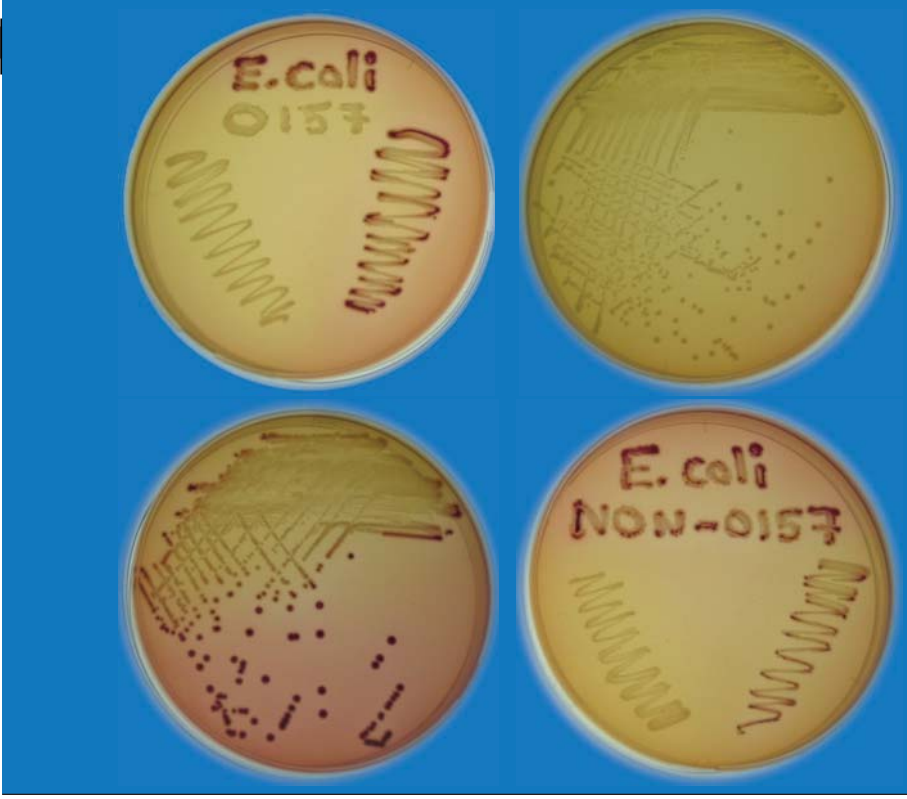


**Marjut Eklund**

# Enterohemorrhagic *Escherichia coli* (EHEC) Findings from Humans in Finland

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Department of Bacterial and Inflammatory Diseases
National Public Health Institute Helsinki, Finland
*and*
Department of Applied Chemistry and Microbiology
University of Helsinki, Finland

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# ENTEROHEMORRHAGIC *Escherichia coli* (EHEC) FINDINGS FROM HUMANS IN FINLAND

A C A D E M I C  D I S S E R T A T I O N

*To be presented with the permission of the Faculty of Agriculture and Forestry, University of Helsinki, for public examination in Auditorium no 1041, Viikinkaari 5, on December 9, at 12 noon.*

Enteric Bacteria Laboratory
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Department of Applied Chemistry and Microbiology
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*To my family*

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# ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC), also known as Shiga toxin-producing *E. coli* (STEC) or verocytotoxigenic *E. coli* (VTEC), is a food- and waterborne pathogen that spreads also by zoonotic or person-to-person transmission route causing severe enteric infections. A typical symptom of EHEC infection in humans is bloody diarrhea but the infection may lead to severe complications as thrombotic microangiopathy, known also as hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP), or even death. Globally, the most significant serotype has been EHEC O157:H7. However, among shigatoxigenic *E. coli*, approximately 450 O:H serotypes have been described. Of these, over 200 have been associated with human infections. During the past decade, the prevalence of human EHEC non-O157 infecions has increased in several countries.

The aim of this study was to gain epidemiological knowledge and data on pheno- and genotypic characteristics of EHEC O157 and non-O157 strains isolated from humans in Finland. For the analysis, all those EHEC strains isolated during 1990–2002 were investigated. In addition, three *E. coli* O157 strains lacking the *stx* genes were included. The strains were differentiated for virulence-associated genes (*stx*, *stx* variants, *eae* and its subtypes, *efa*, *cdt*, EHEC-*hlyA*, *etpD*, *katP*, *espP*, and *sfpA*) by molecular genetic methods. Also, phenotypic tests for other microbial characteristics (O:H serotype, phage type, Stx-, Ehly-, and CDT-production) were used. The pheno- and genotypic properties were analyzed for epidemiological surveys of the EHEC O157 and non-O157 strains. In addition, the virulence profiles of the EHEC strains were studied in relation to the clinical picture of the patients and the geographical prevalence of the domestic infections was investigated. In-depth interviews of the infected people were explored to detect putative sources of human EHEC infections. Furthermore, the clonality of nonmotile, sorbitol-fermenting (SF) *E. coli* O157 strains was compared to the SF *E. coli* O157 strains occurring in Germany.

During the study period, 200 infections caused by strains of EHEC O157 (127 cases; 64%) and non-O157 (73 cases; 36%) occurred in Finland. Most were of domestic origin: 84% of the O157 infections and 78% of the non-O157 infections. Among the domestic infections during 1998-2002, family-related cases accounted for half (49%), followed by sporadic infections (39%), and 11% was associated with three infection clusters. The incidence of the EHEC infections was at its highest in 1998 (0.64 / $10^5$ population), and lowest (0.17 / $10^5$ population) in 2002. Only in 1998

was the incidence of O157 cases higher than that of the non-O157 cases. Geographically, most commonly indigenously acquired EHEC infections were found in the Helsinki Region (28% of the infections; incidence 2.0). However, the population-based incidence was highest in Central Ostrobothnia (8% of the infections; incidence 10.3).

Only strains of the serogroup O157 caused separate infection clusters (C1-C3) affecting 11 people in 1998 and 2001 in Päijät-Häme (C1) and Southwest Finland (Varsinais-Suomi) (C1), and Southeast Finland (Kymenlaakso) (C2, C3). In pheno- and genotypic comparison of the strains, the strain of the subtype O157:H7:PT2:*stx*$_2$:*stx*$_{2c}$:*eae*:1.1 caused three out of four infections in C1. The strain (O157:H$^-$:PT88:*stx*$_2$:*eae*:1.13) was isolated from a child whose mother was infected with a strain of the C1 type. In C2, strains of the subtypes O157:H7:PT4:*stx*$_2$:*eae*:1.57 and O157:H7:PT4:*stx*$_2$:*eae*:1.58 caused four infections. In C3, a strain of the subtype O157:H7:PT14:*stx*$_{1,2}$:*eae*:1.67 caused all three infections. The implicated vehicles for these clusters were putative person-to-person or food-borne transmission. Interestingly, in Finland about 10% SF O157:H7/H$^-$ were detected. The pheno- and genotypic characteristics of these strains shared similar characteristics with strains commonly occurring in Europe, suggesting a transmission of the strains at European Union level. In particular, among the strains of O157:H$^-$:PT88:*stx*$_2$:*eae*, several other determinants also supported their relatedness with similar strains which have recently emerged in Europe.

Among the non-O157 strains, 34 O:H serotypes were detected. As in other European countries, the most common O groups among the isolates were O26, O103, and O145. In addition, two new EHEC serotypes (O102:H7 and O181:H49) associated with human infections were detected, strengthening the importance of EHEC non-O157 strains as human pathogens. Also, seven pheno- and genotypic profiles of non-O157 strains were found from patients suffering from HUS or TTP. However, a particular virulence profile of EHEC O157 group (O157:H7:PT2:*stx*$_2$:*stx*$_{2c}$:*eae*:Ehly) was significantly ($P$=0.02) more frequently associated with a severe clinical picture (bloody diarrhea, HUS, TTP) of the patients than were other profiles; the risk factors for severe symptoms was under 5 year-olds and infection by this type of O157:H7 strain. Strains of this virulence profile also caused the deaths of two children.

For EHEC, a pheno- and genotypically heterogeneous group of strains causing severe infectious diseases, effective surveillance is continuously needed thus allowing detailed early diagnostics of EHEC and preventing the spread of the infection. In particular, pheno- and genotypic characterization of the human EHEC isolates enables the sources of the infections to be swiftly traced both at national and international level, and helps to evaluate the putative virulence ability of the strain.

Keywords: enterohemorrhagic, *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), zoonotic pathogen, epidemiology, virulence characteristics.

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# TIIVISTELMÄ

Enterohemorraaginen *Escherichia coli* (EHEC), josta käytetään myös nimityksiä shigatoksinen *E. coli* (STEC)- tai verosytotoksinen *E. coli* (VTEC)-bakteeri, on elintarvike- ja vesivälitteinen patogeeni, joka leviää myös zoonoottisesti tai henkilöstä toiseen tapahtuvassa tartunnassa aiheuttaen vakavia suolistoinfektioita. Tyypillisimmin EHEC-infektio aiheuttaa ihmisillä veriripulin, mutta tartunta voi johtaa vakaviin komplikaatioihin kuten tromboottiseen mikroangiopatiaan, josta on käytetty myös termejä hemolyyttis-ureeminen syndrooma (HUS) tai tromboottinen trombosytopeeninen purppura (TTP), tai jopa kuolemaan. Maailmanlaajuisesti merkittävä EHEC serotyyppi on EHEC O157:H7, mutta shigatoksisessa *E. coli* -bakteeriryhmässä on kuvattu noin 450 O:H serotyyppiä. Näistä yli 200 on liittynyt humaanitartuntoihin. Viimeisen kymmenen vuoden aikana EHEC non-O157 humaanitartuntojen prevalenssi onkin noussut useassa maassa.

Tämän tutkimuksen tarkoituksena oli saada tietoa Suomessa eristettyjen EHEC O157 ja non-O157 kantojen feno- ja genotyyppisistä ominaisuuksista sekä niiden epidemiologiasta. Tutkimuksessa analysoitiin kaikki aikavälillä 1990–2002 eristetyt EHEC kannat. Tutkimukseen sisällytettiin myös kolme *stx*-negatiivista *E. coli* O157 kantaa. Kannat eroteltiin virulenssiin liittyvien geenien (*stx*, *stx* variantti, *eae* ja sen alatyypit, *efa*, *cdt*, EHEC-*hlyA*, *etpD*, *katP*, *espP* ja *sfpA*) perusteella molekyyligeneettisin menetelmin. Muiden ominaisuuksien (O:H serotyyppi, faagityyppi, Stx-, Ehly- ja CDT-tuotto) karakterisoimiseksi käytettiin fenotyyppisiä testejä. Feno- ja genotyyppiset ominaisuudet analysoitiin EHEC O157 ja non-O157 kantojen epidemiologista tutkimusta varten. Lisäksi EHEC-kantojen virulenssiprofiilit tutkittiin suhteessa potilaiden kliiniseen kuvaan ja kotimaisten tartuntojen maantieteellinen esiintyvyys kartoitettiin. EHEC-tartunnan saaneiden ihmisten syvähaastattelut analysoitiin etsittäessä mahdollisia EHEC-tartunnan lähteitä. Myös liikkumattomien, Suomessa esiintyvien sorbitoli-positiivisten (SF) *E. coli* O157 kantojen samankaltaisuutta verrattiin vastaaviin Saksalaisiin kantoihin.

