types of Stx consist of an A subunit which is linked to a pentamer of identical B subunits. The B subunit binds to the eukaryotic cell surface receptor, glycolipid Gb3, while the A subunit contains the toxic activity. After endocytosis of the bound toxin, this A subunit is translocated to the cytoplasm, where it acts on the 60S ribosomal subunit, resulting in impaired protein synthesis. The Stx's of EHEC are encoded by bacteriophages.

EHEC shows some similarities with EPEC. In particular, the majority of EHEC strains contain the LEE pathogenicity island described for EPEC (46). Both the intimin and the Tir sequences located on the LEE show sequence heterogeneity, resulting in antigenic variation of both intimin and its receptor (47). This variation could account for the differences in colonization patterns observed between EPEC and EHEC. In addition, differences have been observed between the cellular response to EPEC and the response to EHEC, such as the failure of EHEC to induce tyrosine phosphorylation of epithelial cell proteins (48).

The majority of human EHEC strains, including all strains of serotype O157:H7, contain a 60-MDa plasmid which encodes a hemolysin, called enterohemolysin, of which the role in pathogenesis is unknown. In addition, the plasmid may encode adherence factors for EHEC, which have, however, not yet been characterized. EHEC O157:H7 contains an iron transport system which allows the bacteria to use heme or hemoglobin as an iron source. In addition, EHEC O157:H7 produces an extracellular serine protease, called EspP, which cleaves human coagulation factor V (49). These factors may play a role in the survival of EHEC O157:H7 in the intestine during disease.

It is likely that not all virulence factors, essential for EHEC to cause disease, have been identified. However, the LEE locus, including the *eae* gene encoding intimin, appears important for non-bloody diarrhea, while the production of Stx seems essential for the development of hemorrhagic colitis and HUS. It is suggested that Stx translocates from the
damaged large intestine to the blood and then exerts its effect on endothelial cells which contain the Gb3-receptor, such as renal endothelial cells, and on tubular epithelial cells (50).

A diagnosis of EHEC infection can be made in various ways. Sorbitol MacConkey agar is used for detection of EHEC O157:H7 because the large majority of EHEC O157:H7 strains does not ferment D-sorbitol rapidly. The suspected pink colonies should be biochemically identified as *E. coli* and screened for the O157 LPS using specific antisera. Several adaptations have been applied to improve sensitivity and specificity of this culture method. EHEC strains O157:H- strains, associated with HUS, were shown to ferment D-sorbitol on this agar (51).

Several commercially available methods used for detection of EHEC rely on direct detection of the O157 antigen in stool samples, using antisera against the O157 LPS, some of which include an immunomagnetic separation step (52). These methods cannot be used for detection of other serotypes of EHEC, in contrast to cell culture assays or immunological assays which detect Stx's in stool samples (52). DNA probes and PCR have been developed for detection of the genes encoding Stx's (53-55). It should be realized that upon subculture, loss of stx genes may occur. The probe and primers which are used for detection of the conserved region of the eae gene of EPEC can also be used for detection of this gene in EHEC. In addition, a fragment probe and primers have been developed, which detect the presence of the 60 MDa plasmid, containing the genes encoding the enterohemolysin(55). While detection of genes encoding Stx in clinical samples seems sufficient for diagnosis, the presence of additional virulence factors is required for significance of isolates in food or non-clinical samples, in particular when non-O157 strains are concerned. Finally, serum and salivary antibodies against O157 have been determined in patients with HUS (56,57). However, pure LPS for use as antigen is difficult to prepare and such assays cannot be used for detection of antibodies against other EHEC serotypes.

**Enteroaggregative Escherichia coli**

Enteroaggregative *E. coli* (EAaggEC) were shown to be a cause of diarrhea after the development of the HEp-2 adherence test. Epidemiological studies in developing countries revealed that *E. coli* strains which adhered in a characteristic “stacked-brick” configuration to HEp-2 cells, were associated with diarrhea in children (figure). These strains were not of EPEC serotypes and did not possess other known virulence factors associated with other types of diarrheagenic *E. coli* (58). Additional studies supported an association with persistent
diarrhea in certain geographic areas (59,60). In addition, an association with diarrhea in travelers has been suggested (61). EAggEC are probably also a cause of sporadic cases of acute and persistent diarrhea in Europe (62,63) and are sometimes outbreak related (64).

