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Enterohemorrhagic *Escherichia coli* (EHEC)

Microbiological Diagnosis, Characterisation and Clinical Bacteriological Aspects

Christina Welinder Olsson

Göteborg University

Enterohemorrhagic *Escherichia coli*, EHEC

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Opponent: Professor Lars Engstrand, Smittskyddsinstitutet/Karolinska Institutet, Solna

GÖTEBORG UNIVERSITY

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Enterohemorrhagic *Escherichia coli* (EHEC) constitutes a group of *E. coli* that in the past two decades has been the cause of several outbreaks of gastrointestinal disease. The microorganism causes hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in 5-10% of the patients, 30% of the HUS patients develop remaining kidney injuries. The capacity to control EHEC disease and to limit the scale of outbreaks is dependent upon prompt diagnosis and identification of the source of infection.

Cattle are the principal reservoir of EHEC and foodstuffs, which presumably have come into contact with domestic animal manure and/or are inadequately pasteurised, are important vehicles of infection.

The predominating virulence factor of EHEC is the verocytotoxin first identified in an *E. coli* of serogroup O157:H7. In the present study, the PCR technique with primers detecting the verocytotoxin genes was shown to be a fast, sensitive and specific method to screen for and identify EHEC. The study contributes to the knowledge and understanding that also EHEC non-O157 are pathogenic and that all verocytotoxin producing *E. coli* should be looked after among patients with diarrhoea, HC and HUS.

The importance of quick identification was exemplified in an outbreak of EHEC among the staff at the children's hospital in Göteborg, Sweden. As the outbreak was promptly identified, partly thanks to the PCR technique, no further spread to other staff members or patients occurred.

In the present thesis, pulsed-field gel electrophoresis (PFGE) was used and the method was shown to be very useful in the epidemiological work to separate single cases of EHEC from outbreaks.

In order to further characterise EHEC non-O157, the presence of additional chromosomal and plasmid genes probably involved in the pathogenesis were analysed as these genes have been found in EHEC O157. The gene coding for intimin, enabling tight attachment to epithelial cells of the intestine was also often present among EHEC non-O157. Moreover, the gene coding for an enterohemolysin possibly supporting the bacterial need for iron was especially often present among EHEC isolated from patients with severe symptoms.

Further, the study has shown that the gene coding for aerobactin seems to be able to compensate for the absence of enterohemolysin and that also the type 1 fimbriae may be valuable to obtain a niche in the gut.

A comparative study of verocytotoxin producing *E. coli* in cattle showed that there is a significantly lower amount of *E. coli* harbouring the intimin gene and the gene coding for aerobactin, supporting the assumption that not all verocytotoxin producing *E. coli* in cattle are pathogenic.

In conclusion, methods identifying the verocytotoxines or their genes should be used to identify EHEC of all serogroups. PCR is preferable as it is a fast, sensitive and specific method. Genes coding for intimin, enterohemolysin and aerobactin adds to the pathogenicity of EHEC. These genes are not that common among isolates from cattle. PFGE is a most valuable tool for epidemiological tracing of EHEC.

Key words: *Escherichia coli*, EHEC, VTEC, verocytotoxin, PFGE, EHEC-hemolysin, aerobactin, diarrhoea, HUS

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ABBREVIATIONS

A/E	attaching and effacing
bp	base pair
CFU	colony forming unit
CNS	central nervous system
CNF 1	cytotoxic necrotizing factor type 1
EAEC	enteroaggregative *Escherichia coli*
EHEC	enterohemorrhagic *Escherichia coli*
E-hly	EHEC-hemolysin
EIEC	enteroinvasive *Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
HC	hemorrhagic colitis
HUS	hemorrhagic uremic syndrome
kbp	kilo base pair
LEE	locus for enterocyte effacement
MLVA	multiple-locus variable-number tandem-repeats analysis
ND	not done
OMP	outer membrane protein
ORF	open reading frame
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RAPD	randomly amplified polymorphic DNA
RBC	erythrocytes
RTX	repeat-toxins (pore-forming cytotoxin)
SMAC	sorbitol-MacConkey (medium)
VNTR	variable tandem tandem repeat
VT	Verotoxin/Verocytotoxin
VTEC	Verocytotoxin-producing *E. coli*

Genes

chuA	*E. coli* haem-utilization gene
daaE	F1845 fimbriae, major subunit gene
eaeA	*E. coli* attaching and effacing gene, intimin
E-hly	EHEC-hemolysin gene
espP	Extracellular serine protease, plasmid encoded
etpD	EHEC type II secretion pathway
fimA	Type 1 fimbriae, major subunit gene
hlyA	α-hemolysin, toxin precursor gene
hlyCABD	hemolysin operon
iutA	aerobactin, iron uptake transport receptor gene
katP	Catalase-peroxidase, plasmid encoded gene
papC	P fimbriae, pyelonephritis-associated pili, assembly platform gene
sfaD/sfaE	S fimbrial adhesin gene
VT1	Verocytotoxin 1
VT2	Verocytotoxin 2

INTRODUCTION

The recognition of EHEC (enterohemorrhagic *Escherichia coli*) as an etiological agent of diarrhoea with life-threatening complications has made this kind of infection a public health problem of serious concern. The pioneering work leading to the discovery of the *E. coli* verocytotoxins (VTs) was done by Konowalchuk and colleagues during the late 1970s *(Konowalchuk 1977)*. They reported the feature of the production of a toxin with a profound and irreversible cytopathic effect on Vero (African green monkey kidney) cells. O'Brien et al. purified and characterised the cytotoxin and found that it had a similar structure and biological activity as the Shiga toxin produced by *Shigella dysenteriae* type 1 *(O'Brien 1982, O'Brien 1983)*. In the early 1980s, verocytotoxin producing *E. coli* were linked to two conditions of previously unknown cause, hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) *(Karmali 1983, Riley 1983)*. Additionally, isolates of verotoxigenic *E. coli* belonged to a previously rare serogroup O157:H7 *(Karmali 1983, Riley 1983, CDC Centers for Disease Control. 1982)* and this serogroup has been the type most commonly associated with large outbreaks *(Ostroff 1990, Swerdlow 1992, Fukushima 1999)*.

The major justification for continued interest in EHEC infections from a clinical perspective is the high rate of serious complications associated with this infection, especially in children. HUS, which defined by a triad of features: acute renal failure, thrombocytopenia, and microangiopathic haemolytic anemia, occurs in 2-15% of cases *(Carter 1987, Slutsker 1997, Rowe 1998)*. The mortality from HUS is high (3-17%) *(Griffin 1991)*, and has been reported to be even higher among elderly *(Carter 1987)*. A significant number (approximately 30%) suffer a range of permanent disabilities, including chronic renal insufficiency, hypertension, and neurological deficits *(Karmali 1989, Tarr 1995)*.

The pathogenesis involves establishment of the organism in the gut, where it has to compete for space and nutrients with other microorgansims of the normal intestinal microflora. The most important virulence characteristic of the organism, once established, is its ability to produce one or more phage encoded verocytotoxins (VT1 and VT2) *(Paton 1998b)*. Toxin production mediates both local and systemic disease. Local intestinal effects cause the development of bloody

diarrhoea as the toxin has internalised the cells of the gut where it blocks cellular protein synthesis and may lead to apoptosis. HUS results from microvascular disease when the toxins enter the bloodstream and bind to receptors on endothelial cells abundant in kidneys and brain *(Nataro 1998)*.

VT producing *E. coli* can be found in the faecal flora of a wide variety of animals but the most important animal species in terms of human infection is cattle. The bacteria are usually isolated from healthy animals but may be associated with an initial episode of diarrhoea in young animals followed by asymptomatic colonization *(Nataro 1998)*.

The nomenclature has been somewhat confusing with the systems EHEC, VTEC (verocytotoxin *E. coli*) and STEC (Shiga-like toxin *E. coli*) used interchangeably in the literature. In this thesis, EHEC will be used for verocytotoxin producing *E. coli* in humans and VTEC for verocytotoxin producing *E. coli* found in healthy cattle.

REVIEW OF THE LITERATURE

EHEC in humans, pathogenesis and virulence factors

The expression of one or both of the verocytotoxin genes is the main feature of EHEC. Pathogenesis is, however, a multistep process, involving the interactions between several additional bacterial and host factors. Virulence factors studied in this thesis are presented in Table 1.

The infectious dose of EHEC is very low, between 1 and 100 CFU, which is a much lower dose than for most other pathogens of the intestines *(Paton 1998b)*. Ingested EHEC must pass the stomach and then find an ecological niche in the gut competing for space and nutrients with other microorganisms to establish colonization. One of the main determinants of EHEC to cause disease in humans is their ability to adhere to intestinal epithelial cells and to colonize the human gut. The processes of establishment and maintenance of colonization of the intestines are not fully understood but interesting efforts for therapeutic inter-

vention has been made involving interference with these processes *(Dean-Nystrom 2002)*.

VT produced, is transported to receptors on target cell surfaces inducing pathological effects. To reach the receptors the toxins after being produced in the lumen must be absorbed by the intestinal epithelium and then translocated to the bloodstream.

Table 1. The EHEC/VTEC isolates of human and cattle origin were characterized for the following possible virulence factors

Virulence factors	gene	P*/C**	Proven/possible importance	Ref.***
Verocytotoxin 1	*VT1*	C	Inhibit protein synthesis, causing cell death	1
Verocytotoxin 2	*VT2*	C		
Intimin	*eaeA*	C	Mediates intimate adherence to epithelial cells	2
EHEC-hemolysin	*E-hly*	P	Hemolytic to washed sheep RBC	3
Type II secretion	*etpD*	P	Secrete extracellular proteins	4
Catalase-peroxidase	*katP*	P	Defence against oxidative stress etc.	5
Serine protease	*espP*	P	Cleaves coagulation factor V etc.	6
Type 1 fimbriae	*fimA*	C	Adhesins that enable adherence to host cells	7
P fimbriae	*papC*	C		8
S fimbriae	*sfaD/sfaE*	C		9
F 1845	*daaE*	C		10
Aerobactin	*iutA*	C	Mediates bacterial iron acquisition	11
α-hemolysin	*hlyA*	C	Osmotic lysis of target cells	12

* P = plasmid

** C = chromosomal

*** 1 *(O'Brien 1992, Melton-Celsa 1998, Scheutz 2001)*, 2 *(Jerse 1990)*, 3 *(Schmidt 1995, Schmidt 1996b)*, 4 *(Schmidt 1997)*, 5 *(Brunder 1996)*, 6 *(Brunder 1997)*, 7 *(Duguid 1955)*, 8 *(Leffler 1981)*, 9 *(Parkkinen 1983, Ott 1986)*, 10 *(Bilge 1989)*, 11 *(Neilands 1985, Johnson 1988)*, 12 *(Rennie 1974, Seeger 1989, Suttorp 1990)*

Attaching and effacing adherence

A number of enteropathogenic bacteria, including both enteropathogenic *Escherichia coli* (EPEC) and EHEC, cause attaching-effacing (A/E) lesions. A/E bacteria generally infect the small or large intestine and cause diarrhoea. The

mechanism whereby EHEC isolates generate A/E lesions is similar as for EPEC. The A/E lesion, first reported by Staley et al *(Staley 1969)*, is characterized by intimate attachment of the bacterial cell to the intestinal epithelial cell membrane with destruction of the microvilli and disruption of the cellular cytoskeleton at the site of attachment. All A/E bacteria have a chromosomal pathogenicity cassette, called LEE (for locus of enterocyte effacement), which is a 35-kb region of DNA that encodes the gene products necessary for formation of the A/E lesion *(McDaniel 1995, Rosenshine 1996)*.

Donnenberg and Kaper proposed a three-stage model of EPEC pathogenesis *(Donnenberg 1992b)* consisting of: local adherence, signal transduction and phosphorylation of host proteins and intimate adherence. In EPEC local adherence is mediated by so called bundle-forming pili *(Donnenberg 1992a)* encoded by a plasmid gene. However isolates of EHEC do not carry this plasmid and lack genes coding for bundle forming pili *(Willshaw 1992, Beutin 1995, Wieler 1996)*.

One factor part of the LEE cassette that promotes intimate adherence, is the product of the gene *eaeA*, a 94-kDa outer membrane protein (OMP) called intimin *(Jerse 1990)*. The *eaeA* gene has been identified in both EPEC and EHEC and has a sequence homology in the two bacteria of 86% *(Yu 1992)*. Mutation of the *eaeA* locus results in loss of the ability to produce the attaching and effacing effect both in isolates of EPEC and EHEC *(Donnenberg 1991, Donnenberg 1993)*.

The host epithelial receptor for intimin named Tir (translocated intimin receptor) *(Rosenshine 1996)* is also encoded by LEE *(Kenny 1997)* in both EPEC and EHEC. The protein is secreted from the bacteria and for efficient delivery into the host cell, dependent upon the type III secretion system and other LEE-encoded proteins *(Jarvis 1996, DeVinney 1999)*.

Notwithstanding the above, a significant minority of human EHEC isolates, including those from patients with HC and HUS, have been observed not to contain *eaeA*, indicating that intimin is not essential for human virulence *(Barrett 1992, Louie 1994)*.

Fimbriae

Fimbriae or fimbrial adhesins are thread-like structures that reach out from the bacterial surface enabeling bacteria to adhere to host cells.