Tutkimusaikavälillä Suomessa esiintyi 200 EHEC-tartuntaa: 127 (64%) EHEC O157- ja 73 (36%) non-O157-tartuntaa. Suurin osa tartunnoista oli kotimaista alkuperää: 84% O157-tartunnoista ja 78% non-O157-tartunnoista. Kotimaisista tartunnoista vuosina 1998–2002 noin puolet (49%) oli perheensisäisiä tartuntoja, yksittäistapauksia oli 39% ja 11% liittyi kolmeen tartunta-ryppääseen. EHEC

-tartuntojen esiintyvyys oli korkeimmillaan vuonna 1998 (0.64 / $10^5$ populaatio), ja alhaisemmillaan (0.17 / $10^5$ populaatio) vuonna 2002. Vain vuonna 1998 O157 tapausten esiintyvyys oli korkeampi kuin non-O157 tapausten. Kotimaista alkuperää olevat EHEC tartunnat havaittiin pääasiallisesti Helsingin seudulla (28% tartunnoista; esiintyvyys 2.0), mutta esiintyvyys oli korkein Keski-Pohjanmaalla (8% tartunnoista; esiintyvyys 10.3).

Ainoastaan seroryhmän O157 kannat aiheuttivat erillisiä tartuntaryppäitä (C1-C3), jotka liittyivät 11 tartuntaan vuosina 1998 ja 2001 Päijät-Hämeessä (C1), Varsinais-Suomessa (C1), ja Kymenlaaksossa (C2, C3). C1 ryppääseen liittyvien kantojen feno- ja genotyyppisessä vertailussa tyyppiä O157:H7:PT2:*stx*$_2$:*stx*$_{2c}$:*eae*:1.1 oleva kanta aiheutti kolme tartuntaa neljästä. Neljäs, O157:H$^-$:PT88:*stx*$_2$:*eae*:1.13 -kanta eristettiin lapselta, jonka äidillä oli edellämainitun C1 tyypin aiheuttama tartunta. C2 ryppäässä tyyppien O157:H7:PT4:*stx*$_2$:*eae*:1.57 ja O157:H7:PT4:*stx*$_2$:*eae*:1.58 kannat aiheuttivat neljä tartuntaa. C3 ryppäässä tyypin O157:H7:PT14:*stx*$_{1,2}$:*eae*:1.67 kanta aiheutti kaikki kolme tartuntaa. Ryppäiden C1-C3 vehikkeleiksi osoitettiin mahdollinen henkilöstä toiseen tapahtunut tai elintarvikevälitteinen tartunta. Mielenkiintoisesti, Suomessa havaittiin myös 10% SF O157:H7/H$^-$ kantojen aiheuttamia tartuntoja. Näiden kantojen feno- ja genotyyppiset ominaisuudet olivat samankaltaisia Euroopassa yleisesti esiintyvien SF O157:H7/H$^-$ kantojen kanssa, viitaten kyseisten kantojen levinneisyyteen Euroopan Unionin alueella. Erityisesti, O157:H$^-$:PT88:*stx*$_2$:*eae* kannoilla useat tekijät tukivat kantojen sukulaisuutta samantyyppisiin, lähiaikoina Euroopassa esiintyviin kantoihin.

Non-O157 kannat tyypittyivät yhteensä 34 O:H serotyyppiin. Kuten muissakin Euroopan maissa, kantojen yleisimmät O ryhmät olivat O26, O103, ja O145. Myös kaksi uutta humaanitartuntoihin liittyvää EHEC serotyyppiä (O102:H7 ja O181:H49) löydettiin, mikä vahvistaa EHEC non-O157 kantojen tärkeää asemaa humaani-patogeeneina. Myös seitsemän erilaista non-O157 kantojen feno- ja genotyyppiprofiilia löydettiin kannoista, jotka oli eristetty HUS tai TTP-potilailta. Kuitenkin, verrattuna kaikkiin muihin profiileihin, EHEC O157 virulenssiprofiili O157:H7:PT2:*stx*$_2$:*stx*$_{2c}$:*eae*:Ehly liittyi tilastollisesti merkitsevästi ($P$=0.02) useammin potilaiden vakavaan taudinkuvaan (veriripuli, HUS, TTP). Vakavan taudinkuvan riskitekijäksi muodostui potilaan alle viiden vuoden ja tämän O157:H7 kantatyypin aiheuttama tartunta. Kyseisen virulenssiprofiilin omaavat kannat aiheuttivat myös kahden lapsen kuoleman.

EHEC-kannat, jotka ovat feno- ja genotyyppisesti heterogeeninen, vakavia tarttuvia tauteja aiheuttava patogeeninen ryhmä, edellyttävät jatkuvaa tehokasta diagnosointia, joka mahdollistaa yksityiskohtaisen, varhaisen EHEC tartuntojen toteamisen ja uusien tartuntojen ennaltaehkäisemisen. Erityisesti EHEC kantojen feno- ja genotyyppien karakterisointi mahdollistaa tartuntalähteiden nopean jäljitettävyyden sekä kansallisella että kansainvälisellä tasolla ja lisäksi edesauttaa EHEC kantojen mahdollisten virulenssiominaisuuksien arviointia.

Avainsanat: enterohemorraaginen *E. coli* (EHEC), shigatoksinen *E. coli* (STEC), zoonoosi, epidemiologia, virulenssiominaisuudet

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals (I-V). In addition, some unpublished data are presented.

**I.** Saari, M., Cheasty, T., Leino, K., and Siitonen, A. 2001. Phage types and genotypes of Shiga toxin-producing *Escherichia coli* O157 in Finland. J. Clin. Microbiol. 39: 1140-1143.

**II.** Eklund, M., Scheutz, F., and Siitonen, A. 2001. Clinical isolates of non-O157 Shiga toxin-producing *Escherichia coli*: serotypes, virulence characteristics and molecular profiles of strains of the same serotype. J. Clin. Microbiol. 39: 2829-2834.

**III.** Eklund, M., Leino K., and Siitonen, A. 2002. Clinical *Escherichia coli* strains carrying *stx* genes: *stx* variants and *stx*-positive virulence profiles. J. Clin. Microbiol. 40: 4585-4593.

**IV.** Eklund, M., Nuorti, J. P., Ruutu, P., and Siitonen, A. 2005. Shigatoxigenic *Escherichia coli* (STEC) infections in Finland during 1998-2002: a population-based surveillance study. Epidemiol. Infect. 133: 845-852.

**V.** Eklund, M., Bielaszewska, M., Nakari, U-M., Karch, H., and Siitonen, A. 2005. Molecular and phenotype profiling of sorbitol-fermenting *Escherichia coli* O157:H$^-$ human isolates from Finland. *Submitted.*

# AUTHOR'S CONTRIBUTION

**Publication I**
Marjut Eklund (née Saari) set up the phage typing and the nationally standardized pulsed-field gel electrophoresis (PFGE) method with an electronic library of PFGE patterns for *E. coli* O157 at the Enteric Bacteria Laboratory (EBL), National Public Health Institute (KTL). She carried out pheno- and genotypic testing of the strains studied and was responsible for experimental work. She interpreted the results and wrote the paper.

**Publication II**
Marjut Eklund set up the PFGE method with an electronic library of PFGE patterns for *E. coli* non-O157, and modified a polymerase chain reaction (PCR) method detecting $stx_1$ at the EBL. She carried out pheno- and genotypic testing of the strains studied and was responsible for experimental work. She interpreted the results and wrote the paper.

**Publication III**
Marjut Eklund set up the PCR-restriction fragment length (RFLP) for detection of $stx_{2d\text{-}Ount}$ and $stx_{2d\text{-}OX3a}$ at the EBL. She carried out pheno- and genotypic testing of the strains studied. She was responsible for all experimental work. She interpreted the results and wrote the paper.

**Publication IV**
Marjut Eklund carried out pheno- and genotypic testing of the strains studied. She analysed epidemiological and microbiological data on patients, saved the microbiological data of the interviewed patients on the Epi-Info programme. She interpreted the results and wrote the paper together with co-authors.

**Publication V**
Marjut Eklund set up the internationally standardized PFGE method with an electronic library of PFGE patterns for EHEC strains. She carried out pheno- and genotypic testing of the strains studied and was responsible for experimental work performed at the EBL. She interpreted the results and wrote the paper together with co-authors.

# ABBREVIATIONS

| | |
|---|---|
| AAF | Aggregative adherence fimbria |
| A/E | Attaching and effasing |
| AIDA | Plasmidal adhesin |
| ASY | Asymptomatic carriage |
| ATCC | American type culture collection |
| Bdi | Bloody diarrhea |
| BCCM | Belgium Coordinated Collections of Microorganisms, Belgium |
| BFP | Bundle forming pilus |
| bp | Base pair |
| C | Cluster, numbered from C1 to C3 |
| CDC | Centers for Disease Control and Prevention, USA |
| CDT | Cytolethal distending toxin |
| CDT-EC | Cytolethal distending toxin-producing *E. coli* |
| CFs | Fimbrial colonization factors |
| CHO | Chinese hamster ovary cell line |
| ClyA | Cytolysin A |
| CT-SMAC | SMAC plate containing cefiximide and tellurite |
| DAEC | Diffusely adherent *E. coli* |
| DNA | Deoxyribonucleic acid |
| EAEC | Enteroaggregative *E. coli* |
| EAF | EPEC adherence factor |
| EAggEC | Enteroaggregative *E. coli* |
| EAST | Enteroaggregative heat stable toxin |
| EBL | Enteric Bacteria Laboratory of KTL |
| EELA | National Veterinary and Food Research Institute, Finland |
| Efa1/LifA | EHEC factor for adherence |
| EHEC | Enterohemorrhagic *E. coli* |
| Ehly | Enterohemolysin |
| EIA | Enzyme immunoassay |
| EIEC | Enteroinvasive *E. coli* |
| EPEC | Enteropathogenic *E. coli* |
| Esp | *E. coli* secreted protein |
| ET | Electrophoretic type |
| ETEC | Enterotoxigenic *E. coli* |
| ETP | Putative type II secretion system |
| H | Flagellar (H) -antigen |
| HC | Hemorrhagic colitis |
| HNM | Nonmotile ($H^-$) strain, a strain lacking flagella |
| HPA | Health Protection Agency, England |
| HPI | High pathogenicity island |
| HUS | Hemolytic-uremic syndrome |
| IH | Isolate Helsinki, strain in the culture collection of KTL |
| Iha | Adhesion protein |
| IMS | Immunomagnetic separation |
| Int | Adhesion protein intimin encoded by *eae* gene of LEE locus |
| Ipa | Invasion plasmid antigen |
| K | Capsular polysaccharide, K-antigen |
| KatP | Katalase peroxidase |
| KTL | Kansanterveyslaitos, National Public Health Institute |