EAggEC infection causes watery to mucoid and sometimes bloody diarrhea, without obvious fever. EAggEC were shown to induce mucus production in tissue culture models, possibly explaining the often mucoid aspect of diarrhea (65).

The aggregative adherence pattern is attributed to the presence of fimbriae, of which two distinct types have been characterized, called AAF/I and AAF/II (66,67). These fimbriae show some genetic similarities with the members of the so-called Dr family of adhesins, which mediate adherence to the Dr blood group antigen. The genes encoding the AAF fimbriae are located on a 60-65 MDa plasmid. This 60MDa plasmid also carries the genes encoding a heat stable toxin, called EAST1 (68), which shows some amino acid homology with ST of ETEC. Other categories of diarrheagenic E. coli carry these toxin genes as well (69,70) and its role in the pathogenesis of diarrhea is undetermined. In addition, a second heat labile toxin, called plasmid encoded toxin (Pet), has been characterized. This toxin shows some sequence similarities with EspC from EPEC and EspP from EHEC and has protease activity (71). These proteins are all called autotransporter proteins because the protein gene includes sequences which encode secretion of the protein into the periplasmic space and subsequent pore formation, allowing excretion through the outer membrane (so-called type IV secretion system). Both EAST1 and Pet have shown enterotoxic activity in in vitro models (72,73). In addition, Pet showed cytotoxic activity (73).

A three stage model of EAggEC pathogenesis has been proposed, based on the available experimental data. Stage I involves adherence to the intestinal mucosa, mediated by fimbral adhesins such as AAF/I and AAF/II. Stage II involves enhanced host mucus production, which may promote persistent colonization. Stage III includes the production and secretion of enterotoxins and cytotoxins resulting in damage to the intestinal cells. In addition, the release of inflammatory mediators is stimulated, which may contribute to intestinal inflammation (74).

It should be mentioned that the EAggEC virulence factors described above, are found in only a portion of E. coli strains which show aggregative adherence to HEp-2 cells. Additional virulence factors still need to be characterized. For example, α-hemolysin production (75) and Shiga toxin production (76) have been suggested as potential virulence factors of EAggEC. However, it is likely that EAggEC forms a heterogenous group of E. coli
of which only a subset is pathogenic to humans. This suggestion is supported by volunteer studies (77).

EAggEC can be detected using the HEp-2 adherence test which is considered the gold standard. A 1-kb DNA probe has been selected from one of the prototype EAggEC strains (78). This probe represents a part of the 60 MDa plasmid of which the function is unknown. Primers for PCR were derived from the probe sequence (79). Probe and PCR show similar sensitivity and specificity (79).

**Diffusely adherent *Escherichia coli***

Diffusely adherent *Escherichia coli* (DAEC) comprises a category of potentially pathogenic *E. coli* which show diffuse adherence to HEp-2 cells (figure). The epidemiology of DAEC is unclear; a relation with diarrhea has not been confirmed in all studies (80-83). DAEC may cause diarrhea in children in developing countries. DAEC have also been described as a cause of diarrhea in hospitalized patients in Europe (84-86). Due to the epidemiological inconsistencies, the clinical picture associated with DAEC infection remains unclear (87).

The diffuse adherence phenotype is probably mediated by fimbriae, designated F1845, of which the genes are located either on the bacterial chromosome or on a plasmid. F1845 belongs to the Dr-family of adhesins (88). In a minority of strains, a plasmid encoded 100-kDa outer membrane protein is associated with the diffuse adherence phenotype, called AIDA-I (89). In addition, a plasmid encoded 57-kDa outer membrane protein has been identified (90). Recently, a set of DAEC were shown to secrete proteins with high amino acid homology to EspA, EspB and EspD of EPEC (91). These strains also hybridized with a probe for detection of *eae*, the gene encoding intimin. In adherence assays, these strains induced some accumulation of actin in the FAS-test and induced tyrosine-phosphorylation of epithelial proteins, leading to pedestal formation or extended surface structures. However, the distribution of these potential DAEC virulence factors among other DAEC and their association with diarrhea are unclear.