Type 1 fimbriae were the adhesins first described in *E. coli (Duguid 1955)*. They are the most common adhesins produced by these bacteria and mediate adherence to mannose-containing glycoproteins found on the surfaces of many eukaryotic cells *(Johnson 1991)*. More than 90% of *E. coli* isolates from the gut of healthy Swedish adults have the genes for type 1 fimbriae *(Friman 2002)*. However, the type 1 fimbriae have also been shown to play an important role in the colonization of *E. coli* in the human urinary tract and, from their prevalence, it is assumed that they are important in the faecal-oral cycle, for binding within the gastrointestinal tract and/or for transfer between hosts *(Orndorff 1990)*.

Type 1 fimbriae are encoded by the *fim* gene cluster, *fimA-H (Abraham 1987, Abraham 1988)*. The expression is regulated by phase variation within the promotor of *fimA*. Enami M. et al. *(Enami 1999)* showed, however, that VT producing *E. coli* O157 strains appeared unable to express type 1 fimbriae while other serotypes such as O26 and O118 could. The molecular basis of this difference is the presence of a 16 bp deletion within the regulatory region of *fimA* in EHEC O157 but not in other EHEC serotypes tested *(Li 1997, Roe 2001)*. EHEC expressing type I fimbriae may have increased possibility to colonize the gut. Initial adherence by factors such as type 1 fimbriae may make subsequent intimate adherence and A/E lesion formation more likely to occur *(Mack 1999)* as the adhesin, bundle-forming pili, known to be involved in EPEC A/E lesions, is not present among isolates of EHEC.

P fimbriae (Leffler 1980), with the adhesin papG, recognize the Galα1→4Galβ disaccharide of uroepithelial cells and human erythrocytes *(Leffler 1981)*. The *pap* operon consists of 11 genes and the *papC* gene product is located in the outer membrane forming the platform for fimbrial growth *(Kuehn 1994)*. P fimbriae are especially associated with urinary tract infections but have even been found among faecal isolates *(Johnson 1991)*.

S fimbriae are adhesins also expressed by some isolates from urinary tract infections *(Ott 1986)*. The receptor is terminal sialyl-galactoside residues *(Parkkinen 1983, Parkkinen 1989)*. The S fimbrial adhesin is located at the fimbrial tips *(Moch 1987)* and, as with P fimbriae, has a different amino acid sequence than the structural subunit *(Hacker 1985b, Moch 1987)*. The structural subunit shares N- and C-treminal amino acid sequence homology with the P fimbriae and, like other *E. coli* fimbrial subunits, it contains an internal disulfide loop *(Schmoll 1990)*. The expression of S fimbriae is subjected to phase variation by methylation of nucleotides in the regulatory region, similar to the case with P fimbriae *(Saukkonen 1988, van der Woude 1994)*. The *sfa* operon consists of 9 genes and *sfaE* is involved in the transport of subunit proteins *(Schmoll 1990)*.

F1845 is one of four adhesins that belongs to the Dr family of adhesins *(Nowicki 1990)*. These adhesins recognize distinct receptor epitopes on decay-accelerating factor (DAF), a membrane protein involved in regulation of the complement cascade *(Nowicki 1993)*. While three of the adhesins are isolated from patients with urinary tract infections, F1845 is a fimbrial adhesin of diarrhoea-associated *E. coli (Bilge 1989)*. The adhesin confers upon *E. coli* the ability to adhere to cultured epithelial cells in a diffuse pattern, with bacteria distributed evenly over the surface of the cells *(Bilge 1993a)*. The determinant encoding the expression of F1845 consists of at least six genes, designated *daaA-daaF (Bilge 1989, Bilge 1993b)*. The gene (*daaE*) encoding the major fimbrial subunit, which also serves as the adhesin is located at the 3' end of the locus *(Swanson 1991)*.

Bacterial need for iron

It is well known that iron is needed by all living cells *(Weinberg 1978)*. *E. coli* uses iron for oxygen transport and storage, DNA synthesis, electron transport, and metabolism of peroxides *(Neilands 1985, Bagg 1987)*. Almost all iron in biological fluids is, however, complexed with host iron proteins. A host defence mechanism against bacterial infection is to further reduce the amount of iron available to the invading pathogen *(Weinberg 1978)*. Thus, bacteria need effective systems to meet their iron needs during infection.

In *E. coli*, the siderophore *aerobactin* is the most effective of the several iron chelation systems employed by enteric bacteria for iron acquisition *(Warner 1981, Bindereif 1985, Neilands 1985, Williams 1986, Bagg 1987)*. After secretion of aerobactin by *E. coli* cells, it can extract Fe^{3+} from host iron-binding proteins and be taken up through an outer membrane protein (OMP) receptor *(Carbonetti 1984, de Lorenzo 1986b)*. Isolates with the aerobactin system have a growth advantage in low-iron conditions *(Williams 1979, Braun 1984, Montgomerie 1984)* and, in comparison to the other major specialized siderophore, enterobactin, aerobactin is more effective. Aerobactin has a higher affinity constant for iron than enterobactin at neutral pH *(Bagg 1987)*, and aerobactin deferrates transferrin more rapidly in serum or in the presence of albumin, presumably because enterobactin (but not aerobactin) binds to and is inactivated by proteins *(Warner 1981, Braun 1984, Williams 1986, Bagg 1987, de Lorenzo 1988)*. Enterobactin is less soluble and less stable than aerobactin *(Warner 1981, de Lorenzo 1988)* and aerobactin is also continuously recycled without hydrolysis *(Braun 1984)* in contrast to enterobactin where the release of iron requires hydrolysis of the siderophore *(Warner 1981)*. Aerobactin delivers iron directly to bacterial iron centers, whereas enterobactin leaves iron free in the cytosol *(Williams 1986)*. Enterobactin binds to serum proteins, and gives rise to anti-enterobactin antibodies that probably limits its use as a siderophore in vivo *(Bindereif 1985)*. Finally, aerobactin production is stimulated by milder degrees of iron deprivation than are required to stimulate enterobactin production *(Williams 1986, de Lorenzo 1988)*.

In most isolates of *E. coli*, aerobactin is expressed by five operons, *iucA-D* and *iutA (de Lorenzo 1986a)*. *IutA* is coding for the OMP receptor protein. Aerobactin determinants are found both on plasmids and on the bacterial chromosome. The chromosomal location is, however, most common among human isolates *(Valvano 1986, Johnson 1988)*.

Aerobactin has been found to be rarely expressed by isolates of ETEC, on the contrary EPEC and EIEC isolated from humans and from domestic animals with sepsis and mastitis do *(Williams 1985, Linggood 1987)*. The expression of aerobactin is poorly studied among isolates of EHEC.

α-*hemolysin* is a cytolytic protein toxin that lyses erythrocytes of all mammals and even fish *(Rennie 1974, Cavalieri 1984)*. It is encoded by a four-gene operon termed *hlyCABD (Goebel 1982, Johnson 1991)*, which is located on the chromosome in human isolates of *E. coli (Welch 1983, Johnson 1988)*. The *hlyA* gene encodes the structural hemolysin protein *(Waalwijk 1984, Welch 1984)* and this must be activated by the protein encoded by *hlyC (Felmlee 1985, Nicaud 1985)* prior to its secretion. HlyB and D are involved in the secretion of the hlyA determinant *(Hacker 1985a, Wagner 1988)*. The provirulence activity of hemolysin is probably multifactorial and includes the release of iron from erythrocytes, disruption of phagocyte function, and direct toxicity to host tissues *(Johnson 1991)*. Even though α-hemolysin is often expressed among haemolytic *E. coli* isolates *(Cavalieri 1984)*, it seems to be unusual among isolates of EHEC *(Law 1995)*.

The plasmid pO157

Many bacterial pathogens are known to carry plasmids that encode important virulence factors. The plasmids often carry genes responsible for the expression of their group-specific pathogenicity, e.g. the heat-labile and heat-stable toxins of enterotoxigenic *E. coli*. (ETEC), the invasion factors of enteroinvasive *E. coli* (EIEC), the aggregative adherence fimbriae mediating the typical phenotype of enteroaggregative *E. coli* (EAEC) or the virulence factor bundle forming pili of enteropathogenic *E. coli* (EPEC) *(Nataro 1998)*. Virtually all EHEC isolates carry large plasmids about 75-100 kb in size, which were thought to be implicated in the pathogenesis of EHEC *(Bopp 1987, Ostroff 1989, Fratamico 1993, Paros 1993)*. Plasmid pO157, 90 kb large and present in almost all EHEC O157 isolates, was first detected in isolates causing an outbreak of HC *(Wells 1983)*. This plasmid has been the subject of several investigations.

Schmidt et al. characterized the plasmid pO157 by restriction mapping and described a pO157-encoded determinant, designated EHEC-hemolysin *(Schmidt 1995, Schmidt 1996b)*. EHEC-hemolysin is a member of the RTX (Repeats in toxin) family of pore-forming cytolysins and was shown to be cytotoxic for human and bovine cell lines *(Bauer 1996)*. Isolates producing this enterohemolysin are not hemolytic on standard blood agar but produce small, turbid hemo-

lytic zones on washed sheep RBC agar after over night incubation *(Beutin 1989)*. EHEC-hemolysin consists of four open reading frames with 60% homology to the *E. coli* α-hemolysin operon (*hlyCABD*), and the O157 enterohemolysin operon was therefore named *EHEC-hlyCABD (Schmidt 1995, Schmidt 1996b)*. *E-hlyA* is the structural gene for the enterohemolysin. The enterohemolytic phenotype is, however, associated with defective hemolysin secretion. Moreover, by transforming the type strain EDL 933 with the *hlyB* and *hlyD* genes derived from the α-hemolysin operon, this defect could be completed, resulting in an α-hemolytic phenotype. Schmidt et al. *(Schmidt 1995)* analysed isolates of EHEC O157:H7, as well as O157:H45 and some non-O157 EHEC. All EHEC O157:H7 and 50% of *VT* positive EHEC non-O157 harboured the *E-hly* gene, while all isolates negative for *VT* genes were negative for *E-hly*. Thus, there seemed to be a correlation between VT and enterohemolysin production, especially in O157 isolates. The manner in which E-hly may contribute to the pathogenesis of EHEC disease is possibly that hemoglobin released by the action of the hemolysin provides a source of iron, thereby stimulating the growth of EHEC in the gut.

The second pO157-encoded determinant discovered, was the EHEC katP catalase-peroxidase, which is produced in addition to the two chromosomally encoded catalases or hydroperoxidases of *E. coli (Brunder 1996)*. Catalases are part of the bacterial defence mechanisms against oxidative stress *(Farr 1991)*, while the peroxidases are haem binding enzymes that carry out a variety of functions using hydrogen peroxid as the electron acceptor *(Loewen 1985, Triggs-Raine 1988)*. Brunder et al. *(Brunder 1996)* analysed by PCR, representative isolates of the major groups of diarrhoeagenic *E. coli*, including EPEC, EIEC, ETEC, EAEC, EHEC O157 and non-O157 for presence of the *katP* gene. The results of the experiment showed that the *katP* gene was prevalent in EHEC isolates of serogroup O157 that also harboured the EHEC-hemolysin operon and 1/3 of the *E. coli* of two serogroups tested of non-O157 EHEC (O26 and O111) were also *katP* positive. The EPEC, ETEC, EIEC and EAEC isolates were all negative in the PCR assay.

The plasmid also harbours a gene with great similarities to genes of members of the type II secretion pathway systems of Gram-negative bacteria used to secrete

extracellular proteins. The gene encloses 13 open reading frames (ORF) named *etpC-O (Schmidt 1997)*. The ORF *etpD* has a sequence similarity of 70.0% to *pulD*, which is a related gene sequence of *Klebsiella pneumoniae (Schmidt 1997)*. Hybridization experiments with a specific etp probe and various categories of enteric *E. coli* pathotypes revealed that the *etp* gene cluster occurred in 100% of EHEC O157 tested and among 60% of EHEC of other serogroups. It was not found in isolates of EPEC, ETEC, EIEC or EAEC *(Schmidt 1997)*.

Brunder et al. identified and characterized in 1997 an extracellular serine protease espP, which is encoded by pO157 *(Brunder 1997)*. The corresponding *espP* gene showed to consist of one ORF encoding a 1300-amino acid protein. The deduced amino acid sequence had homology to several secreted or surface-exposed proteins of pathogenic bacteria, for example, espC of EPEC. There is 70% homology between the EHEC *espP* and the EPEC *espC*. Functional tests showed that the *espP* gene codes for a protease capable of cleaving pepsin A and human coagulation factor V. Thus Brunder et al. have suggested that secretion of the espP by EHEC in the gut could result in the mucosal hemorrhage observed in patients with hemorrhagic colitis *(Brunder 1997)*.

Verocytotoxin (VT) types and mode of action

The most important virulence characteristic of EHEC, once established in the gut, is its ability to produce VTs. They belong to a group of toxins with the prototype toxin produced by *Shigella dysenteriae* serotype 1. EHEC may produce one or more related toxins designated VT1 and VT2 with five and twelve variants respectively *(O'Brien 1992, Melton-Celsa 1998, Scheutz 2001)*. VT1 is most closely related to the Shiga-toxin produced by *S. dysenteriae*, differing by only one amino acid in the A-subunit of the toxin. VT1 and VT2 are approximately 56% homologous at the deduced amino acid sequence level, while the variants of VT2 are 84-99% homologous to VT2 *(Melton-Celsa 1998)*. The *VT* genes are encoded by lambdoid bacteriophages which lysogenize EHEC *(Schmidt 2001a)*.