| | |
|---|---|
| LA | Localized adherence |
| LEE | Locus of enterocyte effacement |
| LEP | Laboratory of Enteric Pathogens of KTL |
| Ler | LEE-encoded regulator |
| LifA/Efa | EHEC factor for lymphostatin and adherence |
| LPS | Lipopolysaccharide |
| LT | Heat-labile toxin |
| MLE | Multilocus enzyme electrophoresis |
| MLST | Multilocus sequence typing |
| MLVA | Multilocus VNTR analysis |
| NSF | Non sorbitol fermenting |
| O | O polysaccharide, O antigen |
| ONT | O Nontypeable |
| ORF | Open reading frame |
| PAI | Pathogenecity island |
| PCR | Polymerase chain reaction |
| Per | Plasmid-encoded regulator |
| PET | Plasmid-encoded enterotoxin |
| PFGE | Pulsed-field gel electrophoresis |
| PGUA | β-Glucuronidase (GUD) |
| pINV | Invasivity plasmid |
| PT | Phage type |
| RDNC | Reacts but does not conform |
| RFLP | Restriction fragment length polymorphism |
| RH | Reference strain Helsinki, strain in the culture collection of KTL |
| RKI | Robert Koch Institute, Germany |
| RPLA | Reversed passive latex agglutination |
| RTX | Repeats in toxin |
| SF | Sorbitol fermenting |
| ShET1 | Shigella enterotoxin 1 |
| SI | Similarity index |
| SLT | Shiga-like toxin |
| SLTEC | Shiga-like toxin producing *E. coli* |
| SSI | Statens Serum Institute, Denmark |
| SMAC | Sorbitol MacConkey agar |
| Sor | Sorbitol |
| ST | Heat-stabile toxin |
| STEC | Shiga toxin-producing *E. coli* |
| Stx | Shiga toxin |
| Tir | Translocated intimin reseptor |
| ToxB | Clostridial toxin |
| TTP | Thrombotic thrombocytopenic purpura |
| UV | Ultraviolet |
| VT | Verotoxin |
| VTEC | Verotoxin-producing *E. coli* |
| R | O Rough |
| VNTR | Variable number tandem repeats |
| WHO | World Health Organization |

# INTRODUCTION

Diarrheal diseases transmitted by enteric pathogens are major causes of morbidity and mortality in humans (Kaur et al. 2003). Globally, over 2 million deaths related to diarrhea occur each year, particularly among under five-year old children (Clarke et al. 2003, www.who.int). In Finland, 500, 000 people are estimated to suffer from gastrointestinal illnesses annually (Pönkä 1999).

Among diarrheagenic pathogens, Shiga toxin-producing (Stx), enterohemorrhagic *Escherichia coli* (EHEC) are described as a heterogeneous group of highly pathogenic bacteria with very low infective dose; even 1-100 cells are capable of causing disease (Griffin 1998, Jaeger and Acheson 2000). For the first time, EHEC emerged as human pathogens in the USA in the early 1980s during large-scale outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (HUS) caused by shigatoxigenic, non-sorbitol fermenting (NSF) strains of *E. coli* O157:H7 (Karmali et al. 1983a, 1983b, Riley et al. 1983). Ever since, strains of NSF *E. coli* O157:H7 have been epidemiologically, microbiologically, and clinically important worldwide. In addition, a new lineage, a sorbitol-fermenting (SF) nonmotile ($H^-$) O157, was identified as the cause of outbreaks of HUS in Germany in 1988 (Karch et al. 1990, Ammon et al. 1999, Robert Koch-Institut 2002). Recently, strains of this phenotype have emerged in several other European countries (Karch and Bielaszewska 2001), and in Australia (Bettelheim et al. 2002). Currently, among all known Stx producing *E. coli*, over 450 O:H serotypes of human and non-human origin have been detected (Blanco et al. 2004, www.microbionet.com.au). Of these non-O157 serotypes, strains of O26:H11/$H^-$, O103:H2/$H^-$, O111:H2/$H^-$, and O145:H28/$H^-$ deserve special mention by causing outbreaks, as well as in association with severe illnesses (Schmidt et al. 2001).

In addition to EHEC, five well-described pathogroups of *E. coli* with different virulence mechanisms have been associated with human diseases: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Kaper et al. 2004). Virulence mechanisms that characterize the pathogroups of *E. coli* are genetically determined by chromosomally located factors (pathogenecity islands, chromosomally inserted bacteriophages) or extra-chromosomal elements (plasmids) that encode diarrhea-associated characteristics like colonization ability, production of toxins and hemolysins (Aranda et al. 2004, Kaper et al. 2004). The main virulence factor of EHEC bacteria has been concluded to be a production of Stx toxin(s) (Stx1 and/or Stx2) or their variants encoded by the *stx* genes. However, the pathogenesis of EHEC bacteria is considered multifactorial and several augmenting virulence-factors have been described (Paton et al., 2001, Kaper et al. 2004, Paton et al. 2004, Thorpe et al. 2004). Of other diarrheagenic *E. coli*, EPEC have caused, especially in developing countries, large outbreaks of infantile watery diarrhea by causing attaching and effacing lesions (A/E encoded by *eae*) in intestinal epithelial cells

(Donnenberg et al. 1993). ETEC has been the most common *E. coli* group associated with tourist diarrhea with production of heat labile (LT) and/or stable (ST) toxin encoded by respective *lth* and *sta* genes (Holmgren and Svennerholm 1992, Koprowski et al 2000, Olasz et al. 2005). Unlike other diarrheagenic *E. coli* strains, EIEC characteristically cause *Shigella*-like invasive infection also producing plasmid (pINV) mediated proteins encoded by *ipa*, *ial* genes, typically leading to bloody diarrhea (Sethabutr et al. 1993, Nataro and Kaper 1998, Aranda et al. 2004, Schmidt and Hensel 2004). Strains of EAEC group are recognized by aggregative, localized adherence (LA; encoded by *bfpA*) of the bacterial cells on the microvilli and a production of EAEC toxin (EAST). DAEC, also known as cell-detaching *E. coli*, are characterized by diffuse adherence to epithelial cells, possession of adhesin fimbria encoded by a fragment of *daaC* gene, α-hemolysin production, and cytotoxic necrotising factor 1 (Nataro and Kaper 1998, Clarke 2001). In addition, the cytolethal distending toxin (CDT)-producing *E. coli* forms the latest group of enteric pathogens (Clarke 2001).

# REVIEW OF THE LITERATURE

## 1. *Escherichia coli* in health and disease

*Escherichia coli* was first described by the German pediatrician Dr. Theodor Escherich as *Bacterium coli commune* that had been isolated from the feces of a healthy infant (Escherich 1885, Sussman 1985). Even today, *E. coli* is said to be the most abundant of the facultative anaerobic species of the normal human enteric microbiota in feces: the enterobacteria are present at about $10^8$–$10^9$ / g, *E. coli* being the most important species (Bettelheim et al. 2003). This symbiosis provides a nourishing environment for bacteria, offers essential nutrients to the intestinal epithelium and promotes healthy immune responses in the host. In addition, *E. coli* is known to affect the synthesis of vitamin K in the host. Certain *E. coli* strains might also serve as an important factor for inhibition of the growth of enteropathogens (Kruis 2004). Moreover, *E. coli* is also capable of surviving in the environment, water, and food, and spreading efficiently (Faiella-Tommasino and Reigert 2002).

Thousands of serotypes of *E. coli* species, in the *Escherichia* genus, within the family of *Enterobacteriaceae*, form the intestinal bacterial group described as gram-negative, non-sporulating facultative anaerobic rod, usually motile by peritrichous flagella (Ørskov and Ørskov 1983). Pathogenic *E. coli* that do not belong to the normal microbiota, harbor virulence factors, such as adhesins, invasins, entero- and cytotoxins encoded by extrachromosomal plasmids, chromosomal pathogenicity islands, or bacteriophage integrated virulence factors for defeating host defences in order to cause intestinal and extra-intestinal diseases (Kaper et al. 2004). In clinical microbiology, *E. coli* has been the most commonly isolated facultative anaerobic gram-negative rod in feces, and a common cause for intestinal and extra-intestinal infections. The most common clinical outcomes of infection caused by pathogenic *E. coli* have been urinary tract infection, sepsis and meningitis, and enteric diarrheal disease (Kaper et al. 2004).

### 1.2 Classification of diarrheagenic *E. coli*

According to epidemiologic data and pathogenic characteristics, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC) have been validated as the main diarrheagenic *E. coli* pathogroups (Table 1). In addition, with conflicting data, CDT-producing *E. coli* (CDT-EC) are said to be one diarrheal *E. coli* group (Clarke 2001, Kaper et al. 2004, Torres et al. 2005).

**EPEC** were first reported in 1945 by John Bray as "*Bacillus coli nepolitanum*", causative agent of summer diarrhea in infants (Chen and Frankel 2004). Even today, EPEC is a common agent for diarrhea in children in developing countries with a mortality of even 30%, and sporadic EPEC infections appear in industrial countries of considerable concern. However, in industrialized countries, outbreaks caused by EPEC have been rare (Bopp et al. 2003, Kaper et al. 2004), and in Finland, since the 1940s, only one outbreak caused by EPEC has been reported in 1987 (Viljanen et al. 1990). The identification of this pathogen has previously been based on the serotyping, typical O groups being O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 (Bopp et al. 2003, Gomes et al. 2004). Nowadays, the hallmark of EPEC is the ability to form chromosomally by *eae* gene-encoded attaching and effacing (A/E) lesion in host cells and lack of Stx production (Kaper 1996, Chen and Frankel 2004). In addition, strains expressing plasmidal EPEC adherence factor (EAF) mediated by bundle-forming pili (BFP), have been defined as typical EPEC and strains lacking the EAF-plasmid have been designated as atypical EPEC (Kaper 1996, Trabulsi et al. 2002, Chen and Frankel 2004). In industrial countries, the prevalence of atypical EPEC has been higher compared to that in developing countries (Afset et al. 2003, Kaper et al. 2004).

**ETEC** strains were associated with human diarrheal illnesses in 1960s (Taylor et al. 1961, Nataro and Kaper 1998). Ever since, the occurrence of ETEC infection has been high in developing countries, it has been the common agent for tourist diarrhea, and a cause for occasional outbreaks in industrialized countries (Naimi et al. 2003). In Finland, the occurrence of ETEC infections has not been endemic (Keskimäki et al. 2000). During pathogenesis, ETEC colonizes intestinal epithelium using fimbrial colonization factors (CFs) and the related colonization factor antigens (Kaper et al. 2004). In addition, ETEC strains produce plasmid encoded heat-stable (ST) and/or heat-labile (LT) toxin (Nataro and Kaper 1998). The ST and LT toxins have been divided into subgroups Sta, STb and LT-I, LT-II, respectively. Of these, LT-I shares 75% amino acid similarity with cholera toxin (Nataro and Kaper 1998, Kuhnert et al. 2000). Moreover, some strains of the ETEC group have produced EAST1, a toxin characteristic for the EAEC group (Savarino et al. 1996).