DAEC are detected by the diffuse adherence pattern in the HEp-2 adherence assay. A 700-bp DNA probe fragment, derived from the *daaC* gene, which is required for expression of the F1845 fimbriae, can be used for detection of DAEC (92). This probe shows cross-reactivity with related genes from other members of the Dr-family, which are primarily associated with uropathogenic *E. coli*. 
Enteroinvasive *Escherichia coli*

Enteroinvasive *Escherichia coli* (EIEC) is an uncommon cause of diarrhea and is mostly described in relation to outbreaks (93). The incidence of EIEC in developing countries is probably higher than in developed countries (94). EIEC are closely related to *Shigella* sp., strains are generally lysine decarboxylase negative, non-motile and do not ferment lactose. Despite these similarities, EIEC rarely causes a dysenteric syndrome but instead presents most commonly as watery diarrhea (1).

The pathogenesis of EIEC is considered to be virtually identically to the pathogenesis of *Shigella*. Like *Shigella* spp., EIEC possess a large plasmid which carries the genes required for invasiveness, designated plnv. Multiple proteins required for full pathogenicity (Ipa A to IpaD) are secreted by a type III secretion system, the genes of which are located on plnv. After invasion of the intestinal epithelial cell, *Shigella* move within the cytoplasm by formation of an actin tail which extends from one pole of the bacterium, propelling the bacterium in lateral direction through the cytoplasm and thus allowing the bacterium to infect adjacent cells. Regulation of virulence genes in *Shigella* spp. requires both chromosomal and plasmid mediated complex regulatory systems.

A protein, encoded on an EIEC plasmid, has been characterized which could explain the watery diarrhea observed after EIEC infection. This protein has enterotoxic activity in *in vitro* experiments. Why EIEC only rarely cause dysentery remains unclear. EIEC may be misidentified as *Shigella* or non-pathogenic *E. coli* strains, in cases with dysentery.

EIEC can be detected phenotypically in the Sereny test which is the classical test for demonstrating invasiveness of *Shigella* spp. Invasiveness correlates with the ability of the bacteria to cause keratoconjunctivitis after inoculation in the guinea pig eye. Polynucleotide probes and PCR primers have been described for detection of genes from plnv (95,96).

**Identification of diarrheagenic *Escherichia coli* in stool samples**

Identification of diarrheagenic *E. coli* requires differentiation of pathogenic strains from non-pathogenic strains. Before specific virulence factors could be identified and characterized, strains were differentiated on the basis of their serotype, since specific serotypes are associated with clinical disease. The serotype can be regarded as a chromosomal marker that correlates
with certain virulent clones (97). However, serotyping is a laborious and difficult technique, with low sensitivity and specificity. Therefore, detection methods have focused on detection of virulence factors, using either phenotypic or genotypic methods.

A number of _E. coli_-like colonies, grown from a stool sample inoculated on selective agar-plates, are isolated, identified biochemically as _E. coli_, and tested in phenotypic or genotypic assays. The sensitivity of the assay is influenced by the number of colonies tested. Generally, three to five colonies are considered sufficient in case of symptomatic diarrhea.

The HEp-2 adherence test is the most important phenotypic test which is still used for diagnosis of diarrhea caused by localized, aggregative and diffuse adherent _E. coli_. Monolayers of HEp-2 cells, grown on glass coverslips, are incubated with an overnight culture of the bacteria for 3-hours. The cells are then washed to remove non-adherent bacteria, fixed and stained, and the adherence pattern of the bacteria is determined under light-microscopy (figure). A second incubation step for an additional 3 hours has been included by some investigators. This 6-hour assay has shown to improve detection of the aggregative adherence phenotype (37).