All the VTs are AB_5 toxins consisting of a single 32 kDa A-subunit in non-covalent association with a pentamer of identical B-subunits. X-ray crystallographic analysis demonstrated that the B-subunit pentamers form a ring structure

with the carboxy-terminus of the A-subunit inserted into the central pore of B-subunits *(Fraser 1994)*. Toxin binding to cells is mediated by the B-subunits through interaction with globotriaosylceramide (Gb_3) in host cell membrane. Gb_3 is expressed on epithelial and endothelial cells derived from a variety of sites in both humans and animals *(Lingwood 1996)*. Once VTs bind their glycolipid receptors, the toxins are transported through the trans-Golgi network and Golgi apparatus to the endoplasmic reticulum (ER) and nuclear membrane. This pattern of intracellular trafficking is referred to as retrograde transport *(Sandvig 2002)*. During this transport, the A-subunit is cleaved by furin, a calcium-sensitive serine protease localized to the Golgi network. The resultant A-subunit fragments, A_1 + A_2, remain associated by a disulfide bond. An alternative mechanism of A-subunit processing involving the action of the protease calpain has also been described *(O'Loughlin 2001)*. Once in the ER, the disulfide bond linking A_1 + A_2 is reduced, and the A_1 fragment is translocated across the ER membrane into the cytoplasm. The A_1 fragments of VTs catalytically cleave a single adenine residue from the 28S rRNA component of the eukaryotic ribosomal 60S subunit. Following depurination, elongation factor 1-dependent aminoacyl-tRNA binding is inhibited thereby inhibiting the peptide chain elongation step of protein synthesis and ultimately causing cell death *(O'Brien 1992)*.

Role of the verocytotoxin genes in pathogenesis

The characteristic intestinal damage caused by EHEC includes hemorrhage and edema in the lamina propria with focal necrosis and neutrophil infiltration. It has recently been shown that VT1 and VT2 are translocated across polarized intestinal epithelial monolayers and, in this way, penetrate the epithelium *(Philpott 1997, Hurley 1999)*. The VT's can, in this way, get access to and damage the colonic blood vessels. VTs have also been shown to induce the expression of the neutrophil-specific chemokine interleukin-8 by human intestinal epithelial cells in vitro *(Thorpe 1999)*, in other words - it seems like VTs do participate in the process of gut inflammation. It has also been shown that VT1 almost exclusively binds neutrophils when incubated with human whole blood *(Te Loo 2000)*. Moreover VT2, in the bloodstream of HUS patients, has been associated with neutrophils *(Te Loo 2001)*. Additionally, Te Loo et al. *(Te Loo 2000)* have found

that VT1 was easily transferred from neutrophils to the surface of tumor necrosis factor-α (TNF-α)-treated human glomerular microvascular endothelial cells in vitro. From these data Cherla et al. *(Cherla 2003)* made the following conclusions that the toxins (i) possess the mechanism to cross the intestinal epithelial barrier; (ii) induce a neutrophil-rich inflammatory infiltrate in the gut; and (iii) in the bloodstream take a lift on neutrophils which mediate the transport to tissues with high-affinity receptors. The presence of VTs in the circulation appears to be a critical determinant in the development of HUS and CNS complications *(Moxley 1998)*. It is now generally agreed that the major portion of the histopathological lesions associated with both HC and HUS is a consequence of the interaction of VT with glomerular and CNS microvascular endothelial cells *(Richardson 1988)*. An illustration of the process of EHEC infection is shown in Fig. 1.

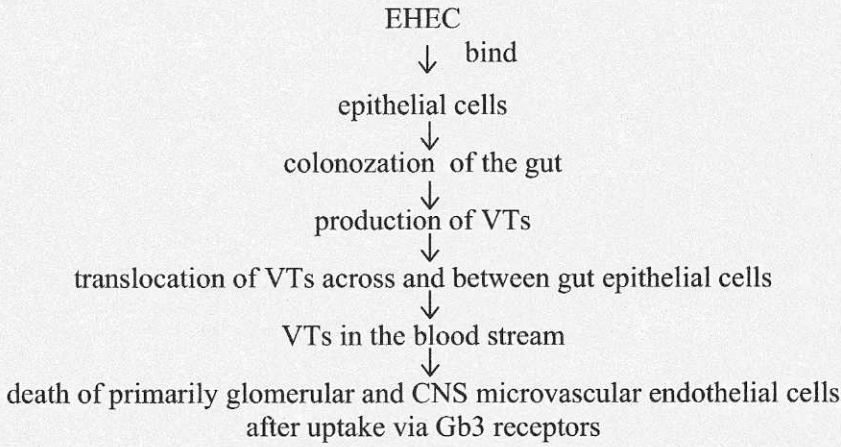

Fig. 1. Schematic illustration showing the main features of the pathogenes of human EHEC infection

Sources of human infections and epidemiology

The first two registered outbreaks in the USA, 1982, in Michigan and Oregon respectively, of EHEC O157:H7 were transmitted by the same source of under-cooked beef. Since then, it has been shown that ruminants and primarily cattle, are the most important reservoir of EHEC *(Chapman 1989, Olsvik 1991, Pierard 1994, Nielsen 2002)*. VT producing *E. coli* are not usually pathogenic for adult cattle, although some serogroups can cause diarrhoea in calves *(Sherwood 1985, Snodgrass 1986, Dorn 1993)*. Through contamination with

faecal material from ruminants, EHEC can be found in many other reservoirs, e.g. water, soil, meat, fruit and vegetable products. Vegetables and fruit, under-cooked meat or inadequately pasteurised dairy products are important vehicles of infection. In recent years rabbits and birds have been reported as reservoir hosts of EHEC that may pose a zoonotic risk for humans *(Garcia 2003, Leclercq 2003)*. Even person to person transmission of EHEC is a well-documented phenomenon during outbreaks and is probably also a reason for a significant portion of sporadic cases *(Beutin 1993, Blanco 1996, Paton 1998b)*.

Verocytotoxin and other virulence factors in *E. coli* isolated from cattle

VT producing *E. coli* can be readily isolated from the gastrointestinal tract of cattle. Several studies in different countries have shown that 10-80% of cattle may carry VT producing *E. coli (Wells 1991, Beutin 1993, Blanco 1996, Cobbold 2000)*. Isolates harboured by cattle have often been characterised in the same way as human isolates using various phenotypic and genetic markers. Individual isolates making up bovine populations of VT producing *E. coli* may be common or diverse both within and between herds and individual animals in keeping with the long-known concept of resident and transient *E. coli* in ecological niches. Of the VT producing *E. coli* excreted by cattle and potentially contaminating meat and other foodstuffs, only a few seem to have the potential to cause human illness. The factors involved in this discrepancy are not yet fully identified but determinants that are important virulence factors in humans and rarely exist in helthy cattle may be candidates.

VTEC does, however, usually not cause disease in cattle and other ruminants which may have helped its successful spread to a large amount of herds of cattle *(Elder 2000, Gansheroff 2000, Synge 2000)*. Studies have shown that the presence of the *eaeA* gene and the plasmid, coding for the *E-hly* gene, is more seldom present among non-O157 EHEC isolated from cattle than from humans *(Barrett 1992, Jenkins 2002)*. On the contrary verocytotoxin positive O157 EHEC isolated from cattle are, however, in a Danish study, always *eaeA* positive and also harbour the plasmid pO157 *(Nielsen 2002)*.

Another potential virulence factor is the α-hemolysin. It has in two studies however been shown to occur at a low frequency among bovine VTEC *(Law 1995, Sandhu 1997)* and VTEC isolated from healthy cattle have only to a low extent the ability to use heme or hemoglobin as the source of iron *(Law 1995)*.

The capacity to use iron by the presence of aerobactin was identified by Fectau et al. to be an important feature among *E. coli* (not necessarily VTEC) found in the blood of bacteremic calves *(Fecteau 2001)*. The presence of aerobactin producing VTEC, especially in healthy cattle is, however, not well studied.

Most *E. coli* has type 1 fimbriae. For cattle, it has been established that type 1 fimbriae bind well to rumen epithelium *in vitro (Galfi 1998)*. Concerning the adhesin genes, S and especially P fimbriae, seems to be common among *E. coli* isolated from cattle with septicemia and diarrhoea. These findings were, however, made with no correlation to verocytotoxin-positivity *(Bertin 1998)*. F1845 has, however, in one study been shown to be uncommon among VTEC isolates of diarrhoeic cattle *(Mainil 1993)*.

Methods for laboratory identification and isolation of EHEC

Because of their capacity to cause severe disease, EHEC infections of humans have in many countries become notifiable by public health authorities for the prevention of outbreaks and further spread of the pathogen among human beings. One of the major problems of identifying EHEC is that they resemble commensal *E. coli* in very many aspects apart from producing the VTs. Additionally, they often occur as a minority of the coliforms in the faecal flora of patients. The only way to identify all types of EHEC is the detection of VTs or of the genes associated with VT production. A number of biological (cytotoxicity tests) and serological tests have been developed for identification of VTs produced by the bacteria and genetic methods are used for identification of VT1 and VT2 specific sequences, some of which will be discussed here. Screening methods, which are not directed to identification of VTs or their genes, for example, plate indicator media, such as sorbitol MacConkey (SMAC) or enterohemolysin agar and serological typing of putative EHEC isolates, can certainly help in isolation of these pathogens but the suspected EHEC isolates

have always to be confirmed as such by identification of the VT production itself or by the presence of VT genes.

EHEC of serogroup O157 is usually, but not always, unable to ferment sorbitol. This has led to the development of SMAC agar medium, from which pale yellow colonies can easily be selected from the predominantly red colonies of sorbitol-fermenting *E. coli (March 1986)*. However, together with seroagglutination for serogroup O157 this will only detect sorbitol negative EHEC O157 and thus perpetuate the myth that EHEC O157 are the only ones that matter.

Most of the methods currently in use to identify whether EHEC are present in a faecal specimen or food, now involve DNA-based techniques. Generally, PCR-based methods have been successfully applied. Specific oligonucleotides (primers) are used to amplify short selected sections of DNA specific for the *VT* genes. Different laboratories use a variety of systems *(Thomas 1994, Welinder-Olsson 2002, Pulz 2003)*. There is, however, not yet any standardized, ready to use, PCR kit available.

The cytotoxicity assay method use tissue cultures (Vero cells) to which faecal and other supernatant preparations are applied, for observation of characteristic cytopathic effects *(Konowalchuk 1977, Karmali 1983)*. After suitable incubation time, 2-4 days, the cells are examined for the specific cytopathic effects. The specificity of the tissue culture tests can be improved by employment of VT1 and VT2 specific neutralizing antisera in order to determine if the cytotoxic effect is caused by VTs or by another non-neutralizable toxin present in the sample *(Karmali 1999)*. The method should be seen as the gold standard with which other tests are compared while it will reveal the presence of hitherto unknown variants of VTs *(Cermelli 2002)*. It is however a very labour-intensive and time consuming test and not really appropriate for the routine diagnostic laboratory.

There are several immunological methods for detection of VTs and, in contrast to DNA-based and cell culture assays, they are often commercially available as ready to use test kits *(Scheutz 2001)*. These tests are often easy to use and do not require specific materials and skills for employment. Even though the assay is

easy to use, it can prove very time consuming and require the use of many wells of a microtitre plate until the EHEC is isolated. However, for the rapid screening of human faeces, where generally few samples are expected to be positive, these test systems are valuable.

The isolation of EHEC from a positive sample may cause problems, particularly as EHEC are often present in small numbers in the sample and resemble commensal *E. coli* in their phenotypic and growth characteristics. Colony immunoblot or colony hybridisations are recently described techniques, now commercially available *(Gallien 1996, Timm 1996, Rüger 2001)*. The basis for these techniques is that colony blots are made from primary or secondary isolation plates and the presence of EHEC on these plates is then detected by either immunological or nucleic acid probes. When positive blots are noted, they can easily be referred back to the original agar plate and the positive colonies and studied further.

Another possibility to isolate potential EHEC from a positive sample is the fact that many EHEC produce the EHEC-hemolysin *(Beutin 1989)*. This enterohemolysin will show hemolysis on washed sheep-blood agar plates. The general utility of this test was futher demonstrated *(Bettelheim 1995)*. However, not all EHEC produce enterohemolysin, although most of the important human pathogenic ones do, and not all enterohemolysin-producing *E. coli* are EHEC.

Epidemiological typing of EHEC

Transmission of EHEC follows three main routes: food- or water-borne infections, acquisition of disease by direct or indirect contact with animals, and person-to-person spread. Typing of EHEC bacteria is an important part of identifying the route of transmission and preventing further spread. It is also valuable to be aware of the clonal relatedness between isolates when other traits, for example possible virulence factors are investigated. There are quite a few different methods available and some of the most often used will be described here.

For serogrouping animal sera reactive with the so-called O, K and H antigens on the bacterial cell wall, capsule and flagella respectively are used. Suspensions of

heat-killed bacteria mixed with antiserum are incubated over night at 50°C and agglutination is inspected by microscope *(Lidin-Janson 1977)*. The need for antisera against several different O, K and H antigens, however, makes the method complicated to perform at most laboratories and is often not discriminative enough. There are commercially serogrouping test kits available. These, however, only detect a few serogroups.

Random amplified polymorphic DNA (RAPD) PCR is a method based on the use of one or a few short primers of arbitrary sequence that are allowed to bind under low stringency conditions to various sites on both strands of the template DNA *(Welsh 1990)*. The PCR reaction yields a series of products of varying size, which if separated by gel electrophoresis, gives a band pattern that represents a "fingerprint" characterising a particular bacterial isolate. The method is fast to perform (1-2 days), but the discriminatory power is not always sufficient.