**EIEC** was first described in 1944 as a paracolon bacillus, later identified as *E. coli* O124 (Lan et al. 2004). In 1971, EIEC strains were associated with diarrheal diseases (DuPont et al. 1971), bloody diarrhea being typical of EIEC infection, and the occurrence being high in poor countries (Beutin et al. 1997, Nataro and Kaper 1998, Sarantuya et al. 2004). EIEC strains are generally nonmotile and only a few O groups including O28ac, O29, O112ac, O121, O124, O135, O136, O143, O144, O152, O159, O164, O167, and O173 have been found (Cheasty et al. 1983, Lan et al. 2004). EIEC strains invade and multiply within enterocytes, causing the death of these hosts (Escobar-Paramo et al. 2003, Thiem et al. 2004). The invasive character of EIEC is governed by a 140 MDa invasivity plasmid (pInV), which encode a type III secretion system (TTSS) and respective TTSS effector proteins. The TTSS is also homologous to that of Salmonella and Yersinia (Sansonetti 1992, Sethabutr et al.

1993, Nataro and Kaper 1998, Lan et al. 2004, Schmidt and Hensel 2004). In addition, striking phenotypic and genotypic similarities have related EIEC strains with *Shigella* spp (Escobar-Paramo et al. 2003, Lan et al. 2004).

**EAEC**, previously known as EAggEC, has been recognized as an important agent of acute and prolonged diarrhea especially in children of developing world, a common cause of travelers' diarrhea, and increasingly an agent of outbreaks in developed countries since 1987 (Itoh et al. 1997, Nataro and Kaper 1998, Okeke and Nataro 2001, Huang et al. 2004, Nataro 2005). However, in Finland, only sporadic infections have occurred (Keskimäki et al. 2001). EAEC strains are a highly heterogeneous group consisting of about 90 identified serotypes, the most common being O15:H18, O44:H18, O77:H18, O111:H12, O125, and O126 (Okeke and Nataro 2001). EAEC are defined by their aggregative adherence to intestinal epithelial cells in a characteristic "stacked-brick" pattern (Nataro and Kaper 1998). During pathogenesis, EAEC have adhered to the intestinal mucosa by plasmid encoded aggregative adherence fimbria (AAF/I–AAF/III), increased production of a mucus biofilm, mucosal toxicity due to inflammation, and cytokine release (Huang et al. 2004, Torres et al. 2005). Other virulence factors of EAEC have been plasmidal enterotoxin (PET) (Eslava et al. 1998) and enteroaggregative heat stable toxin 1 (EAST-1) (Kuhnert et al. 2000, Okeke and Nataro 2001, Zhou et al. 2002). Also, some strains of EHEC have produced EAST-1 (Karch et al. 1999b).

**DAEC**, also known as cell-detaching *E. coli*, have caused diarrhea particularly in < 12 months-old children and has been an important pathogens in developed countries (Nataro and Kaper 1998, Clarke 2001, Kaper et al. 2004). Little is known of their exact pathogenesis but DAEC strains are characterized by their diffuse adherence pattern on epithelial cells, production of α-hemolysin and cytotoxic necrotising factor 1 (Clarke 2001). In addition, fimbrial adhesin F1845 mediated by both chromosome and plasmid, and plasmid encoded adhesin (AIDA-1) have been involved in diffuse adherence phenotype (Kaper et al. 2004, Torres et al. 2005).

**CDT-EC** has been designated as enteric pathogens with conflicting evidence for pathogenesis (Clarke 2001). In pathogenicity model, the CDT toxin has caused cell cycle arrest and subsequent death in eukaryotic cells (Clarke 2001, Bielaszewska et al. 2005). Also other diarrheagenic *E. coli* strains, EPEC, EAEC (Clarke 2001, Clark et al. 2002), and SF EHEC O157:H$^-$ strains (Janka et al. 2003) have produced this toxin. In contrast, CDT has rarely been characteristic for strains of NSF *E. coli* O157:H7 (Janka et al. 2003).

**EHEC** bacteria were first discovered in 1977 by the production of cytotoxin, verotoxin (VT), lethal to Vero (African green monkey) cells, which led to these pathogens being called verocytotoxigenic *E. coli* (VTEC) (Konowalchuk et al. 1977). In following studies of purificaton and characterization of verotoxin, O'Brien et al. (1982, 1983a, 1983b, 1984, 1987) observed a striking similarity with a structure and biological activity of the verotoxin and Stx toxin produced by *Shigella dysenteriae* type 1. This new toxin could also be neutralized by anti-Stx, and

subsequently it was called Shiga-like toxin (SLT). It was also observed that there were two major classes of SLT toxins: SLT-I and SLT-II known also as VT1 and VT2. Currently, these toxins have been also designated as Stx1 and Stx2, and have been found to include numerous variants (Nataro and Kaper 1998, Paton and Paton 1998, Kaper et al. 2004).

In early studies, *E. coli* strains isolated from diarrheal patients produced Stx but no significant proof of etiological significance of EHEC in diarrheal diseases or the possible pathogenetic significance of Stx was available (Karmali et al. 1989). In 1982, EHEC was clearly associated with human infectious diseases when shigatoxigenic *E. coli* serotype O157:H7 was identified as a cause of two outbreaks of hemorrhagic colitis in the USA (Riley et al. 1983). Almost simultaneously, a severe clinical outcome of the patients, hemolytic uremic syndrome (HUS), was associated with fecal cytotoxin and cytotoxin-producing *E. coli* in stools (Karmali et al. 1983a, 1983b). Moreover in Canada, Johnson et al. (1983) independently reported that *E. coli* O157:H7 strains isolated from patients with hemorrhagic colitis produced a cytotoxin active on Vero cells. Ever since, EHEC have been of considerable concern not only because of severity of the illness they can cause, but also the low infectious dose and increasing incidence worldwide: currently about 450 O:H serotypes of shigatoxigenic *E. coli* have occurred (Duffy et al. 2001, Blanco et al. 2004).

EHEC bacteria have acquired several chromosomal and plasmidal virulence factors. However, production of Stx1 and/or Stx2 or their variants encoded by phage-mediated chromosomally located *stx* genes is considered a main virulence factor (O'Brien et al. 1983a 1983b, Duffy et al. 2001). Adhesion mechanisms or production of other toxins, such as enterohemolysin (Ehly) or CDT, have been identified as augmenting virulence factors (Kaper et al. 2004). However, the nomenclature of EHEC and the Stx family is a complex issue (Acheson et al. 1999, Scheutz et al. 2001). The term enterohemorrhagic *Escherichia coli* (EHEC) was originally used to denote the Shiga-like toxin-producing *E. coli* (SLTEC) or Shiga toxin-producing *E. coli* (STEC), synonymous with the verocytotoxigenic *E. coli* (VTEC) (Nataro and Kaper 1998, Feng et al. 2005). For example, EHEC have been designated as a subset of STEC most of which carry an *eae* gene encoding intimin and are pathogenic to humans (Tarr et al 2005). According to the World Health Organization (WHO 1998), EHEC is defined as Stx-producing *E. coli* bacteria.

Table 1. Characteristics and vehicles or sources for outbreaks of the main diarrheagenic *E. coli* groups[1].

| Group | Year associated with diarrhea | Vehicles, sources, and factors for outbreaks | Main virulence factors: *chromosomal*[2] / plasmidal | Biological effect of the virulence factor |
|---|---|---|---|---|
| EPEC | 1945 | Untreated water | *Attaching and Effacing ability (A/E)* | Adhesion, destruction of microvilli, ion secretion |
| | | Food, weaning food | EPEC Adherence Plasmid (EAF) | Adhesion |
| | | Dust and aerosols | Bundle forming pilus (BFP) | Adhesion |
| | | Fomites | *LifA/Efa* | Adhesion |
| ETEC | 1961 | Untreated water | Heat-labile/stable enterotoxin (LT, ST) | Ion secretion |
| | | Food, weaning food | Heat-stable enterotoxin (EAST) | Ion secretion |
| | | | Fimbrial colonization factors (CFs) | Colonization |
| EIEC | 1971 | Water, food | *Vir/IcsA cluster* | Membrane ruffing, intracellular movement |
| | | Person-to-person | IpaA, IpaB, IpaC, IpaD, IpaH | Invasion, possible inflammation |
| | | Restaurant meals | Shigella enterotoxin 1 (ShET1) | Ion secretion |
| EAEC | 1987 | Water | Heat-stable enterotoxin (EAST) | Ion secretion |
| | | Person-to-person | Aggregative Adherence Fimbria (AAF) | Adhesion |
| | | Restaurant-meals | Plasmidal enterotoxin (PET) | Serine protease, ion secretion, cytotoxicity |
| EHEC | 1982 | Meats sold by butchers | *Shiga toxin (Stx)* | rRNA inhibiting protein synthesis, apoptosis |
| | | Hamburgers, ground-beef | *Attaching and Effacing ability (A/E)* | Adhesion, destruction of microvilli, ion secretion |
| | | Fermented sausages | Enterohemolysin (Ehly) | Lysis of erythrocytes |
| | | White radish sprouts | *E. coli* secreted protein (EspP) | Serine protease, cleaves coagulation factor V |
| | | Unpasteurized food items | Katalase Peroxidase (KatP) | Reduction of peroxides of immune response |
| | | Unchlorinated water | ToxB | Adhesion |
| | | Salads | *Efa-1/LifA* | Adhesion |
| DAEC | 1993 | Environment, wet season | *Fimbrial adhesin F1845* | Adhesion |
| | | | Adhesin in diffuse adherence (AIDA-I) | Adhesion |

[1] The information presented is derived from several publications (Karch et al. 1999a, 1999b, Kuhnert et al. 2000, Sharma et al. 2003, Kaper et al. 2004, Varnado et al. 2004, Torres et al. 2005).

[2] The chromosomal genes are presented in italics and underlined.

## 2. EHEC infection in humans

The infectious dose of EHEC bacteria has been estimated to be low: just 1-100 cells of the strains of serogroup O157 have been reported to cause infection (Griffin 1998, Jaeger and Acheson 2000). The low infectious dose enables the effective spreading of this pathogen via food, water or animal transmission. In addition, person-to-person transmission of EHEC bacteria has been important (Bell 2002). The high mortality associated with EHEC infection has differentiated this organism from the infections caused by, for example, EPEC, ETEC, and EAEC (Law 2000).