A number of polynucleotide or oligonucleotide DNA-probes and PCR primers have been described for detection of genes encoding specific virulence factors, such as adherence, toxin production and invasiveness. These probes and primers are mentioned in the sections describing each category of diarrheagenic _E. coli_. Genotypic assays have not only been applied to isolated colonies, but also to blots of stool samples (98) or, in case of PCR, to DNA isolated directly from stool samples. However, identification of diarrheagenic _E. coli_ using genotypic methods requires verification of phenotypic expression of the identified virulence factor, in particular when the adherence to HEp-2 cells is concerned. Verification of phenotypic expression requires isolation of single colonies.

**Treatment of infection with diarrheagenic _Escherichia coli_**

Diarrhea due to _E. coli_ is a self-limiting disease. Rehydration is the mainstay of treatment in case of severe diarrhea. Antibiotic treatment of ETEC and EPEC diarrhea has been shown to decrease duration of disease and shedding of bacteria (99). However, antibiotic resistance is common among all categories of _E. coli_ (60,100,101). Controversy exists about the use of antibiotics in case of EHEC infection. It has been suggested that antibiotic treatment of EHEC enhances release of Shiga toxin and promotes absorption of Shiga toxin by killing
other colonic bacteria, while others reported a decreased incidence of HUS in patients who were treated with antibiotics early in the course of disease.

Antimotility agents can be used in case of diarrhea without visible blood or mucus. Antimotility agents have been shown to increase the risk for development of HUS during EHEC infection and should be avoided when such infection is suspected or proven.

**Prevention of infection with diarrheagenic *Escherichia coli***

Studies on the prevention of infection with diarrheagenic *E. coli* have been focused on ETEC and EHEC. Prevention of ETEC diarrhea by antimicrobial prophylaxis is commonly used by travelers to endemic areas. Although antimicrobial prophylaxis by travelers is not generally recommended, particularly because of possible side-effects and the development of antimicrobial resistance opposed against a relatively low disease burden, certain groups of patients, for example those on immunosuppressive therapy, may benefit (102). The oral prophylactic drugs used to prevent traveler’s diarrhea in adults vary from bismuth subsalicylate to trimethoprim-sulfamethoxazole, doxycycline or fluoroquinolones, depending on the prevalence of resistant strains at the travel destination (102).