Pulsed-field gel electrophoresis (PFGE) utilizes restiction enzymes that cut DNA infrequently. Relatively large fragments (50-750 kb) of chromosomal DNA are generated. The fragments are then separated by special electrophoretic procedures, as DNA fragments larger than 40 kb are not efficiently resolved by conventional agarose gel electrophoresis. The DNA fragments cleaved by XbaI are too large to be sieved through even the largest pores of the gel matrix. However, the fragments can be resolved by cyclically altering the orientation of the electric field during electrophoresis *(Schwartz 1984, Rietman 1997)*.

Because intact DNA is required for PFGE analysis, conventional DNA isolation methods that cause shearing of DNA are not suitable. DNA isolation procedures for PFGE involve entrapment of bacteria in agarose plugs. The bacterial cell walls are lysed by treatment of the plug with enzymes and detergent. After extensive washing of the plug restriction of the remaining DNA is performed with enzymes chosen to yield a relatively small number of large restriction fragments. XbaI is the enzyme often used for the cleavage of EHEC DNA, and agarose plugs containing the restricted DNA are subjected to PFGE.

AIMS OF THE STUDY

The main purpose of this study was to perform molecular biological characterisation of EHEC to improve the diagnostics of the organism and characteristics of the infection in order to contribute to the control of EHEC disease in humans. The specific aims were:

1. To show that screening, using PCR, for the *VT* genes is a well-suited method for the diagnosis of EHEC.

2. To contribute to the understanding that also *E. coli* non-O157 are pathogenic.

3. To study clinical features such as period of incubation and duration of faecal shedding of EHEC in the human gut.

4. To show that PFGE is a valuable tool for epidemiological typing of EHEC.

5. To identify especially virulent isolates by demonstrating genes that, beyond the *VT* genes, may contribute to the bacterial virulence.

MATERIAL AND METHODS

Patients, clinical specimens and isolates from cattle

In paper I stool specimens were obtained during 1997 and 1998 from 3,948 patients. All routine specimens sent to the Bacteriological laboratory, Sahlgrenska University Hospital in Göteborg, Sweden, from patients under 15 years of age with diarrhoea or from older patients with sorbitol-negative colonies on sorbitol-Mac-Conkey agar and/or a reported diagnosis of severe or bloody diarrhoea were included. Some individuals were symptom free family members of patients with diagnosed EHEC.

Paper II included EHEC isolates from paper I that were EHEC positive by both culture and PCR. It also included an additional 30 isolates from patient specimens sampled as described in paper I, between 1998 to 1999, but the age of the included children was below 11 years.

Paper IV included EHEC isolates described in paper I and II all epidemiologically unrelated with different PFGE patterns. 13 isolates were of serogroup O157 and 18 of the serogroups O8, O22, O46, O76, O91, O117, O118, O121, O145 and E43478/86, as well as four OR (rough isolates) and four ON (non-type able isolates). This paper IV also includes 12 VTEC isolates from healthy cattle. One specimen was of serogroup O157 and 11 of serogroup O1, three O75, three OR and four ON, respectively. The cattle isolates all had different PFGE patterns, one specimen had, however, a pattern similar to one of the patient specimens. The cattle were sampled during a prevalence study in 1999 performed by the National Veterinary Institute in Uppsala.

Paper III describes an outbreak where 59 staff members of the children's hospital in Göteborg with gastrointestinal symptoms were sampled, 37 of whom had attended a party. All six kitchen staff members were sampled for EHEC, as well as patients at the hospital with symptoms suggesting EHEC infection. The buffet served at the party consisted of peanuts, prawns in warm sauce, grated carrots, chicken and lettuce served with mangojuice and wine. No food remained to be sampled, however, carrots and lettuce from the wholesaler who had supplied the

vegetables for the party were analysed for verocytotoxin-producing bacteria with PCR and cultured for detection of EHEC.

All stool specimens were also cultured for *Salmonella, Campylobacter, Shigella*, and *Yersinia* species.

Isolation of chromosomal DNA and plasmids

Bacterial growth from the primary agar plate culture of the stool specimens was suspended in 4 ml of double-distilled, sterile water to McFarland 4. One millilitre of the suspension was transferred to a centrifugation tube. This sample was heated to 100°C in a block thermostat for 15 min. before centrifugation at 7000 x g for 1 min. A 5 µl aliquot of the supernatant was used for each PCR. Single *VT1/VT2* positive colonies were analysed by PCR after suspension and boiling in 100 µl double-distilled, sterile water. Five microlitres of the supernatant was used for each PCR.

Plasmids were prepared from the isolates according to the manufacturers' (Qiagen, VWR, Göteborg, Sweden) instructions for large plasmids. One millilitre of overnight culture of EHEC resulted in a plasmid preparation dissolved in 50 µl of 10 mM Tris-HCl, pH 8.5.

Screening for *VT1*, *VT2* and *eaeA* genes by PCR

Primers used for detection of the *VT1/VT2* and *eaeA* gene sequences were designed by S. Löfdahl, Smittskyddsinstitutet, Sweden (Table 2). In paper I, the three PCR reactions were performed separately under the same conditions except for the primer content. The 50 µl reaction mixture contained 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9.0), 0.01% (w/v) Tween, 1.5 mM $MgCl_2$, 100 µM (each) dNTP and 1.25 U *Taq* DNA polymerase (Advanced Biotechnologies, UK). 10 pmol of each primer belonging to each primer pair was added. The amplification was performed using an Automatic Cycler (DNA thermal cycler 9600; Applied Biosystems, Sweden), using an initial modifying denaturation step of 4 min. at 96°C, followed by 20 sec. at 94°C, 45 sec. at 55°C and 10 sec. at 72°C. The amplified fragments were separated in 2% agarose gel stained with ethidium bromide. Primers VT1*l* and VT1*r*, VT2*l* and VT2*r*, and eaeA*l* and

eaeA*r* gave PCR fragmenst of 130 bp, 298 bp and 376 bp in length, respectively as seen in Table 2.

To improve the method of analysis, a multiplex PCR was used in papers II-IV and primers detecting both *VT1* and *VT2* gene sequences were added to the same reaction mixture. The $MgCl_2$ concentration was changed to 2.5 mM, and 1.25 U Gold *Taq* (Applied Biosystems) was used. The thermocycling then started with 10 min. of incubation at 94°C. The sensitivity of this new protocol was the same as before (data not shown). Even detection of the *eaeA* gene was by this time performed with Gold *Taq* with a prolonged incubation time initialising the thermocycling.

Methods to detect VT1 and VT2

For verocytotoxicity testing, crude faecal cultures were tested for verocytotoxicity essentially performed as described by Clarke et al. *(Clarke 1989)*. Crude cultures from sorbitol MacConkey agar plates were suspended in nutrient broth and incubated overnight at 37°C and then centrifuged at 7000 x g for 10 min. Fifty microlitres of supernatant was added to wells in a 96-well, flat-bottomed, microtitre plate. Each well contained fresh Vero cells and 100 µl of Eagle's minimal essential medium (Gibco, Sweden) supplemented with 5% foetal calf serum. The plates were incubated at 37°C in a 5% CO_2 incubator for 48 hours and were examined directly under an inverted microscope to estimate the degree of destruction of the Vero cell monolayer. Wells having at least 50% destruction of the monolayer as compared to a standard control well, were considered positive.

The direct toxin test (Premier Meridian Diagnostics, USA) was performed according to the supplier's instructions. The plate method described in the instructions was used.

PCR detecting *VT1* and *VT2* genes applied to other bacteria

To test the specificity of the PCR method for detection of EHEC, suspensions of species related to EHEC and other potential pathogens and commensals of the intestines were examined. These bacteria were all from the Culture Collection,

University of Göteborg, Sweden: *Acinetobacter*, CCUG 38560; *Citrobacter freundii*, CCUG 418; *VT* negative *E. coli*, CCUG 24; *Enterobacter cloacae*, CCUG 6323; *Hafnia alvei*, CCUG 38858; *Klebsiella pneumophila*, CCUG 2715; *Pseudomonas aeruginosa*, CCUG 17619; *Proteus mirabilis*, CCUG 4637; *Salmonella enteritidis*, CCUG 32352; *Shigella boydii*, CCUG 9564; *Shigella dysenteriae* type 1, CCUG 39701; *Shigella flexneri*, CCUG 9506; *Shigella sonnei*, CCUG 9567; and *Yersinia* type 3, CCUG 8233. *E. coli* CCUG 29197, which gave a positive signal in PCR for both VT1 and VT2 producing sequences, was used as a positive control.

PCR detecting the plasmid genes *E-hly*, *etpD*, *katP* and *espP*

PCR for detection of the plasmid genes *E-hly*, *etpD* and *katP* was essentially performed as described earlier by Schmidt et al. *(Schmidt 1999)* in a thermal reactor (Omnigene, UK) using 2.0 U of Ampli *Taq* DNA polymerase (Applied Biosystems). Concentrations of primers, dNTP and $MgCl_2$ in the reaction mixture were as described above and in Table 2. Even the *espP* gene was detected by PCR. Primers espP1 and espP3 were designed according to the nucleotide sequence reported by Brunder et al. *(Brunder 1997)* and found in the EMBL database library under accession number X97542. Amplification gave rise to an 800 bp fragment. The PCR was performed in a total volume of 10 µl containing 1 µM of each primer and 1 µl Taq DNA polymerase (5U/µl; Applied Biosystems) diluted 1:12.5 in enzyme dilution buffer (10 mM Tris [pH 8.3], 2.5 mg of bovine serum albumin/ml [Labora, Göteborg, Sweden]). The concentrations of $MgCl_2$ and dNTP were as described above and in Table 2. The amplification was performed in a thermal reactor (Rapid cycler; Idaho Technology, Biotech.IgG A/S, Copenhagen, Denmark) under conditions also described in Table 2.

Positive control for detection of the plasmid genes was kindly provided by Dr. H. Schmidt, Institut für Hygiene und Mikrobiologie, Würzburg, Germany.

PCR for detection of *fimA*, *papC*, *sfaD/sfaE*, *daaE*, *iutA* and *hlyA*.

PCR to indicate P, S and type 1 fimbriae, as well as the genes coding for aerobactin and α-hemolysin, was mainly performed as described by Nowrouzian et al. *(Nowrouzian 2001)*. Briefly, the reaction mixture contained the same ingredients as described above although the amount of primers detecting the *hlyA* gene was 20 pmol. The thermal reactor used was Omnigen (UK).

Primers to detect F1845 in the DR family of adhesins by PCR were designed in house. The primers were designed according to the nucleotide sequence reported by S. Bilge *(Bilge 1993a)*. The PCR was performed in a total volume of 50 µl using 2 U of Taq DNA polymerase and the other conditions for the PCR reaction as described above and in Table 2. The thermal reactor used for amplification was an Omnigen (UK). Positive control for detection of these adhesin genes and the genes encoding aerobactin and α-hemolysin was kindly provided by F. Nowrouzian, Department of Clinical Bacteriology, Göteborg University, Sweden.

Table 2.

Target	Primer designation	Nucleotide sequence of primers (5' to 3')	PCR conditions			$MgCl_2$ konc	Length of PCR product	Ref no.*
			denaturing	annealing	extension			
VT1	VT1 *l*	GAA GAG TCC GTG GGA TTA CG	94°C, 20s	55°C, 45s	72°C, 10s	2.5 mM	130 bp	1
	VT1 *r*	AGC GAT GCA GCT ATT AAT AA						
VT2	VT2 *l*	ACC GTT TTT CAG ATT TT(AG) CAC ATA					298 bp	1
	VT2 *r*	TAC ACA GGA GCA GTT TCA GAC AGT						
eaeA	eaeA- *l*	CAC ACG AAT AAA CTG ACT AAA ATG					376 bp	1
	eaeA- *r*	AAA AAC GCT GAC CCG CAC CTA AAT						
E-hly	hlyA1	GGT GCA GCA GAA AAA GTT GTA G	94°C, 30s	57°C, 60s	72°C, 90s	3 mM	1551 bp	2
	hlyA4	TCT CGC CTG ATA GTG TTT GGT A						
etpD	D 1	CGT CAG GAG GAT GTT CAG	94°C, 30s	52°C, 60s	72°C, 70s	1.5 mM	1062 bp	2
	D13R	CGA CTG CAC CTG TTC CTG ATT A						
katP	wkat-B	CTT CCT GTT CTG ATT CTT CTG G	94°C, 30s	52°C, 60s	72°C, 150s	1.5 mM	2125 bp	2
	wkat-F	AAC TTA TTT CTC GCA TCA TCC						
espP	espP 1	AGG CAC TTG AAC GTT ACG GGG T	94°C, 1s	59°C, 1s	72°C, 32s	4 mM	800 bp	1
	espP3	ACC GTT GTA TTC ACC GCC AGA C						
fimA	type 1-331f	CGA CGC ATC TTC CTC ATT CTT CT	94°C, 120s	65°C, 60s	72°C, 120s	1.5 mM	721 bp	3
	type 1-1052r	ATT GGT TCC GTT ATT CAG GGT TGT T						
papC	pap1	GAC GGC TGT ACT GCA GGG TGT GGCG					328 bp	3
	pap2	ATA TCC TTT CTG CAG GGA TGC AAT A						
sfaD/sfaE	sfa 1	CTC CGG AGA ACT GGG TGC ATC TTA C					410 bp	3
	sfa 2	CGG AGG AGT AAT TAC AAA CCT GGCA						
daaE	1845 f	AAC TTA CTT ATA TGC AAT GAA CAG	94°C, 120s	60°C, 60s	72°C, 120s	3 mM	149 bp	4
	1845 r	ATT GAT TAC CTG TTC AGT TTT GTG A						
iutA	aer-851f	GGC TGG ACA TCA TGG GAA CTG G	94°C, 120s	65°C, 60s	72°C, 120s	3 mM	301 bp	3
	aer-1152r	CGT CGG GAA CGG GTA GAA TCG						
hlyA	hly1	AAC AAG GAT AAG CAC TGT TCT GGCT					1177 bp	3
	hly2	ACC ATA TAA GCG GTC ATT CCC GTCA						

* 1 *(Welinder-Olsson 2002)*, 2 *(Schmidt 1999)*, 3 *(Nowrouzian 2001)*, 4 *(Welinder-Olsson 2004a)*

Serogrouping

For O157, the *E. coli* test kit, Oxoid LTD (Hampshire, England), was used. Positive control isolate of serogroup O157 was CCUG 29197B. Serogrouping of other than O157 was performed as previously described by Lidin-Janson et al. *(Lidin-Janson 1977)* or by the Laboratory of Enteric Pathogens, Central Public Health Laboratory, Colindale, England *(Gross 1985)*.