The main symptom of human EHEC infection is watery and bloody diarrhea but the disease may progress especially in children and elderly people to HUS, thrombotic thrombocytopenic purpura (TTP), and even death (Karmali et al. 1983a, 1983b, Riley et al. 1983, Karmali 1989, Ludwig et al. 2001, Caprioli et al. 2005). HUS or TTP cause complications in about 10% of the cases of *E. coli* O157 infection and are responsible for a mortality rate of 2–10% (Ahmed and Donaghy 1998). However, asymptomatic carriages have also been reported mainly in adults (Stephan and Untermann 1999). In the treatment of EHEC-infection, no specific antimicrobial medication is recommended (Tarr et al. 2005), since the use of antimicrobial agents may lead to an increase of Stx production (Herold et al. 2004).

### 2.1 Occurrence of EHEC infections

Since the first EHEC O157:H7 outbreaks in the USA in the early 1980s, there has been a dramatic increase in the number of reported infections caused by this O group in the developed countries (Nataro and Kaper 1998), and reports from Africa (Effler et al. 2001) have shown that rates of O157:H7 infections in countries lacking diagnostic capabilities might be underestimated (Tarr et al. 2005). Annual incidence rates of $\geq 8 / 10^5$ of population have been reported in regions of Scotland, Canada, and the USA (Mead and Griffin 1998). Also, outbreaks with hundreds of cases of EHEC infection have been reported from the United Kingdom (Gillespie et al. 2005) and North America, and outbreaks affecting several thousands of individuals have occurred in Japan, where over 9,000 children were infected (Michino et al. 1998). In addition, in Scotland severe, fatal outbreaks with 21 deaths among over 400 individuals infected with the *E. coli* O157:H7 have occurred (Ahmed and Donaghy 1998). Moreover, an international outbreak caused by EHEC O157 has occurred among tourists belonging to five nationalities visiting Fuerteventura, Spain (Pebody et al. 1999). Of European countries, the incidence of O157 infections has been high ($1.3–2.1 / 10^5$ of population during 1995–1998) in England and Wales (Tozzi et al.

2001, Willshaw et al. 2001). The first outbreak of EHEC O157 in Scandinavia occurred in Sweden in 1995, affecting about 100 people (Ziese et al. 1996). In Finland, with a population of 5.1 million, the incidence rate of EHEC O157 infections has ranged from 0.06 in 1990 to 1.0 in 1997 (Keskimäki et al. 1998). In 1997, the first outbreak of EHEC O157 also occurred (Paunio et al. 1999).

The emergence of SF EHEC O157:H$^-$ occurred in Germany in the late 1980s in the form of several outbreaks (Karch et al. 1990, Ammon et al. 1999, Karch and Bielaszewska 2001, Robert Koch-Institut 2002). Even recently, these variants have been identified as the second most common cause of sporadic cases of HUS in Germany (Friedrich et al. 2002, Gerber et al. 2002). In addition, these organisms have occurred in the Czech Republic (Bielaszewska et al. 1998), Austria (Allerberger et al. 2000, Gerber et al. 2002), and the UK (Allison 2002, Taylor et al. 2003). In Finland, the first SF STEC O157:H$^-$ strain was detected in 1997 (Keskimäki et al. 1998). Outside Europe, SF *E. coli* O157:H$^-$ strains have been detected especially in Australia (Bettelheim et al. 2002). In addition, nonmotile strains of O157 group have occurred in the USA (Mohle-Boetani et al. 2001, Feldman et al. 2002) and the UK (O'Brien et al. 2001). However, commonly the strains especially in the USA have been pheno- and genotypically different from the German isolates.

The implication of EHEC non-O157 has been increasing over the past 10 years in human infections (Scheutz et al. 2004). Currently, among 450 EHEC non-O157 serotypes (Blanco et al. 2004, www.microbionet.com.au), the predominant O groups associated with human diseases have been O26, O91, O103, O111, O128, O145, and O121 (Bettelheim 2000, Prager et al. 2002, Blanco et al. 2004). The prevalence of EHEC non-O157 infections has been higher than those of O157 especially in continental Europe (Caprioli and Tozzi 1998, Gerber et al. 2002), Australia (Elliott et al. 2001), Latin America (Blanco et al. 2004), and Denmark (Scheutz et al. 2005). In Australia, EHEC strains of serotype O111:H$^-$ have caused an outbreak affecting more than 200 people (Paton et al. 1996). With regard to other continents, it has been estimated that 25–50% have been caused by strains of the EHEC non-O157 group in the USA (Thorpe 2004), and strains of EHEC non-O157 associated with human illnesses have recently been detected in developing countries (Khan et al. 2002a, 2002b). In addition, non-O157 infections have played an important role in Argentina, Chile, and South Africa (Blanco et al. 2004). In Germany, Italy, and the United Kingdom, strains of EHEC non-O157 have caused 10 to 30% of the HUS cases (Caprioli et al. 1997). Also, in other studies, dozens of serotypes have been associated with the EHEC infections or HUS (Beutin et al. 1998, Goldwater and Bettelheim 2000, Evans et al. 2002, Jenkins et al. 2003, Blanco et al. 2004). With an addition to the growing list of strains of human pathogenic EHEC non-O157, recent studies have identified O117:H7 as a new EHEC non-O157 serotype (Olesen et al.

2005). Moreover, variants not serotyping with antisera against the estabilished O groups have occurred (Jenkins et al. 2003).

## 2.2 Sources and vehicles of EHEC infections

The principal reservoir of EHEC O157 and non-O157 bacteria is considered to be the gastrointestinal tract of healthy cattle and other ruminants like sheep and goats. In addition, domestic animals such as cats, dogs, and rabbits (Garcia and Fox 2003) have been sources of EHEC. Human infections or outbreaks have also been associated with a wide variety of food items; undercooked ground beef, hamburgers, dry fermented sausage, unpasteurized milk or surface contaminated fruit and vegetables. In addition, untreated or contaminated drinking water or swimming water have been causative agents for the EHEC infection, and also person-to-person transmission has been important (Nataro and Kaper 1998, Paton and Paton 1998, Jaeger and Acheson 2000, Kaper et al. 2004). Nosocomial and laboratory-acquired infections have also been reported (Coia 1998a, 1998b, Welinder-Olsson et al. 2003). In Finland, the first outbreak of EHEC O157:H7 in 1997 was associated with swimming in a lake (Paunio et al. 1999). In addition, human EHEC O157 (Lahti et al. 2002) and non-O157 infections (Heinikainen et al. 2004) have been traced to cattle.

# 3. Evolution and clonality of EHEC

The carriage of EHEC-associated virulence (Figure 1) has enabled studies on several research areas such as on pathogenicity, evolution, clonality and epidemiology. In the evolutional studies of EHEC, the key research elements have been the chromosomally located LEE locus encoding the attachment and effacement phenotype, and the *stx*-phage integrated chromosomal *stx* loci (Feng et al. 1998, Whittam 1998, Jores et al. 2004). According to a stepwise evolutionary model, the pathogenic lineage of EHEC O157 has arisen during the past 50 years from an EPEC ancestor of serotype O55:H7 possessing the LEE locus (Figure 2) (Whittam et al., 1993; Feng et al. 1998, Whittam 1998, Wick et al. 2005). Key events were the acquisition of the new serogroup O157 antigen -encoding *gnd-rfb* region, integration of the $stx_2$-phages, and the large virulence plasmid (designated as pO157) (Duffy and Garvey 2001, Kim et al. 2001). In subsequent steps, the EPEC O157 strains were lysogenized by $stx_1$ phage, lost the ability to ferment sorbitol, mutated leading to inactivation of the *uidA* gene, which resulted in a loss of β-glucuronidase activity. In addition, two separate clades resulted: the groups of NSF EHEC O157:H7, and SF nonmotile O157:$H^-$ strains, the latter also known as a German clone (Whittam et al. 1993, Karch et al. 1993, Feng et al. 1998, Whittam 1998, Kim et al. 2001,

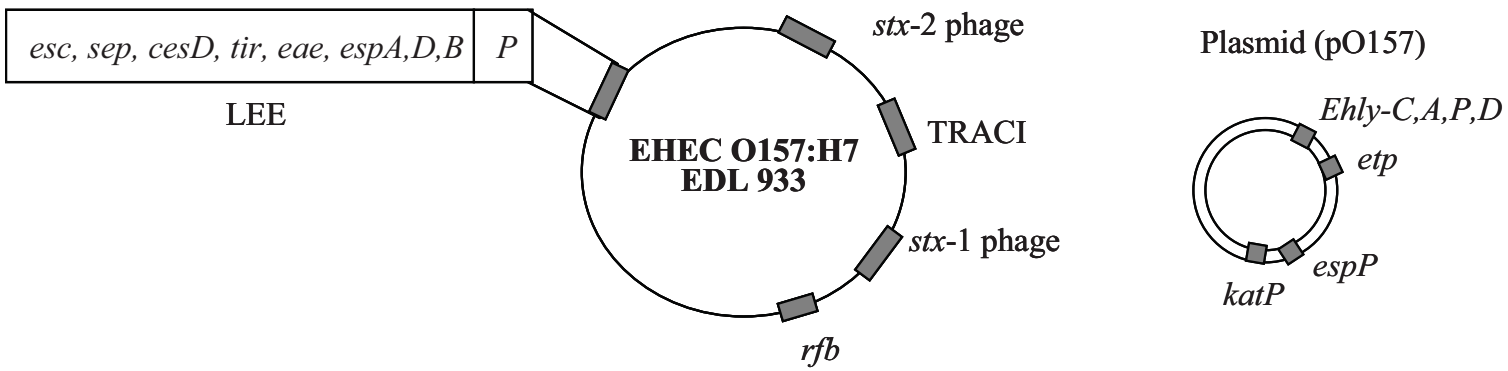
Monday et al. 2004, Wick et al. 2005,). Geographically, the lineage of EHEC O157:H7 is known as a disseminated complex of highly related genotypes (Kim et al. 2001), although some geographical variations of the strains have been documented in Europe, the USA, and Australia (Kim et al. 2001, Brüssow et al. 2004).

In genetic analyses, most EHEC non-O157 strains have been distantly related to the typical *E. coli* O157:H7 group called EHEC1, and form separate clades: EHEC2, STEC1, and STEC2 (Whittam 1998, Shaikh and Tarr 2003). EHEC2 has been considered the most common group among non-O157 strains, including strains of serotype O111:H8/H11/H$^-$, O26:H11, or nontypeable O-groups. The STEC1 group includes many different O-groups and usually with H21 flagellar antigen. The most common serotypes have been O113:H21, O174:H21 (previously OX3:H21), and O91:H21. These strains have typically been negative for the carriage of the LEE. STEC2 is composed of strains belonging to serotypes O103:H2/H6 and O45:H2 (Whittam 1998, Donnenberg and Whittam 2001). However, in other studies, similarities have occurred also between strains of the O groups O26, O15, and O103 (Jores et al. 2004). Moreover, among the strains of O26:H11 important findings of genetic changes have been made: this clone has been shown to carry a pathogenicity island (HPI), which has homologous sequences to pathogenic *Yersinia* sp., and this HPI island is not found in the closely related O111 strains (Karch et al. 1999b), which may have been due to the rapid acquisition or loss of genetic material, or other unsolved events (Donnenberg and Whittam 2001).