The high incidence of disease due to ETEC in infants and children in developing countries and the importance of ETEC as a cause of traveler’s diarrhea stressed the need for the development of an effective vaccine against ETEC. An oral vaccine seems the most appropriate approach since oral vaccination is the most efficient way of eliciting mucosal immunity. A variety of approaches has been used for the development of effective vaccines, including the use of whole killed cells, toxoids, and purified fimbriae (103). Advantage has been taken of the antigenic similarity between ETEC LT and cholera toxin, by applying the extensively tested oral B-subunit/whole-cell cholera toxin vaccine for the prevention of traveler’s diarrhea (11). This vaccine showed partial protection against diarrhea in Finnish travelers to Morocco, particularly in case of mixed infection (11). None of the candidate ETEC vaccines has been tested in large field trials in children in developed countries but an oral killed ETEC plus cholera toxin B-subunit vaccine was recently shown to be safe and immunogenic in adult volunteers and in children in Egypt (12,104). The development of ETEC vaccines with broad protection is greatly hampered by the antigenic variability of ETEC adhesins and by the fact that purified native ST is not immunogenic (5).
The large outbreaks caused by EHEC O157:H7 demanded the development of an effective vaccine against EHEC. The development of such vaccine is still in an experimental stage but Stx is considered a crucial component of a candidate vaccin, in addition to intestinal colonization factors (1).
<table>
<thead>
<tr>
<th>Category of E. coli</th>
<th>Clinical manifestations</th>
<th>Virulence factors</th>
<th>Predominant serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ETEC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enteropathogenic</td>
<td>Acute gastro-enteritis</td>
<td>Entero-adherence factor, Bundle forming pilus, Locus of enterocyte effacement (LEE)</td>
<td>O55:H6/NM; O86:H34/NM; O111:H2/12/NM; O119:H6; O126:H27/NM; O127:H6; O128:H2/12; O142:H6</td>
</tr>
<tr>
<td>(EPEC)</td>
<td></td>
<td>Intimin, Translocated intimin receptor (Tir)</td>
<td></td>
</tr>
<tr>
<td>Enterohemorrhagic</td>
<td>Acute diarrhea, hemorrhagic colitis, hemolytic uremic syndrome</td>
<td>Shigatoxin 1 (Stx1), Stx2, LEE, intimin, Tir</td>
<td>O26:H11/32/NM; O55:H7; O111:H8/NM; O113:H21; O117:H14; O157:H7</td>
</tr>
<tr>
<td>(EHEC)</td>
<td></td>
<td>hemolysin, EHEC-plasmid</td>
<td></td>
</tr>
<tr>
<td>Enteroaggregative</td>
<td>Acute and persistent diarrhea</td>
<td>EAegEC-plasmid, Entero-aggregative heat-stable toxin 1</td>
<td>O3:H2; O15:H18; O44:H18; O77:H18; O86:NM; O111:H21; O127:H2; ONT:H10</td>
</tr>
<tr>
<td>(EAegEC)</td>
<td></td>
<td>Plasmid encoded heat-labile toxin</td>
<td></td>
</tr>
<tr>
<td>Diffuse adhering (DAEC)</td>
<td>Undetermined</td>
<td>Fimbia</td>
<td>Not available</td>
</tr>
<tr>
<td>Entero-invasive (EIEC)</td>
<td>Acute watery diarrhea</td>
<td>Invasiveness-plasmid</td>
<td>O29/NM; O112:HNM; O124:H30; O124:NM; O136:NM; O143:NM O144:NM; O152:NM; O159:H2/NM; O164:NM; O167:H4/5/NM</td>
</tr>
</tbody>
</table>
Scope of the thesis

The studies described in this thesis were performed to investigate the role of diarrheagenic *Escherichia coli* in persistent diarrhea. Although diarrheagenic *E. coli* are increasingly recognized as an important cause of persistent diarrhea in developing countries, their role in the etiology of persistent diarrhea in the developed world is still unclear. The currently available data on the role of diarrheagenic *E. coli* in persistent diarrhea are reviewed in chapter 2.

The studies presented are particularly focused on three patient groups:
The first group concerns travelers who have returned with persistent diarrhea from (sub)tropical areas where diarrheagenic *E. coli* are endemic. Methods were developed for the detection of enterotoxigenic *E. coli*, the main cause of traveler's diarrhea, which are described in chapter 3. The association of different categories of diarrheagenic *E. coli* with acute and persistent diarrhea in these returned travelers is described in chapter 4. To search for possible virulence factors associated with diarrhea in travelers with persistent diarrhea, the isolated diarrheagenic *E. coli* strains were further characterized with respect to their potential to induce interleukine 8 release in epithelial cells, of which the results are described in chapter 5.

The second patient group consists of patients with inflammatory bowel disease (IBD). The occurrence and persistence of diarrheagenic *E. coli* in the large intestine of patients with ulcerative colitis and Crohn's disease, in relation to disease activity, was investigated and the results are presented in chapter 6. In order to further clarify the potential role of the intestinal flora, not limited to *E. coli*, for IBD disease activity, additional studies were performed to further locate the intestinal bacteria in the intestinal mucosa, using in situ hybridization. This study is presented in chapter 7.

Human immunodeficiency virus type 1 (HIV) infected patients formed the third patient study group. Results of a study investigating the association of the presence of diarrheagenic *E. coli* with diarrhea in these patients are presented in chapter 8.

In the final chapter, the results presented in the previous chapters are placed together in a wider context, to form a general discussion.
Reference List


72. Savarino SJ, Fasano A, Watson J, Martin BM, Levine MM, Guandalini S, Guerry P. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1...