PFGE (pulsed-field gel electrophoresis)

PFGE was used to establish clonal relatedness and diversity among isolates (papers I-IV). Each isolate was grown overnight in 5 ml tryptic soy broth, and 250 µl of the suspension was prepared according to the method described by Gautom *(Gautom 1997)*. The DNA was digested with 20 U of *XbaI* enzyme in 200 µl of the appropriate restriction enzyme buffer following electrophoresis performed with the Gene Path system (Bio-Rad Laboratories, Sweden). The program used, was recommended by the supplier: no. 22 (Eco 157), with initial switch time 2.2 sec., final switch time 54.2 sec., run time 22 hours, angle 120°C, gradient 6.0 V/cm, temperature 14°C and a linear ramping factor.

The PFGE patterns were interpreted according to the methods of Tenover et al. *(Tenover 1995)*. Patterns differing by three bands or less were considered related to or probably related to each other. The gels were also digitised for computer-aided analysis. The Molecular Analyst software package (Bio-Rad Laboratories) was used for analysis. Calculation of the similarity matrix was done with the Jacquard algorithm after defining each band between sizes 145 and 582 kbp. The clustering was achived with the unweighted pair group method.

Statistical methods

Rates of the presence of virulence factors in comparison to different groups of *E. coli* isolates were calculated using Fisher's exact test (results, this thesis and papers I and IV).

RESULTS

Methodological investigations

Adjustment of the PCR assay (I, II, III, IV)

In paper I, amplification of the genes coding for VT1 and VT2 was carried out in separate reactions. 1.0 pg of EHEC template DNA was clearly visible when visualized in an ethidiumbromid stained agarose gel. This is equivalent to approximately 200 genome copies, assuming a genome size of 4,600 kbp. Even the primers eaeA*l* and eaeA*r* detecting the *eaeA* gene sequence gave a clearly visible band of the expected size after amplification of this amount of template DNA.

To test the specificity of the PCR method for detection of EHEC, even other human pathogens or species genetically related to *E. coli* and commensals of the intestines were examined. None of the bacteria tested gave any DNA fragment except *Shigella dysenteriae* type 1.

In papers II, III and IV the amplification reaction detecting the *VT* genes was improved with a multiplex PCR detecting both *VT1* and *VT2* genes simultaneously. This changes in methodology had no negative influence on the sensitivity.

PCR for screening of EHEC among patients with clinical symptoms (I, II, III, IV)

Table 3 shows the results of PCR versus culture, direct toxin test, verocytotoxin test, exposure to EHEC-positive patients and development of clinical symptoms of EHEC infection among 3,948 patients (paper I). Stool specimens for 55 (1.4%) of the patients were positive by PCR for either or both VT1 and VT2 producing sequences. EHEC were also detected by culture in 39 of the 55 stool-specimens. Among the 16 patients whose first stool specimens were positive by PCR but negative by culture, eleven had symptoms of HUS, bloody diarrhoea, or diarrhoea. Five had no or only slight symptoms, but they had been exposed to EHEC positive patients who had been diagnosed by culture as well as by PCR. Five patients had clinical symptoms of EHEC together with positive verocyto-

toxin test or direct toxin test (Table 4). No specimen was positive by culture but negative by PCR. When we assume that also the 16 patients for whom no positive culture was given but clinical data, verocytotoxin test result, direct toxin test result and/or epidemiological data indicated positivity the results gave a positive predictive value of 98% (55/56), a negative predictive value of 100% (3,892/3,892), sensitivity 100% (55/55) and specificity 99.97% (3,892/3,893).

Table 3. Results of PCR versus culture, toxin test, verocytotoxin test, exposure to EHEC-positive patients and development of clinical symptoms (from paper I) *(Welinder-Olsson 2000)*

		No. of patients		
		EHEC diagnosed by culture, direct toxin test, verocytotoxin test, exposure or clinical symptoms	**EHEC culture-negative**	**Total**
PCR	Positive	55	1	56 [a]
	Negative	0	3892	3892 [b]
	Total	55 [c]	3893 [d]	3948

[a] Positive predictive value, 98% (55/56)
[b] Negative predictive value, 100% (3892 of 3892)
[c] Sensitivity, 100% (55 of 55)
[d] Specificity, 99.97% (3892 of 3893)

Table 4. Clinical symptoms and toxintest results among the 16 PCR positive culture negative specimens

Clinical symptoms and/or exposure to EHEC-positive patients (n = 11)	**Clinical symptoms and positive direct toxin test or verocytotoxin test** (n = 5)
2 HUS	
2 bloody diarrhoea	5 diarrhoea including
3 diarrhoea	1 with abdominal pain
4 symptom-free	

In the papers II and IV, the screening method was used to identify additional patients and EHEC isolates for further studies, as well as VTEC isolates from healthy cattle.

In paper number III, the power of the screening method during an outbreak situation in 1999 is presented. Fifty-nine members of the staff with more or less severe diarrhoea at a children's hospital in Göteborg were sampled. Eleven stool specimens were positive for *VT2* by PCR, indicating EHEC infection (Fig. 2). No one was positive for *VT1* and only staff members who had attended a party were EHEC positive. In 9 of the 11 specimens, individual colonies of verocytotoxin-producing *E. coli* were isolated by culture and these all agglutinated positive for serogroup O157. Thirty-seven stool specimens were also analysed from in-patients during the time for the outbreak. Of these, two children who were admitted to the hospital due to diarrhoea and HUS respectively during the first week of September were diagnosed with *E. coli* O157 infection.

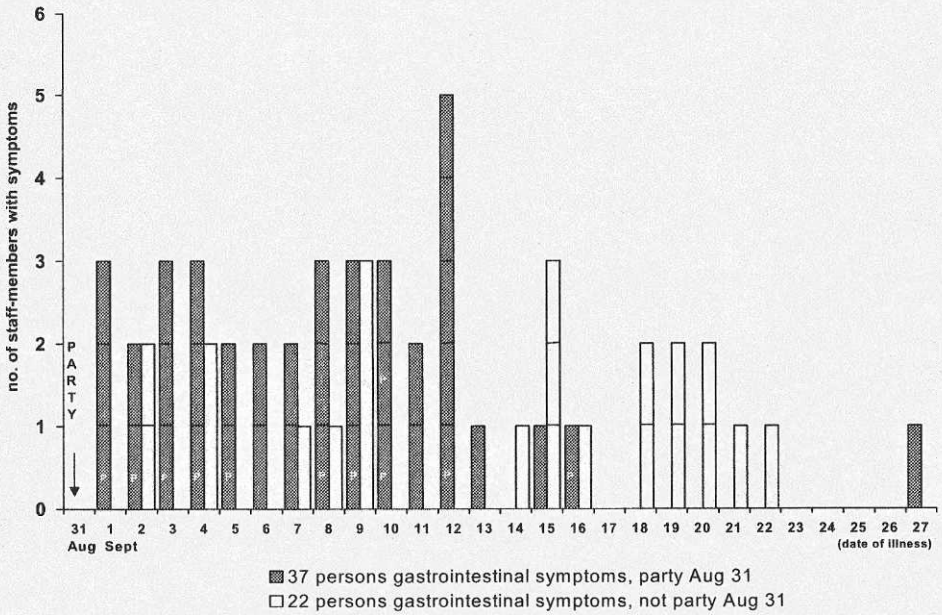

Fig. 2. EHEC outbreak among staff at a children's hospital. Date for onset of gastrointestinal symptoms for 59 of the staff members sampled. Thirty-seven of the 59 members had attended a party on 31 August. P in the columns indicate EHEC diagnosed by culture and/or PCR. (From paper III) *(Welinder-Olsson 2004b).*

PFGE for epidemiological typing (I, II, III, IV)

In paper I, PFGE has been used for epidemiological typing of EHEC. 55 isolates from different patients were studied. Fig. 3 shows that EHEC O157 with seven different PFGE patterns were identified during May – September 1997. Among these, we found 18 isolates with the same PFGE pattern, A. These isolates originated from a clone that caused an epidemic outbreak of EHEC on the west coast of Sweden during the late summer months. At the time for this epidemic outbreak additionally EHEC O157 with three different PFGE patterns (C, E and F) from seven patients were isolated. Isolate E was both *VT1* and *VT2* positive while the isolates C and F were just *VT2* positive as the clone with pattern A. Isolates with PFGE patterns G, H and I were isolated from patients who had arrived in Sweden from other European countries no more than 2 weeks prior to producing their stool specimen. Patients harbouring strains with PFGE pattern A, E and F had not been outside Sweden the month before giving their stool specimen. No information was available from the patient with the PFGE pattern C.

Time-axis showing month for isolation of EHEC O157 of different PFGE patterns

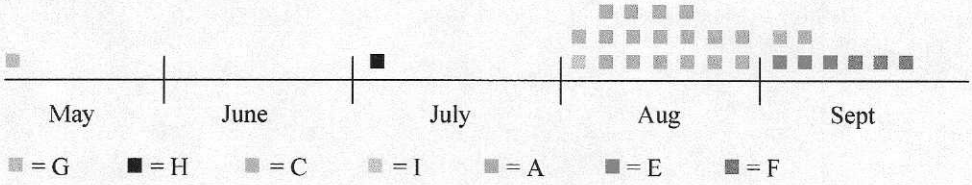

Fig. 3. Capital letters indicates PFGE patterns. G, H and I are from patients having been abroad.

In paper II, additional isolates of EHEC have been studied according to their PFGE pattern together with their serogroups (Fig. 4). Isolates with an identical or related PFGE pattern belong to the same serogroup. This is shown in PFGE patterns O117 and O121 as well as O157. Typing with PFGE is, however, more discriminating than serogrouping as variants of a PFGE pattern are possible to discern within a serogroup. Isolates with different PFGE patterns belong to different serogroups. Eight isolates were non-typable into serogroup even though they were sent to the Laboratory of Enteric Pathogens (Colindale, UK), while three were not typeable into PFGE patterns.

The PFGE method has also been used to establish clonal relatedness and diversity among strains when an outbreak seemed to have occurred at the children's hospital in Göteborg, paper III. All isolates from the staff that had attended the same party had the same PFGE pattern (Fig. 5). Isolates of EHEC from two children, who were admitted to the hospital due to diarrhoea and HUS at the time of the outbreak had, however, different PFGE patterns compared to the outbreak strain and differed to that with nine and five bands respectively.

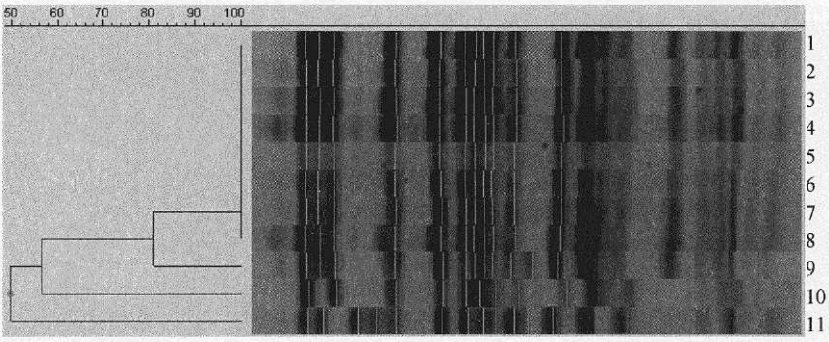

Fig. 5. PFGE patterns and dendrogram of EHEC O157 isolated from 9 (no. 1-9) of the staff members at the children's hospital and from two children (no.10-11) hospitalised at the time of the outbreak.

In paper IV, the PFGE method has been used to establish that isolates of different clonality were studied.