In the evolutionary studies, two highly divergent phylogenetic gene clusters (*stx*$_1$ and *stx*$_2$) were also described, which separated long ago and underwent most of their sequence evolution outside *E. coli* (Whittam 1998). Of these, *stx*$_1$ has been highly conserved, and it has been postulated that this gene region first moved from *S. dysenteriae* into the ancestral bacteria of EHEC2 group, accumulated several mutations, and then moved by transduction recently into EHEC1 O157:H7 (Whittam 1998). Instead, the *stx*$_2$ genes have expressed several variants, and in addition to *E. coli*, have moved among divergent species like *Citrobacter freundii* and *Enterobacter cloacae* (Whittam 1998). In general, both *stx*$_1$ and *stx*$_2$ have provided an example of the rapid exchange of genetic cassettes between different *E. coli* strains (Brüssow et al. 2004) and other bacteria.

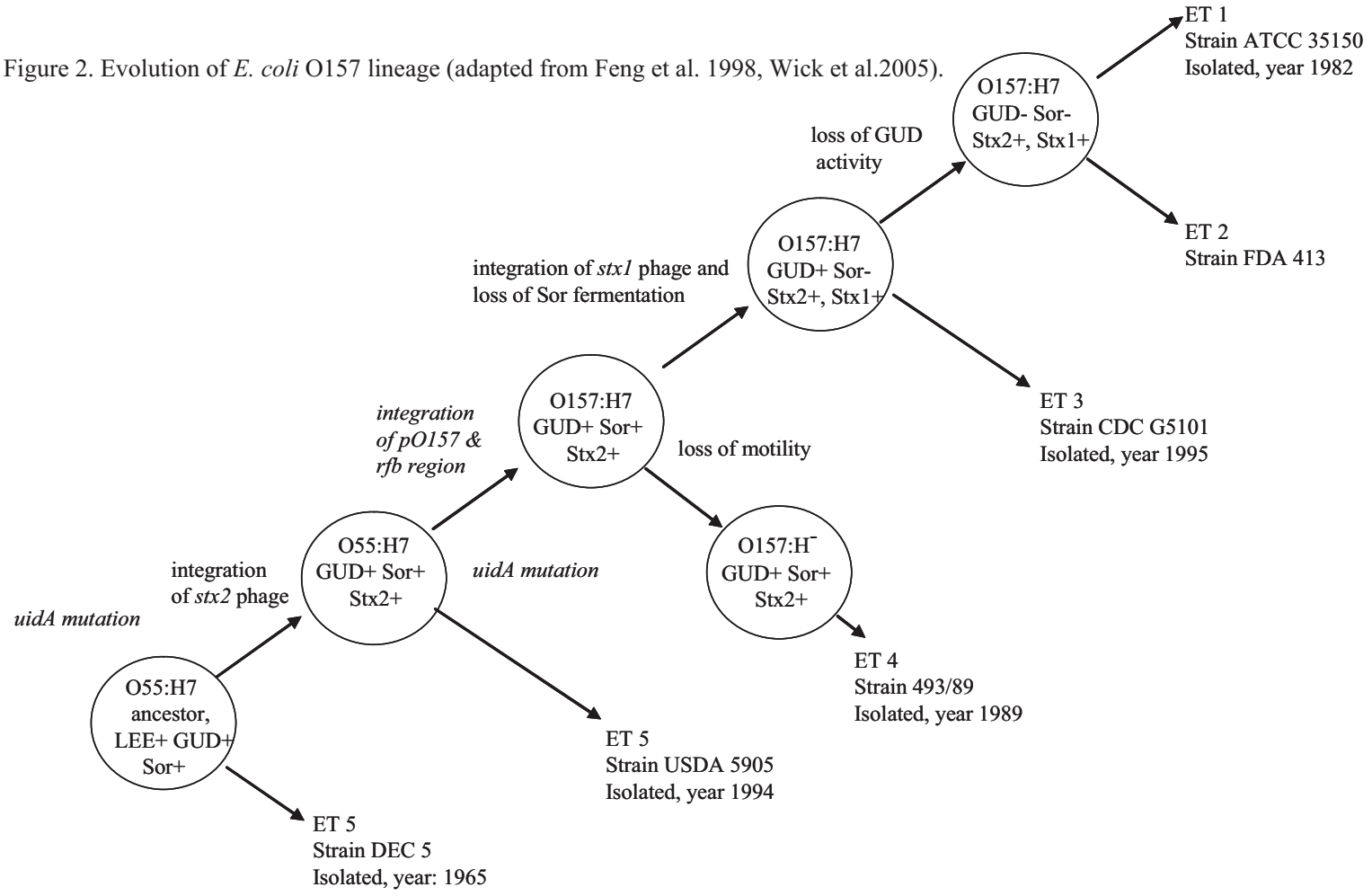
The clonality and epidemiological relationships of the EHEC O157 (Liesegang et al. 2000, Karch and Bielaszeska 2001, Kim et al. 2001, Beutin et al. 2002) and various non-O157 strains (Schmidt et al. 1999a), such as O103 (Prager et al. 2002), O118 (Wieler et al. 2000), or O145 (Sonntag et al. 2004) have also been exploited in studies of outbreaks or sporadic infections. In addition, the nearly 1,400 genes identified in *E. coli* O157:H7 have enhanced studies of the comparability of these strains (Perna et al. 2001).

Figure 1. Genetic loci of virulence factors in the international reference strain *E. coli* O157:H7 (adapted from Karch et al., 1998, Tschäpe and Fruth 2001).




Abbreviations:
LEE, Locus of Enterocyte Effacement; *esc*, *sep*, *cesD*, genes for the type III apparatus; *tir*, gene for translocated intimin receptor; *eae*, gene for intimin; *espA*, *D*, *B*, genes for effector proteins; P, promotor; *stx*, genes for Shiga toxin; TRACI, telluride-resistance-and adhesion-conferring island; *rfb*, gene for LPS synthesis; *Etp*, gene for type II secretion system; *Ehly –C,A.P,D*, genes for EHEC- hemolysin; *katP*, gene for katalaseperoxidase; *espP*, gene for serine protease.

Figure 2. Evolution of *E. coli* O157 lineage (adapted from Feng et al. 1998, Wick et al.2005).




Abbreviations: *uidA*, gene for ß–glucuronidase activity; LEE, Locus of Entrocyte Effacement; GUD, ß –glucuronidase; Sor, Sorbitol; *stx*, genes for Shiga toxin; pO157, plasmid pO157; *rfb*, gene for LPS synthesis; ET, electrophoretic type.

# 4.Virulence characteristics of EHEC

To date, several virulence factors for EHEC bacteria have been suggested, such as 1) Stx1 and/or Stx2 production, 2) formation of the attaching and effacing phenotype, 3) enterohemolytic activity, 4) production of serin protease Esp, 5) presence of a special catalase system KatP, 6) production of ToxB protein with homolygy to large clostridial cytotoxins, 7) StcE metalloprotease which is transported by the *etp* into the extracellular environment, 8) a heat-stable enterotoxin (EAST) 9) adhesion protein Iha, 10) fimbria-associated colonization factors, 11) iron supply by hemin-receptor and high pathogenicity island HPI, and 12) lipopolysaccharides (LPS) endotoxins (Tschäpe and Fruth 2001, Schmidt and Hensel 2004). In addition, the sequencing of the EHEC O157 bacteria has rapidly enabled the discovery of genes (over 1,300 genes) as their new candidates as virulence factors, alternative metabolic capacities, prophages, and other new functions (Hayashi et al. 2001, Perna et al. 2001).

## 4.1 Chromosomally located virulence characteristics

The most important bacterial virulence factor of EHEC has been suggested to be the production of Stx1, Stx2, or their variants encoded by lysogenic, lambdoid bacteriophages (Melton-Celsa and O'Brien 2000). The other important virulence factor is possession of chromosomally located *eae* gene encoding intimine (the *E. coli* attaching and effacing protein) that mediates the attaching of EHEC bacteria to the surface of epithelial cells of the intestine.

### 4.1.1 Shiga toxin (Stx) and *stx* variants

The cardinal feature of EHEC strains is the production of Stx1 and Stx2, which are antigenically distinct, major groups of Stx toxin (Melton-Celsa and O'Brien 2000). Stx1 of EHEC bacteria is closely related to the Shiga toxin 1 produced by *Shigella dysenteriae* serotype 1 (Melton-Celsa and O'Brien 2000). Among the Stx1 and especially Stx2, tens of variants have been identified, the main variant groups being Stx1c, Stx1d, Stx2c, Stx2dac (i.e. activatable by elastase), Stx2e, Stx2f, or Stx2g (Melton-Celsa and O'Brien 1998, Scheutz et al. 2001, Friedrich et al. 2002, Cherla et al. 2003, Leung et al. 2003). Stx1 and Stx2 toxins are approximately 59% homologous at the amino acid sequence level, while the variants of Stx2 share 84-99% similarity with the Stx2 (Cherla et al. 2003). The genes for the Stx group are either located on lambdoid bacteriophages or on the chromosome (Melton-Celsa and O'Brien 2000, Tschäpe and Fruth 2001, Cherla et al. 2003). In addition, EHEC may

express more than one Stx if carrying more than one Stx-encoding bacteriophage (Cherla et al. 2003).

Stx toxins are holotoxins consisting of an A subunit and a pentameric B subunit. The five B subunits form a doughnut-shaped structure and the C-terminus of the A–subunit is inserted non-covalently into this ring. In eukaryotic cell membranes, the receptor for Stx family members is globotriaosylceramide (Gb3) except for the pig edema disease toxin, Stx2e, which preferentially binds to the longer glycolipid globotetraosylceramide (Gb4) (Melton-Celsa and O'Brien 2000, Law 2000). Once bound to the target cell membrane, Stx toxin molecules are internalized via vesicles by endocytic mechanism of the intestinal epithelial cells, transported through the *trans*-Golgi network and Golgi apparatus to the endoplasmic reticulum and nuclear membrane (Cherla et al. 2003). Subsequently, the Stx A subunit is nicked by protease, which generates catalytically active $A_1$ and $A_2$ fragments. The released $A_1$ subunit of the toxin is able to inhibit the peptide chain elongation step of protein synthesis leading to cell death (Law 2000). However, in some cells, the toxin vesicles have been degraded by lysosomes (Law 2000). In the bloodstream the Stx toxin is further transported into organs (for example kidneys) rich with specific Gb3 receptors (Arab and Lingwood 1998). Although Stx1 and Stx2 have similar modes of action, their toxicities appear to be different. Compared to Stx1, Stx2 has been 1,000 times more cytotoxic towards human renal microvascular endothelial cells, the putative target of Stx in the development of HUS (Louise and Obrig 1995, Law 2000).

In 1983, it was found that the genetic information for the production of Stx of *E. coli* O26:H11 and *E. coli* O157:H7 outbreak strains was carried in lambdoid bacteriophages integrated in the chromosome (Melton-Celsa and O'Brien 2000, Herold et al. 2004). In recent years, Stxs have been found to be encoded by several lambdoid bacteriophages of approximately 60 Kb in size (*stx*A encoding the A-subunit, *stx*B encoding the B-subunit), which are able to cause lysogenization of the host cells of various enteric bacteria, in particular *E. coli* (Tschäpe and Fruth 2001, Gamage et al. 2003). The *stx* genes have been associated with a specific gene region (late phase region) of the lambdoid phage (Neely and Friedman 1998, Unkmeir and Schmidt 2000, Tschäpe and Fruth 2001). When lambdoid phage DNA have integrated into the chromosomal genome of the bacterial host cells, the *stx* genes have remained silent under genetic control (Tschäpe and Fruth 2001) but have become highly expressed when phage enter the replicative or lytic cycle, resulting in phage production and bacterial lysis (Johnson et al. 1981, Wagner et al 2001, Gamage et al. 2003, Gamage et al. 2004). Induction to the lytic cycle can occur after exposure of the bacteria to DNA damaging agents such as antibiotics or ultraviolet (UV) light (Gamage et al. 2004), resulting in the release of a new generation of bacteriophage particles and Stx into the environment (Tschäpe and Fruth 2001).

The numerous variants of $stx_1$ and $stx_2$ have been identified, differing by only one or two amino acids in either the A or B subunit (Lin et al. 1993, Paton and Paton 1998, Bastian et al. 1998, Pierard et al. 1998). However, many different styles of designations have been used in nomenclature to differentiate these toxins, which has created a non-uniform nomenclature (Scheutz et al. 2001). Nevertheless, based on the genomic restriction profiles, some *stx* designations can be expressed as $stx_1$/$stx_{1vO111}$ (hereafter $stx_1$), $stx_2$, $stx_{2c}$/$stx_{2vha}$/$stx_{2vOX393}$ (hereafter $stx_{2c}$), $stx_{2vhb}$, $stx_{2vO111}$/$stx_{2vOX392}$ (hereafter $stx_{2vO111}$ and $stx_{2vOX392}$), $stx_{2e}$, and $stx_{2ev}$ genes (Lin et al. 1993, Bastian et al.1998). In addition, based on the synonyms of Stx and VT toxins, for example the new toxins by Pierard et al. (1998) can be designated VT2d-Ount (hereafter $stx_{2d\text{-}Ount}$) and VT2d-OX3a (hereafter $stx_{2d\text{-}OX3a}$). Of the toxin types, Stx2, Stx2c and Stx2dac have been the most prevalent associated with HUS patients or patients with severe symptoms (Friedrich et al. 2002, Prager et al. 2005). Instead, Stx2d-Ount variant of the B-subunit of the Stx, has been associated with human disease but with mild symptoms (Pierard et al. 1998). Stx2e has typically caused edema disease in pigs (Gyles et al. 1988, Weinstein et al. 1988). Stx2f has been associated mainly with feral pigeons (Schmidt et al. 2000), and Stx2g has recently been identified as a toxin found in bovines (Leung et al. 2003).

However, although the Stx production is considered a main virulence factor of EHEC, strains of non-shiga toxin producing O157:H$^-$ *E. coli* have been found among strains isolated from patients with HUS or diarrhea (Schmidt et al. 1999b). Thus, yet unknown, non-Stx virulence traits causing severe symptoms in susceptible patients may have been present. However, also the loss of *stx* genes can rapidly occurr among some strains and a putative loss of *stx* genes of STEC strains during the storage or isolation of the strains might complicate the study of the virulence of these strains. These strains can appear in diagnostics only as *stx* negative variants but clinically and epidemiologically they have been *stx* positive strains.

### 4.1.2 Locus of Enterocyte Effacement (LEE)

Most EHEC strains harbour the pathogenicity island (PAI) of locus of enterocyte effacement (LEE), which encodes a type III secretion system (TTSS), responsible for the secretion and translocation of virulence determinants, and effector proteins that are homologous to those produced by EPEC (McDaniel et al. 1995, Kaper et al. 2004). LEE is involved in the production of proteins associated with the intimate adherence to intestinal epithelial cells, initiation of host signal transduction pathways, and the formation of A/E lesions (Perna et al. 1998, Clarke et al. 2003, Schmidt and Hensel 2004, Torres et al. 2005). The overall sequence identity of LEE-encoded proteins of EHEC and EPEC has been up to 94%, although the similarity of *E. coli* secreted proteins, such as EspA, EspB, EspD, intimin, and translocated intimin receptor (Tir), have ranged from 67% to 88% (Law 2000, Torres and Kaper

2002, Schmidt and Hensel 2004). Components (Esc/Sep proteins) of the TTSS form an apparatus for translocation of EspB, Tir and possibly other proteins into the eukaryotic cell (Kaper et al. 1998). Furthermore, a second cryptic TTSS, *E. coli* type III secretion system 2, has been identified, which can potentially influence the expression of genes within the LEE locus of *E. coli* O157:H7 and non-O157 (Makino et al. 2003, Zhang et al. 2004).

During pathogenesis, the *E. coli* secreted protein EspA creates a tube-like structure (translocon) through which other proteins, such as EspB, EspD and Tir are suspected to pass into the host membrane (Law 2000). After translocation, Tir spans the host membrane, creates a hairpin loop structure featuring both its N and C termini in the host cytoplasm and central domain binding the intimin outer-membrane protein encoded by the *eae* gene (Torres et al. 2005). The attachment activates cell-signaling pathways, causing alterations in the host cell cytoskeleton and resulting in the depolymerization of actin and the loss of microvilli showing as a pedestal structure and the eventual death of host cells (Kaper et al. 2004).

In EHEC strains, a LEE-encoded regulator (Ler) has been required for expression of the LEE-encoded proteins (Elliott et al. 2000). The Ler is part of the EPEC and EHEC-associated plasmid-endoced regulatory (Per) cascade (Porter et al. 2005). The Ler has also been noticed to regulate the expression of additional virulence factors not essential for the A/E lesion, which are mediated either by LEE or outside LEE (Elliott et al. 2000).

Of the LEE associated genes, *eae* and its subtypes encoding the intimate adhesion protein (intimin), have offered also a diagnostic and epidemiological value. Depending on the *E. coli* lineage carrying the LEE locus, the size (36 to 111 kb) of the LEE regions has varied (Jores et al. 2004), and currently 17 different *eae* intimin types have been found (Blanco et al. 2005). Among the EHEC serotypes O157:H7/H$^-$, O111:H$^-$, O26:H11/H$^-$, commonly recovered from outbreaks of HUS and hemorrhagic colitis, the most typical intimin subtypes have been *eae*-α, -β, and -γ (Blanco et al. 2005). Although the pathogenicity of LEE is supported by the presence of the *eae* gene in important EHEC serogroups like O157, O26, O103, O111, and O145 isolated from HUS patients (Karch 2001, Kaper et al. 2004). However, LEE-negative serogroups of Stx producing strains have also occurred that have been associated with disease, for example also in the O groups O103 (Kaper et al. 2004) and O174 (Keskimäki et al. 1997, Paton et al. 2001), thereby complicating the pathogenesis. In addition, newly recognized adhesion factors and fimbria encoded outside the LEE have been suspected to promote the pathogenicity of EHEC (Tschäpe and Fruth 2001, Paton et al. 2001, Kaper et al. 2004, Toma et al. 2004, Torres et al. 2005), although LEE is still considered to be one of the most fundamental adhesion mechanisms.

## 4.2 Plasmid mediated virulence characteristics

EHEC strains have carried numerous plasmid-mediated determinants like Ehly, encoded by EHEC-*hlyA* (Karch and Bielaszewska 2001, Kim et al. 2001, Beutin et al. 2002), the Type II Secretion System (ETP) encoded by *etpD* (Schmidt et al. 2001), hemolytic protein (cytolysin A) encoded by *clyA* (Ludwig et al. 2004), and esterase inhibitor-specific metalloprotease encoded by *stcE* (Lathem et al. 2003). However, strains of SF O157:H$^-$ alone have been shown to carry a gene cluster (*sfp*) specific for the expression of fimbriae (Brunder et al. 2001, Friedrich et al. 2004), Instead, unlike other typical EHEC stains, SF O157 isolates have lacked the plasmid mediated catalase peroxidase (KatP, *katP*) and serine protease (EspP, *espP*) (Karch and Bielaszewska 2001).

### 4.2.1 Enterohemolysin (Ehly)

EHEC strains have in common a 60 Mda plasmid (Whittam 1998) that carries four open reading frames (ORFs) responsible for the enterohemolytic phenotype (Karch et al. 1998). These genes encoding the Ehly protein have been termed EHEC-*hlyC*, EHEC-*hlyA*, EHEC-*hlyB*, and EHEC-*hlyD* that are highly related to the genes of the *E. coli* α-hemolysin, the naming of the genes thus being analogous to the respective genes of the *E. coli* α-hemolysin operon (Karch et al. 1998). Ehly protein acts as pore-forming, repeats in toxin (RTX) cytolysin on eukaryotic cells, lyses erythrocytes, and is considered a putative pathogenetic mechanism of EHEC bacteria (Beutin et al. 1989, Beutin et al, 1994, Kaper et al. 2004). However, mutations of the open reading frames (ORFs) of the EHEC-*hly* gene have resulted in the loss or reduction of the enterohemolytic phenotype (Karch et al. 1998). Among EHEC strains, the carriage of EHEC-*hlyA* and production of Ehly have been typical of the O157:H7 strains. Instead, of the SF O157:H$^-$ strains, the strains have typically possessed the EHEC-*hlyA* but have not clearly shown enterohemolytic activity (Karch and Bielaszewska 2001).

### 4.2.2 Putative type II secretion system (ETP)

The type II secretion pathway system has frequently been involved in the transport of pathogenicity factors outside the bacterial cell in gram-negative bacteria (Brunder et al. 2001, Friedrich et al. 2004). In EHEC plasmids, DNA analysis has lead to the finding that the ETP system of EHEC strains includes 13 open reading frames, the respective genes called *etpC* to *etpO* (Schmidt et al. 2001). However, the total function and specificity of the ETP system are not well understood (Caprioli et al. 2005).

### 4.2.3 Katalase peroxidase (KatP)

KatP is a bifunctional, bacterial catalase peroxidase (Schmidt et al. 2001). It harbours an aminoterminal signal peptide, suggesting that it is transported through the cytoplasmic membrane. KatP has been present mainly in periplasm in wildtype *E. coli* O157 strains but unlike NSF O157 strains, SF O157 isolates have typically lacked *katP* genes (Karch and Bielaszewska 2001, Schmidt et al. 2001).