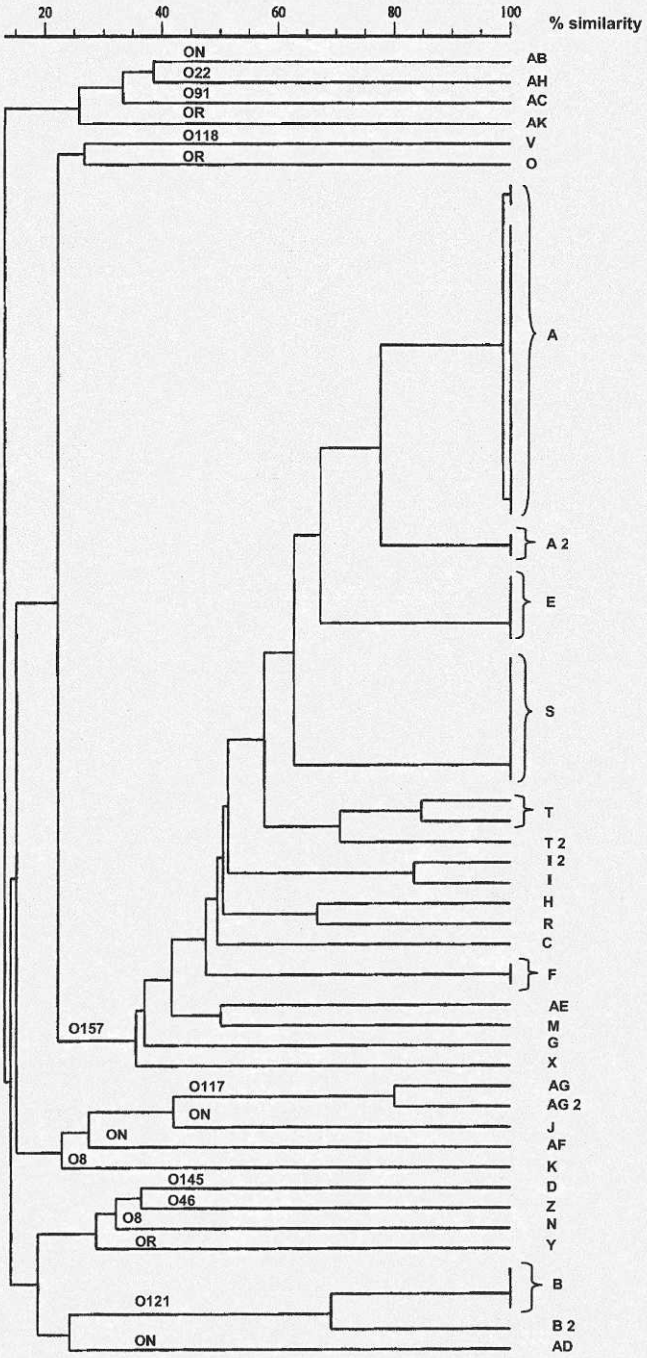

Fig. 4. Dendrogram of EHEC strains, both O157 and non-O157, isolated from routine fecal specimens sent to the Bacteriological Laboratory of the Sahlgrenska University Hospital in Göteborg. The dendrogram was constructed with the use of the unweighted pair group method by comparison of XbaI PFGE patterns. (From paper II) *(Welinder-Olsson 2002)*

Patients and bacterial characteristics

In this study, including specimens sampled during the years 1997-1999, 66% (44/67) of the specimens positive by both culture and PCR were of serogroup O157 and 34% (23/67) non-O157. During this time, there was however two outbreaks of serogroup O157, which increased the amount of this serogroup. Other serogroups identified were O8, O22, O46, O76, O91, O117, O118, O121, O145, E43478/86 and 5 OR (rough isolates) and four ON (non-type able isolates). In paper I, including 55 patients with PCR positive specimens both culturable and not culturable, EHEC caused 7 cases (13%) of HUS, 19 cases (35%) of bloody diarrhoea, 20 cases (36%) of diarrhoea and 9 persons (16%) were symptom-free. In paper II, which includes specimens from 67 patients both PCR and culture positive, 6 patients (9%) had HUS, 27 patients (40%) had bloody diarrhoea, 24 patients (36%) diarrhoea and 8 persons (12%) were symptom-free or with unknown symptoms. Table 5 shows that it is a similar proportion of patients with severe symptoms among those who were contaminated by EHEC O157 compared to non-O157.

The age of the patients spanned between 4 months and 74 years, but 38% (21/55) in paper I and 45% (30/67) in paper II were 3 years or less. In paper I, there was an equal amount of male and female patients. In paper II, 46% (31/67) of the patients were male and 54% (36/67) were female.

Table 5. Gastrointestinal symptoms among patients compared to whether EHEC O157 or non-O157 were isolated.

Symptom	O157 (n = 46)	Non-O157 (n = 23)
HUS	9%	9%
Bloody diarrhoea	43%	39%
Diarrhoea	39%	30%
Symptom-free	9%	13%
Symptoms not known	-	9%

Period of incubation and duration of symptoms (I, III)

The outbreak in 1999 gave the opportunity to study the period of incubation for EHEC. The source of infection was identified as the food at a party, and party-

members were interviewed about the onset of gastrointestinal symptoms. In nine of the EHEC-positive individuals, the incubation time was between 8 hours and 10 days. One person, however, who was also EHEC-positive by both PCR and culture, reported first symptoms 16 days after the party (Fig. 2).

In paper I, we show for how long patients' stool specimens may give a positive PCR result for the *VT1* and *VT2* genes together with positive results for culture, as most patients stool-specimens were analysed for EHEC more than once. About 70% (9/13) of the patients are still EHEC positive one week after their first stool specimen but within a month most specimens are negative. The longest period of time for a patient to harbour EHEC in the gut was 32 days. We did not obtain any additional samples from that patient and we do not know for how long the patient was EHEC-positive. One patient had two positive stool specimens, using both PCR and culture, three months apart but a negative specimen in-between and may have been reinfected otherwise the case was an example of long time faecal shedding.

The presence of the *eaeA* gene in correlation to patient's symptoms (I, II)

Most of the EHEC-positive patients had isolates that were positive for the *eaeA* gene sequence (including all of the HUS patients), i.e. 100% (44/44) of the EHEC O157 and 61% (14/23) of the non-O157 (paper II). Among eleven patients with bloody diarrhoea just seven (64%) were, however, identified to be *eaeA* positive (Table 6). Furthermore, additionally one patient with bloody diarrhoea, from whom we did not manage to isolate the EHEC bacteria, had a stool specimen negative for *eaeA*, but positive for *VT2* (paper I).

The presence of the four plasmid genes in relation to patients' symptoms (II)

All EHEC O157 possessed the EHEC *hly* gene as well as the *etpD* gene cluster (Table 6). 95% (42/44) of the O157 EHEC harbour the *katP* gene and 89% (39/44) the *espP* gene. The two isolates, that failed to produce an amplification product with primers detecting the *katP* gene, had different PFGE patterns. However, among the five isolates negative for the *espP* gene three have identical PFGE patterns. The O157 *E. coli* were both from patients with milder symptoms

(or diarrhoea only) and from patients with severe symptoms such as bloody diarrhoea and HUS, even if they had isolates of EHEC possessing all four plasmid-encoded determinants, together with *eaeA* positivity and one or both of the *VT* genes.

Among the 23 EHEC non-O157, presence of the plasmid genes is somewhat different than described above for the EHEC O157. 14/23 (61%) were *E-hly* positive, 2/23 (9%) were positive for the *etpD* gene cluster, 3/23 (13%) were positive for *katP* and 11/23 (49%) were *espP* positive. There is, however, a significant correlation for the presence of *E-hly* and severe symptoms for EHEC non-O157 (p=0.0094). Eleven of the patients from whom EHEC non-O157 was isolated had HUS or bloody diarrhoea. Ten of these (91%) harboured EHEC with the *E-hly* gene, while only 1/11 (9%), 1/11 (9%) and 6/11 (55%) harboured the *etpD* gene cluster, *katP* or *espP* genes respectively (Table 6).

Table 6. *VT* and plasmid genes in EHEC O157 and non-O157.

Virulence gene	**O157** (n=44)	**Non-O157** (n=23)	**Non-O157 with BD and/or HUS*** (n=11)	
VT1	14%	52%	45%	NS**
VT2	100%	52%	55%	NS
eaeA	100%	61%	64%	NS
E-hly	100%	61%	91%	p=0.0094
etpD	100%	9%	9%	NS
katP	95%	13%	9%	NS
espP	89%	49%	55%	NS

* BD (bloody diarrhoea) or HUS (hemorrhagic uremic syndrome)
** NS (not significant)

Presence of other virulence genes in EHEC isolates of human origin (IV)

Human EHEC isolates were analysed according to an additional six potential virulence genes beside the verocytotoxin genes, the *eaeA* gene and the plasmid borne genes *E-hly*, *etpD*, *katP* and *espP*. The EHEC isolates analysed, all have different PFGE patterns and 13 belong to serogroup O157, while 18 are non-O157 isolates. The six genes include four genes coding for proteins involved in the bacterial adhesion to host cells namely the P, S, F1845 and type 1 fimbriae.

The two other genes are *iutA* and *hlyA*, coding for aerobactin and α-hemolysin, involved in the bacterial competition for iron (Table 7).

All isolates of serogroup O157 have the gene coding for type 1 fimbriae and 89% (16/18) among the non-O157 EHEC harbour this gene. The two EHEC isolates not harbouring the gene coding for type 1 fimbriae have, however, the *eaeA* gene, and seven isolates not harbouring the *eaeA* gene have the gene coding for type 1 fimbriae. Just three isolates of human origin harbour any of the genes coding for P, S or F 1845 fimbriae.

The gene *iutA* coding for aerobactin is more often present among non-O157 than EHEC O157. Aerobactin is coded for by 44% (8/18) of the non-O157 isolates and 8% (1/13) of the O157 isolates. The chromosomally encoded α-hemolysin is about as uncommon among isolates of serogroup O157 as non-O157, 1/13 and 1/18 respectively. However, 97% (32/33) of the human isolates harbour at least one of the genes *E-hly*, *iutA* or *hlyA* possibly involved in the support of iron.

Table 7. EHEC O157 and non-O157 with different PFGE patterns isolated from humans and cattle compared to presense of known and possible virulence factors.

				Chromosomal genes									Plasmid genes			
Human isolates						Adhesin genes					Iron support					
No.	Symptoms [a]	PFGE[b]	Serogroup[c]	*VT1*	*VT2*	*eaeA*[d]	*fimA*[e]	*papC*[f]	*sfaD/E*[g]	*daaE*[h]	*hlyA*[i]	*iutA*[j]	*E-hly*[k]	*etpD*[l]	*katP*[m]	*espP*[n]
1	D	H	O157	+	+	+	+	-	-	-	-	-	+	+	+	+
2	BD	M	O157	+	+	+	+	-	-	-	-	-	+	+	-	+
3	BD	X	O157	+	+	+	+	-	-	+	-	+	+	+	+	-
4	D	E	O157	+	+	+	+	-	-	-	-	-	+	+	+	-
5	BD	F	O157	-	+	+	+	-	-	-	-	-	+	+	+	+
6	BD	C	O157	-	+	+	+	+	+	-	+	-	+	+	+	+
7	D	G	O157	-	+	+	+	-	-	-	-	-	+	+	+	+
8	D	I	O157	-	+	+	+	-	-	-	-	-	+	+	+	+
9	HUS	A	O157	-	+	+	+	-	-	-	-	-	+	+	+	+
10	BD	R	O157	-	+	+	+	-	-	-	-	-	+	+	+	+
11	D	AE	O157	-	+	+	+	-	-	-	-	-	+	+	-	-
12	BD	S	O157	-	+	+	+	-	-	-	-	-	+	+	+	+
13	HUS	T	O157	-	+	+	+	-	-	-	-	-	+	+	+	+
				31%[p]	100%	100%	100%	8%	8%	8%	8%	8%	100%	100%	85%	77%
1	D	AC	O91	+	+	-	+	-	-	-	-	+	-	-	+	-
2	HUS	O	OR	+	-	+	+	-	-	-	-	-	+	-	-	+
3	D	AD	ON	+	-	+	-	-	-	-	-	-	+	-	-	+
4	NK	AF	ON	+	-	+	+	+	-	-	-	+	+	+	-	+
5	BD	V	O118	+	-	+	+	-	-	-	-	+	+	-	+	+
6	D anemia	NT	E43478/86	+	-	+	+	-	-	-	-	+	-	-	-	-
7	BD	NT	ON	+	-	-	+	-	-	-	-	-	+	-	-	-
8	BD	Y	OR	+	-	+	+	-	-	-	-	-	+	+	-	-
9	BD	NT	O76	+	-	-	+	-	-	-	-	-	+	-	-	-
10	D	AB	ON	+	-	+	+	-	-	-	+	-	-	-	-	-
11	SF	AG	O117	+	-	-	+	-	-	-	-	+	-	-	-	-
12	D	Z	O46	-	+	+	+	-	-	-	-	+	-	-	-	-
13	D	AH	O22	-	+	-	+	-	-	-	-	-	-	-	-	-
14	NK	K	OR	-	+	-	+	-	-	-	-	+	-	-	-	+
15	BD	N	O8	-	+	-	+	-	-	-	-	-	+	-	-	-
16	BD	B	O121	-	+	+	+	-	-	-	-	-	+	-	-	+
17	BD	D	O145	-	+	+	+	-	-	-	-	-	+	-	-	+
18	SF	AK	OR	-	+	+	-	-	-	-	-	+	+	-	+	+
				61%	38%	61%	89%	6%	0%	0%	6%	45%	61%	11%	17%	44%

Isolates from cattle				Chromosomal genes										Plasmid genes		
						Adhesin genes					Iron support					
No.	Symptoms [a]	PFGE[b]	Serogroup[c]	*VT1*	*VT2*	*eaeA*[d]	*fimA*[e]	*papC*[f]	*sfaD/E*[g]	*daaE*[h]	*hlyA*[i]	*iutA*[j]	*E-hly*[k]	*etpD*[l]	*katP*[m]	*espP*[n]
1	-	AN	O157	-	+	+	+	-	-	-	-	-	+	+	-	+
1	-	AQ	ON	+	-	-	+	-	-	-	-	-	-	-	-	-
2	-	AT	O75	+	-	-	+	-	-	-	-	-	+	-	-	+
3	-	AU	ON	+	-	+	-	-	-	-	-	-	+	-	-	+
4	-	AM	OR	+	-	+	+	-	-	-	-	+	+	-	-	+
5	-	AL	ON	-	+	-	+	-	-	-	-	+	+	-	-	+
6	-	AO	O1	-	+	-	+	-	-	-	-	-	-	-	-	-
7	-	AP	O75	-	+	-	+	-	-	-	-	-	-	-	-	-
8	-	AR	ON	-	+	-	+	-	-	-	-	-	-	-	-	-
9	-	AS	OR	-	+	-	+	-	-	-	-	-	-	-	-	-
10	-	K2	OR	-	+	-	+	-	-	-	-	-	-	-	-	+
11	-	AV	O75	-	+	-	+	-	-	-	-	-	+	-	-	+
				36%	64%	18%	91%	0%	0%	0%	0%	18%	45%	0%	0%	55%

[a] D, diarrhoea; BD, bloody diarrhoea; SF symptom-free; NK, not known

[b] NT, not typeable in PFGE pattern.

[c] OR, rough; ON, not typeable in serogroup; E43478/86, provisional serotype

[d-n] virulence factors; [d] *eaeA*, intimin; [e] *fimA*, type 1 fimbriae; [f] *papC*, P fimbriae; [g] *sfaD/sfaE*, S fimbriae; [h] *daaE*, F1845; [i] *hlyA*, α-hemolysin; [j] *iutA*, aerobactin; [k] *E-hly*, EHEC hemolysin; [l] *etpD*, type II secretion; [m] *katP*, bifunctional catalase peroxidase; [n] *espP*, serine protease

[p] percent positive

Presence of virulence genes in VTEC isolated from healthy cattle (IV)

Verocytotoxin producing *E. coli* isolates from cattle (one O157 and 11 non-O157) were also analysed by PCR concerning possession of the same eleven gene determinants also tested for among the human isolates (Table 7), namely the plasmid genes *E-hly*, *etpD*, *katP* and *espP*, the genes coding for the adhesins intimin, P, S, F1845 and type 1 fimbriae and the genes coding for α-hemolysin and aerobactin. The isolate of serogroup O157 is, besides *VT2* positive, also positive for *eaeA*, *E-hly*, *etpD*, *espP* and for the genes coding for type 1 fimbriae. Concerning EHEC non-O157, 18% (2/11) were *eaeA* positive and 45% (5/11) were *E-hly* positive. Six isolates (55%) were *espP* positive. None of the isolates originating from cattle was *katP* positive.

No isolate originating from cattle has the genes coding for P or S fimbriae or the fimbrial adhesin F1845. All but one isolate have, however, the gene coding for type 1 fimbriae. No isolate has the gene coding for α-hemolysin and 17% (2/12) have the gene coding for aerobactin. 50%, (6/12) isolates with cattle origin, harbour any of the genes *E-hly*, *iutA* or *hlyA*.

Differences concerning virulence factors between human and cattle isolates (IV)

Enclosing both EHEC O157 and non-O157, the *eaeA* gene is to a significantly higher degree more often present among isolates of human than cattle origin (24/31 vs 3/12, p=0.0035). Additionally, 97% (30/31) of the human isolates have at least one of the genes *E-hly*, *iutA* or *hlyA*, which is to a significantly higher extent than found among isolates from cattle, among these 50% (6/12) harbour any of these genes (p=0.0009).

DISCUSSION

Several recent outbreaks of gastrointestinal disease caused by EHEC have highlighted the threat these organisms pose to public health *(Bell 1994, Cowden 1997, Fukushima 1999, Welinder-Olsson 2004b)*. Outbreaks of this art have the potential to overwhelm acute-care resources, even in countries with advanced health care systems. Our capacity to control EHEC disease in humans and to limit the scale of outbreaks is dependent upon fast and correct diagnosis and identification of the source of infection. In this thesis, molecular biological techniques have been tested and shown to be very useful in this purpose. As it has been obvious that EHEC is a heterogeneous group of bacteria, which beyond the production of verocytotoxins probably also have other virulence factors, PCR has been used for screening of other potential virulence genes. The thesis also includes a description of an outbreak of EHEC, which caused a great deal of trouble both among staff and patients as well as to the health care system. With the use of prompt molecular biological methods, it could be quickly identified and the consequences could be limited.

Importance of fast and adequate identification of EHEC

PCR is increasingly accepted to be the most sensitive means of determining whether a faecal specimen or a food sample contains EHEC. Although direct extracts of faeces or foods can be used as templates for PCR, the best results are usually obtained by testing extracts after primary culture *(Gannon 1992, Paton 1993, Begum 1995)*. Even though the laboratory looses some time for diagnosis, it is important to have a high sensitivity as the amount of EHEC is often very low in human faeces. In this thesis, we use a PCR, which detects 200 genome copies. This has shown to be sensitive enough to detect one single EHEC colony spiked in human faeces specimens.

PCR, with the primers used in this thesis, has a high sensitivity and specificity. Assuming that not only the patients with positive cultures were infected with EHEC but also the 16 patients for whom only clinical data, the verocytotoxin test result, the direct toxin test result and/or epidemiological data verified the PCR results, the overall sensitivity of PCR was 100% (55/55) as well as the specificity (3,892/3,893). The positive predicted value was 98% (55/56) and the

negative predicted value was 100% (3,892/3,892). The sensitivity of culture was only 71% (39/55) even though the culture procedure is usually preceded by a positive PCR result and great efforts were made to find the bacteria harbouring the *VT* genes.

There should be no doubt anymore, that it is important to be able to identify not only EHEC O157 but also the non-O157 EHEC *(Paton 1998b, Schmidt 1999, Welinder-Olsson 2000, Welinder-Olsson 2002)*. Nevertheless there are still studies published that do not include these serogroups. As Bettelheim and Beutin *(Bettelheim 2003)* argue, there should be no excuse for a reasonable sized hospital, veterinary or food laboratory not to be able to detect the presence of EHEC of any serogroup in a sample. Methods which only rely on detection of EHEC O157 and not on the other EHEC serogroups *(Feldsine 2002a, Feldsine 2002b)* perpetuate the false myth of the uniqueness of this serogroup and the true incidence and ecology of EHEC will be underestimated.

Papers I and II contribute to the understanding and knowledge that EHEC of several serogroups are pathogenic. They identify at least ten additional pathogenic serogroups and approximately just as many that are non-typeable regarding serogroups. Methods that are directed to identify the VTs or their genes are important and identification of sorbitol negative *E. coli* of serogroup O157 is not sufficient. In paper II, only 30% of the non-O157 EHEC were sorbitol negative. If the only identification procedure was culture for sorbitol negative isolates on SMAC agar plates followed by positive O157 agglutination, we would have misidentified 26 patients specimens, as seen in paper I. Four of these patients had HUS, seven had bloody diarrhoea, eleven had diarrhoea, and four had no symptoms. In paper II, which only includes both PCR and culture positive specimens even enclosing the PCR and culture positive specimens from paper I, 44 EHEC were O157 isolates and 23 EHEC were non-O157. There was a larger number of EHEC O157 partly because there had been additionally one outbreak of EHEC of this serogroup. It also illustrates that, sorbitol negative EHEC O157 are easier to identify and distinguish from the other commensals in the faeces flora. The proportion of patients with HUS, bloody diarrhoea, diarrhoea or patients who are symptom-free are almost the same in patients having EHEC of serogroup O157 or EHEC non-O157.

Over time, several different PCR methods have been used *(Johnson 1990, Pollard 1990, Brian 1992, Gannon 1992, Begum 1993)*. The PCR method has to be easy to perform to be useful for screening for EHEC positive specimens. Complex PCR reactions involving primers detecting several virulence genes at the same time *(Paton 1998a)* increase the risk for mismatches and loss of sensitivity, together with problems when analysing the agarose gel. However, in recent years new developments in PCR technology, such as using fluorescence for the identification and verification of PCR products have made the PCR-technique even more useful, and easy to perform *(Heid 1996, Wittwer 1997, Bellin 2001, Pulz 2003)*. It is, however, still important to identify the *VT* gene harbouring organism by culture, as even *Shigella dysenteriae* type 1 harbour the gene sequence identified by the primers in PCR.

Other techniques that involve the identification of VTs, are either protein detection by cytotoxicity assays or enzyme-linked immunosorbent assay (ELISA). Cytotoxicity assays are the most sensitive methods for detecting active VTs *(Paton 1998b)*, but they are cumbersome to perform and are therefore not very useful for routine microbiology diagnosis. VT specific ELISAs are an alternative, they are however less sensitive than PCR *(Beutin 1998)* and are also known to generate some false positive results *(Ball 1996, Beutin 1996a)*.

Period of incubation and duration of symptoms

For proper epidemiological considerations, it is important to know the average period of incubation. The incubation period earlier reported ranges from 1 to 9 days (mean is 3.1 to 3.9 days) during community outbreaks *(Riley 1983, Ostroff 1990, CDC Centers for Disease Control. 1991)* and from 1 to 14 days (mean is 4 to 8 days) in institutional settings *(Ryan 1986, Carter 1987, Su 1995)*. In the outbreak situation described in paper III, the period of incubation was between 1 and 16 days with a median of 8 days. Such a long incubation period as 16 days is not earlier reported, still it was found unlikely that person to person transmission between personnel has occurred a this institutional setting.

E. coli infection has been linked to ingestion of food and drink contaminated with faecal material from bovine sources *(Riley 1983, Swerdlow 1992)*. In some cases, however, EHEC has been transmitted directly from person to person. One important factor in direct transmission is how long an infective person sheds the pathogen. Only a few studies have however focused on this. From the studies in paper I, it can be concluded that 70% (9/13) of the patients from whom a second specimen was analysed within a week of the first positive specimen were still EHEC positive as determined by PCR. One month after the first positive specimen, most patients were negative. Karch et al. *(Karch 1995)* have done a similar analysis of serial stool specimens collected from patients infected with EHEC O157. Positivity was, however, only analysed by culture. It revealed that in 68% of the patients, only the first culture was O157 positive, and the three cultures that followed, collected at 2- to 4-day intervals, were negative. In other words 2-4 days after the first positive specimen, 68% of these patients were EHEC culture negative. The longer period of shedding shown in our study is probably explained by the more sensitive PCR procedure that we used.

Karch et al, like us, identified patients who were long-term shedders. Three weeks or more after the onset of symptoms, they were still able to identify EHEC O157 from 7 out of 53 patients (13%). We identified about the same proportion, 5/40 (12.5%) including one specimen from a patient sampled the fifth week after the first positive specimen and one patient with a positive specimen 3½ months after the first positive specimen with a negative specimen inbetween. To identify healthy carriers of EHEC by screening for bacteria harbouring the genes coding for the VTs, we identified only 2 positive out of 400 patients specimen (0.5%), indicating that there are few healthy carriers in Sweden.

PFGE for epidemiological typing of EHEC

Typing of EHEC for epidemiological purposes is critical to identify an outbreak and to stop further spread of the organism. It was speculated by MP Doyle (University of Georgia) that EHEC O157:H7 probably emerged some 30-40 years ago when *E. coli* acquired bacteriophages that carried genes encoding two Shiga-like toxins *(Konowalchuk 1977, Rowe 1995)*. If this is true, it is not sur-

prising that these phylogenetically young organisms have only a few characteristics that can be used to distinguish one isolate from another. Grif et al. 1998 *(Grif 1998)* made a comparative study of five different techniques for epidemiological typing of *E. coli* O157. They found that the methods phage typing, ribotyping two types of RAPD and PFGE, all had their shortcomings but underlined the great value of PFGE as a subtyping system with patterns that tend to be stable in outbreaks. Although single-band differences have previously been described with PFGE among isolates from epidemiologically associated cases *(Bender 1997)*, the discriminating power was as high as for phage typing.

In the present thesis, we have used PFGE both for EHEC O157 as well as for non-O157. The method was used for epidemiological purposes as well as to secure that different clones of EHEC were studied when possible virulence factors of different isolates were compared.

In paper I and II, it was shown that between May and September 1997 there were seven different clones of EHEC O157 isolated. Five of these were isolated during August and September, one of which was from a patient who had been abroad.

PFGE was especially useful during an outbreak situation at the children's hospital in Göteborg. It was important to assess that it was an ongoing outbreak among the staff and that neither of the two children who were admitted to the hospital at the time for the outbreak were infected during their stay at the hospital.

The EHEC isolates described in paper I and II showed a large variety of PFGE patterns among both EHEC O157 and non-O157, which demonstrated a great clonal diversity. This is in agreement with the findings reported by Rios et al. *(Rios 1999)*, who have studied the clonal diversity among isolates of Chilean isolates of EHEC, serogroup O26, O111 and O157, from patients with different severities of symptoms. Consequently, PFGE provides sufficient information about clonal diversity and is one of the better methods of choice for epidemiological investigations both for isolates of EHEC O157 and non-O157. Inte-

restingly there was some correlation between PFGE patterns and plasmid gene composition as shown in paper II.

PFGE is especially useful for epidemiological investigations in a limited geoghraphic area handled by a laboratory in close connection with the personnel who have the epidemiological knowledge. International comparison of EHEC, which may be of interest, places high demands on inter-laboratory reproducibility that are not so easy to achieve. Although there is software which transform bands mathematically to numerical values, PFGE and other banding pattern-based methods are not communicateable enough.

However, sequencing of the complete bacterial genomes, including the sequence of two *E. coli* O157:H7 isolates has given Lindstedt et al. *(Lindstedt 2003)* the opportunity to search the genomes for Variable Number of Tandem Repeats (VNTRs) in *E. coli* O157:H7 that could be used as a source of genetic polymorphisms and be used in a typing assay. VNTRs for typing purposes have in recent years also been tested in some other bacterial species for example *Yersinia pestis (Adair 2000, Klevytska 2001, Le Fleche 2001)* and *Mycobacterium tuberculosis (Frothingham 1998, Supply 2000, Le Fleche 2002, Skuce 2002)*. Lindstedt et al. found multiple-locus variable-number tandem-repeats analysis (MLVA) to be faster than PFGE and, accordingly, less labour intensive with yet slightly higher discriminatory power. Additionally, it has the benefit compared to PFGE and other banding pattern based methods that it has the potential for fully automating the analysis. Additionally, it leaves no moments for bias due to manual handling and gives the opportunity to be highly communicateable.

Additional virulence factors beside the verocytotoxin genes

Virulence factors in EHEC have mainly been studied in EHEC O157. As we believe that all VT producing *E. coli* with the opportunity to find a niche in the human intestine could be harmful, we have characterised both EHEC O157 and EHEC non-O157 concerning the presence of the two different *VT* genes and other presumptive virulence factors encoded by chromosomal as well as plasmid genes. The fact that the incidence of EHEC infection in humans is relatively low even though a fairly high amount of cattle, the principal reservoir of EHEC,

harbour VT producing strains, suggests that Shiga-like toxin production alone may not be sufficient for EHEC infection. Additional factors that appear necessary for virulence of EHEC O157 are the locus for enterocyte effacement (LEE) and the large plasmid pO157. Characterization of EHEC isolates by use of PCR provides detailed information about genetic variability. This information together with epidemiological investigations are important to improve our understanding of the distribution of possible virulence genes among various EHEC serogroups of animal and human origin.

In our investigation, all EHEC O157 harboured the gene coding for VT2, and a few also harboured the VT1 coding sequence. The *eaeA* gene coding for intimin was also always present and, with few exceptions, the plasmid harbouring the *E-hly*, *etpD*, *katP* and *espP* genes. The genes coding for VT1 and VT2 are more equally distributed among the non-O157 EHEC. About 60% harboured the *eaeA* gene coding for intimin and 50% of them were *VT1* positive and 50% *VT2* positive. Accordingly, there was no correlation between *eaeA* positivity and the presence of the *VT2* gene sequence as suggested by Boerlin et al. *(Boerlin 1999)*. In contrast to EHEC O157, the non-O157 EHEC often failed to give an amplification product of the four plasmid-borne determinants, at least not all four simultaneously. The most predominantly determinant was the *E-hly* sequence (61% positive), followed by the *espP* sequence (48% positive) the *katP* sequence (13% positive) and the *etpD* sequence (8.7% positive). Still, EHEC non-O157 seems to be as pathogenic as EHEC O157. There was approximately the same procentual amount of patients with HUS and bloody diarrhoea among patients infected with EHEC O157 as with non-O157. There is, however, a significant correlation ($p=0.0094$) between severe symptoms such as HUS or bloody diarrhoea and the presence of the *E-hly* gene sequence among patients infected with EHEC non-O157. Thus, the results indicate, what has also been shown by others, that the EHEC-hemolysin may represent an interesting virulence marker for EHEC involved in severe human disease *(Schmidt 1995, Beutin 1996b, Schmidt 1996a, Sandhu 1997)*.

The presence of *eaeA* did not clearly correlate to pathogenicity. Even though the two HUS patients had EHEC positive for *eaeA*, there were five patients with bloody diarrhoea not harbouring the gene. It has been generally believed that

there is a strong association between carriage of *eaeA* and the capacity of EHEC to cause severe human disease. Studies have shown that the proportion of *eaeA* positive isolates is much higher in isolates from patients with severe disease in comparison to VTEC from animals *(Barrett 1992, Beutin 1993, Beutin 1995, Sandhu 1996)*. The *eaeA* gene is, however, present among a relatively high degree of the patient isolates (61%), which probably illustrates that it is important not necessarily for the severity of the symptoms but to contribute to the colonization of the human gut. This is in accordance with earlier observations that there are isolates of EHEC from patients suffering from HC and HUS that do not contain the *eaeA* gene *(Barrett 1992, Louie 1994, Paton 1999)*.

No correlation could be found between the presence of the *espP* gene and severity of symptoms even though there were quite a few EHEC non-O157 harbouring this gene or the *etpD* and *katP* genes which, however, are relatively rare among these kind of isolates. Even though many non-O157 EHEC have a plasmid similar to pO157 our results are in agreement with Brunder et al. in that the plasmids of EHEC are not uniform genetic elements but heterogenous in their gene composition *(Brunder 1999)*.

As we have isolated EHEC from patients with more or less severe symptoms, not harbouring the genes coding for intimin or enterohemolysin, efforts have been made to identify other genes that could possibly be a substitution for these genes. In this thesis, there has been a focus on genes involved in the adherence of EHEC to enterocytes and to genes possibly involved in the bacterial competition for iron.

PCR with primers detecting the genes coding for P, S, F1845 and type 1 fimbriae was used to identify adhesin genes other than *eaeA*, coding for intimin. The P, S and F1845 fimbriae were all very rare among both EHEC O157 and non-O157. On the contrary, the majority of EHEC harbour the type 1 fimbriae, 100% (13/13) of the O157 and 89% (16/18) of the non-O157 EHEC. Even if the majority of EHEC have the gene coding for type 1 fimbriae in coexistence with the *eaeA* gene, nine EHEC non-O157 had either the gene coding for type 1 fimbriae or the gene coding for intimin and there was no isolate of EHEC that did not have at least one of these two genes. Type 1 fimbriae cannot be a substitute

for intimin that mediates the intimate attachment of EHEC to the intestinal epithelial cell membrane, which also includes loss of microvilli. Type 1 fimbriae may, however, contribute to the ability to cause disease by supporting the bacterial presence in the human intestine where it has been shown to bind to small and large intestinal epithelial cells *(Wold 1988, Adlerberth 1995)*.

PCR, with primers detecting the genes coding for aerobactin and α-hemolysin, was performed to detect factors other than E-hly possibly involved in the bacterial competition for iron. Among EHEC O157, isolates harbouring genes coding for aerobactin or α-hemolysin are rare. Only 1 out of 13 isolates have either the gene *iutA* or *hlyA*. This is not surprising since these isolates all have a plasmid harbouring the gene coding for EHEC-hemolysin. Probably, at least one of the ways that the enterohemolysin contributes to the pathogenesis of EHEC is that haemoglobin released by the action of E-hly provides a source of iron. This is also supported by Torres and Payne who, in 1997, identified and cloned a gene encoding an iron-regulated haem-transport protein and showed that this *E. coli* haem-utilization gene (*chuA*) encoded an outer membrane protein that was synthesized in response to iron limitation. Expression of this protein in a laboratory strain of *E. coli* O157:H7 was sufficient for utilization of haem or haemoglobin as iron sources and it was highly homologous to the *shuA* gene of *Shigella dysenteriae (Torres 1997)*.

On the contrary we have found (paper IV) that the gene coding for aerobactin is rather common in EHEC non-O157. Interestingly, these isolates usually have either the *iutA* gene coding for aerobactin or the *E-hly* gene, rarely (in 3 out of 18 isolates) both genes simultaneously. Taking EHEC O157 and non-O157 together 97% of the isolates possess at least one of the three genes coding for aerobactin, α-hemolysin or EHEC-hemolysin coding for traits affecting the iron support. A similar phenomenon was identified by Valvano et al. *(Valvano 1986)* when studying virulent neonatal *E. coli* K1 isolates. By comparison of the α-hemolysin production with possession of the aerobactin system they found that all of the aerobactin-producing isolates did not synthesize α-hemolysin, whereas 11 of 12 aerobactin-nonproducing isolates were haemolytic. Of the K1 strains examined, 92.5% possessed either the aerobactin system or the ability to produce α-hemolysin or both.

To strengthen the hypothesis concerning possible virulence factors in EHEC, verocytotoxin producing *E. coli* isolated from healthy cattle were analysed, as beef and dairy products are the most common source of human EHEC infection. There is a relatively low incidence of EHEC in comparison to a high presence of VTEC in cattle. The present thesis presents the results that VTEC non-O157 isolated from cattle only in 18% harbour the *eaeA* gene in comparison to 61% among the human isolates. This is in agreement with previously reported results *(Barrett 1992, Jenkins 2002)*. However, we have also shown among isolates from both humans and cattle that isolates not having the gene coding for intimin at least have the gene coding for type 1 fimbriae and vice versa. This may indicate that type 1 fimbriae is an important adhesin in the gut of both humans and cattle, while the *eaeA* gene is less valuable to obtain a niche in the intestines of cattle. As the gene coding for type 1 fimbriae and the *eaeA* gene are also often present in the same organism, it may also be the case that type 1 fimbriae can initiate adherence and make subsequent intimate adherence and A/E lesion formation more likely to occur. It has however been shown that the type 1 fimbriae is not expressed in EHEC O157 because of a gene deletion, while it is expressed in some studied EHEC non-O157 *(Sherman 1987, Enami 1999, Roe 2001)*. The presence of fimbriae on the surface of *E. coli* O157:H7 has been reported by several investigators *(Karch 1987, Toth 1990, Fratamico 1993, Elliott 2000)*, but their genetic representation and their molecular structure are still unknown. Some putative adhesin factors are now under investigation. In recent years, investigators have identified gene clusters both chromosomal, sfp and Iha-reactive protein, and in a plasmid, $lpfA_{O113}$, of isolates of EHEC that may function as adhesins *(Brunder 2001, Schmidt 2001b, Doughty 2002)*. The chromosomal gene clusters are encoded by *eaeA* negativ EHEC O113 and O91:H$^-$ respectively and the plasmid gene was identified in a sorbitol fermenting EHEC O157:H$^-$. Even if the significance of these gene clusters is not yet fully understood, there are obviously additional adhesin gene clusters beside the LEE and type 1 fimbriae that could add to the capacity to adhere to human epithelial cells.

None of the cattle isolates in this study harboured the gene sequences coding for P, S and F1845 fimbriae. Mainil et al. used a probe technique to study the

presence of F1845 and they made the same observation that F1845 is rare among VTEC isolates of cattle with diarrhoea *(Mainil 1993)*. In a study performed by Bertin et al. P and S fimbriae are found to be usual among *E. coli* isolated from cattle that suffer from diarrhoea. These isolates, however, belong to a group of *E. coli* that also produce CNF1 the cytoxic necrotizing factor type I but not verocytotoxin *(Bertin 1998)*. Osek et al. have however made observations similar to the ones presented in this study as they found only a few isolates of VTEC with P fimbriae *(Osek 2000)*.

The most interesting difference between the human and cattle isolates as shown in the present thesis is, however, that only 45% of the cattle isolates harbour the genes *E-hly*, *iutA* and/or *hlyA* coding for traits affecting the iron support whereas 97% of the human isolates possesses at least one of these determinants. We postulate that it is important to have any of these traits for the competition for iron in the human gut, while it seems less important in the intestines of cattle. This difference is maybe also one of the reasons to the rather few incidences of human EHEC infection, in comparison to the relatively high frequency of VTEC in cattle. The difference also indicates the importance of aerobactin as a virulence factor of EHEC non-O157. It has previously been shown that the EHEC-hemolysin production and heme or hemoglobin utilization are factors more common to human isolates than to isolates from cattle *(Law 1995)*. Moreover, Osek et al. found the same low number of VTEC harbouring *E-hly* as presented in our study among calves with diarrhoea *(Osek 2000)*. The presence of the gene sequence enabling aerobactin syntheseis was however not studied and we believe that the expression of aerobactin or, to some extent, α-hemolysin contributes to the pathogenicity of EHEC non-O157.

CONCLUSIONS

This thesis shows the importance of screening for EHEC among children and older patients with severe symptoms as HUS or bloody diarrhoea, identifying the *VT* genes. Using culture and O157 agglutination only, half the amount of patients, probably even fewer, would have been identified. The PCR method detecting the *VT* genes appeared to be a fast, sensitive, and specific method.

The period of incubation, which has said to be fairly short, was shown to be between 8 hours and 16 days. Within one week after the first positive EHEC specimen, about 70% (9/13) of the patients were still positive.

PFGE was a well-suited method for epidemiological typing of EHEC both O157 and non-O157, and was more discriminative than serogrouping which had little value.

Knowledge about virulence markers except for the *VT* genes is scarce. The *eaeA* and *E-hly* genes coding for intimin and EHEC-hemolysin seemed in these studies to be markers, beside the *VT1* and *VT2* genes, for the most virulent isolates of EHEC O157 and non-O157.

The *eaeA*, *E-hly* and *iutA* genes coding for intimin, EHEC-hemolysin and aerobactin, respectively, were present to a higher extent among human isolates than isolates from cattle. As the low frequency of EHEC in humans does not correspond to the high numbers of VT producing *E. coli* in the main source of infection, cattle, this fact may be one of the reasons why not all VT producing *E. coli* are pathogenic. Furthermore, aerobactin encoded by the gene *iutA* seemed to some extent to be able to compensate for the absense of EHEC-hemolysin in some isolates and the expression of aerobactin may therefore contribute to the virulence especially of EHEC non-O157.

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