### 4.2.4 Serine protease (EspP)

Unlike other Esp proteins (EspA, EspB, EspD), the secreted serine protease EspP is plasmid mediated. The EspP can cleave human coagulation factor V *in vitro* (Schmidt et al. 2001) and might be an accessory virulence factor exacerbating hemorrhagic colitis (Brunder et al. 1999, Schmidt et al. 2001). For example, sera from five of six children suffering from EHEC infections reacted with purified EspP protein (Schmidt et al. 2001). However, unlike NSF O157 strains, SF O157 isolates have typically lacked *espP* genes (Karch and Bielaszewska 2001).

### 4.2.6 Other chromosomal and plasmidal virulence factors

Virulence factors of EHEC include, for example, surface structure proteins such as endotoxins. In addition, among EHEC bacteria, numerous other virulence factors have been characterized (Prager et al. 2005). In some strains, the production of a lymphostatin and EHEC factor for adherence (Efa1) encoded by *efa1* (Klapproth et al. 2000, Nicholls et al. 2000, Janka et al. 2002) have been detected. In addition, novel STEC autoagglutinating adhesin Saa encoded by the virulence plasmid pO113, might contribute to pathogenesis (Paton et al. 2001). This pO113 plasmid, also known as large hemolysin plasmid, has been isolated from a LEE-negative strain O113:H21, which was responsible for outbreak of HUS (Paton et al. 1999). Recently, the nucleotide sequence of the pO113 has been determined, showing a high degree of similarity with pathogenic transporter regions, for example EspP (Leyton et al. 2003). This plasmid also carries a novel type IV pilus biosynthesis locus that is required for efficient plasmid transfer (Srimanote et al. 2002). Of other virulence factors, a heat stable enterotoxin (EAST) typical of EAEC group has been detected among strains of EHEC (Karch et al. 1999b). Moreover, production of a new virulence toxin, subtilase (Paton et al. 2004), or cytolysin A (ClyA) (Ludwig et al. 2004) might be a contributing factor(s) to the virulence of some EHEC strains or the Stx-negative *E. coli* O157 strains. Furthermore, in strains of SF EHEC O157:H$^-$ a novel plasmidal *sfp* gene cluster (*sfpA*, *sfpH*, *sfpC*, *sfpD*, *sfpJ* and *sfpG*), which mediate mannose-resistant hemagglutination and the expression of fimbriae, has

recently been characterized (Brunder et al. 2001). Interestingly, this cluster has taken over the plasmidal loci of *katP* and *esp* (Brunder et al. 2001, Bettelheim et al. 2002, Friedrich et al. 2004). Also, most of the SF O157:H$^-$ strains have possessed a chromosomal gene cluster encoding a novel type of CDT toxin designated CDT-V, which has rarely been found in *E. coli* O157:H7 (Janka et al. 2003). Among newly recognized plasmid pO157 mediated toxins, ToxB belonging to a large clostridial toxin family has been characterized. These AB-toxins mediate cell entry, which may lead to altered regulation of cell cytoskeleton and eventually diarrhea (Schmidt et al. 2001).

### 4.3. Transmission of virulence factors and bacterial species

Genes encoding important virulence factors are often located on mobile genetic elements, such as insertion sequences, transposons, gene cassettes, integrons, plasmids or bacteriophages and can therefore be transferred horizontally from one cell to another (Karch et al. 1999a, 1999b, Brunder and Karch 2000). This transposition event is defined as a DNA recombination reaction resulting in translocation of a discrete DNA segment such as the insertion sequence or transposon from a donor site to target site, that can cause changes in gene expression (Brunder and Karch 2000). Furthermore, gene cassettes and integrons allow natural cloning and expression of cells, and the exchange of plasmids between bacteria is an important source of genomic variation (Brunder and Karch 2000). For example, the chromosomally integrated pathogenecity island LEE locus occurrs among strains of EPEC and EHEC (Karch 2001, Schmidt and Hensel 2004). Some plasmids are associated with phenotypes, such as antibiotic resistance of several bacterial species (Dougan et al. 2001). Among EHEC, Stx toxins are encoded by several different lambdoid bacteriophages, which can cause lysogenization of various enteric bacteria, particularly *E. coli* and horizontal spread of Shiga toxinogenicity (Unkmeir and Schmidt 2000, Tschäpe and Fruth 2001). For example, Stx-producing strains have been found among *S. dysenteriae* type 1, *S. sonnei* (Strauch et al. 2001), *C. freundii* (Schmidt et al. 1993, Tschäpe et al. 1995), and *E. cloacae* (Paton and Paton 1996, Herold et al. 2004). In addition, free *stx*-phage particles have been detected in nature, such as in a natural water environment (Muniesa et al. 1999).

## 5. Screening for EHEC

Early diagnosis of EHEC infections in routine microbiological laboratories creates a basis for crucial acts, such as treatment of the patient and prevention of additional cases of EHEC infection (Karch et al. 1999a). The identification of strains of *E. coli* O157 has worldwide been well-covered in routine microbiological laboratories with

the use of differentiating media, especially Sorbitol-MacConkey agar (SMAC) (March and Ratnam 1986, Paton and Paton 2003), and commercial detection kits based on the agglutination and immunological reaction (Table 2). The SMAC plate differentiates the NSF isolates of serogroup O157 as colorless colonies compared to the other *E. coli* flora, which typically ferment sorbitol and have a red phenotypic appearance. However, some enterobacterial genera or species, such as *Proteus*, *Providencia*, *Hafnia*, *Enterobacter*, and *E. hermanii*, also grow in colorless colonies, of which some species share common epitopes with the O157 antigen causing crossreactions (March and Ratnam 1986, Lior and Borczyk 1987, Karch et al. 1999a). In addition, *Yersinia enterocolitica* O:9 has cross-reacted with human anti-O157 sera (Chart et al. 1991). An SMAC plate containing inhibitory components, such as cefiximide and tellurite (CT-SMAC) for other bacterial species, has also been used in the detection of strains of NSF O157. However, this medium has also been inhibitory particularly for strains of SF O157 (Karch and Bielaszewska 2001), which limits its use. A considerable number of strains within the O157 and non-O157 serogroups have been sorbitol-fermentative, thus complicating phenotypic detection of these pathogens on SMAC. However, rapid agglutination or immunomagnetic separation (IMS) tests using the serological capture and detection of the most common serogroups of non-O157 strains (O26, O91, O103, O111, O128, O145) have been exploited for the detection of these pathogens.

Based on the vast phenotypic diversity of EHEC bacteria, the most effective detection method for detection of all EHEC bacteria has been the detection of the Stx production or the investigation of the *stx* genes of the isolates. As the definition of EHEC bacteria is the production of Stx or the related *stx* genes, several methods have been developed for detection of the toxin(s) (Table 2). Of these methods, enzyme-immunoassays (EIA-assays) detect the presence of Stx1 and/or Stx2 toxin(s) in bacterial cultures or stool. Most EIA tests are based on the sandwich-method, where immobilized monoclonal or polyclonal antibodies have been used as ligands for Stx molecules. The bound Stx toxin and antibody-enzyme-conjugate of the kit forms a visible color reaction, thus indicating the presence of the Stx toxin in the sample. In addition, reversed passive latex agglutination (RPLA) tests detect Stx1 and/or Stx2 in bacterial cultures. In the RPLA, the presence of Stx(s) is detected with anti-Stx bound to latex particles. During incubation, the presence of Stx(s) in the sample is indicated by agglutination of the Stx toxin and the latex bound anti-Stx molecules in the walls of the sample dwell in a microtiter plate. However, other bacterial genera, such as *Campylobacter*, *Citrobacter*, *Pseudomonas*, and *Edwardsiella*, have also been observed to produce Stx, which might produce bias towards false positive reactions in Stx testing and search for EHEC bacteria (Beutin et al. 1996a, Kehl et al. 1997, MacKenzie et al. 1998, Pickett 2000). Thus, the isolation of the Stx-producing strain is very crucial for microbiological confirmation of the causative strain's species and pathogroup.

In specialized laboratories, molecular genetic methods like polymerase chain reaction (PCR) and hybridization methods have been of great value in detection of the *stx* genes of EHEC bacteria and the subsequent isolation of the EHEC strain. Currently, many of these methods have also become commercially available. Of the detection methods, the PCR enables the testing of bacterial culture or direct patient sample, such as stool, for the virulence genes to be searched. However, generally, the stool cultures or pure bacterial cultures are preferred because stool typically contains inhibitory substances for the PCR-reaction, lowering the specificity and sensitivity of the test (Paton and Paton 2003). Also, in the detection of EHEC, timelapse between the sampling of the specimen and onset of EHEC infection has affected the rates of recovery and overall detection of EHEC isolates. Human fecal specimens collected two days after the onset of symptoms have had an isolation rate of 100% for *E. coli* O157, but the rate has decreased to 33% for specimens collected after seven days (Thomson-Carter 2001). For the recovery of *E. coli* O157, especially, enrichment procedures have been used succesfully, in particular with the combination of the IMS technique (Karch et al. 1999a, Thomson-Carter 2001).

Table 2. Methods for the detection of EHEC bacteria.

| Diagnostic Principle | Test method |
|---|---|
| ***Phenotypic methods*** | |
| Detection of *E. coli* O26, O91, O111, O103, O111, O145, O157 antigens in bacterial culture | Latex agglutination, immunomagnetic separation (IMS) |
| Selective isolation of *E. coli* O157 bacteria based on color reaction on agar plate | Chromogenic agars |
| Detection of enterohemolysin production of bacterial culture on agar plate | Enterohemolysin agar |
| Screening for Stx toxin or O157 antigen in bacterial culture or stool | Enzyme immuno-assays (EIA) |
| Screening and separate detection of Stx1 and Stx2 toxins in bacterial culture or stool | Reverse passive latex agglutination (RPLA) |
| ***Genotypic methods*** | |
| Detection of genes encoding Stx, O specific regions or accessory virulence factors in bacterial culture or stool | PCR, PCR combined with restriction fragment length polymorphism (PCR-RFLP), PCR combined with hybridization, colony hybridization, immunoblotting |

Data based on Karch et al. 1999a, Perelle et al. 2004, Beutin et al. 2005, Feng et al. 2005, Prager et al. 2005, and an additional Internet search using the following search terms: diagnostics, sltec, stec, ehec, or vtec.

# 6. Subtyping of EHEC

In the epidemiological surveillance of human infectious diseases, the detailed pheno- and genotypic characterization of the causative human isolate allows targeted investigations not only between human infections, such as person-to-person contacts, but also between human and non-human sources like contaminated food, cattle, or the environment (Karch et al. 1999a, Lahti et al. 2002, Heinikainen et al. 2004). In the differentiation of EHEC, the methods used must have high typeability, reproducibility, and high discriminatory power. In addition, interlaboratory harmonization of the methods used has been preferable. The cornerstones in both the national and international epidemiological study of EHEC O157 and non-O157 infections has been characterization of the isolates for determinants, such as the O:H serotype, phage type, *stx* gene possession or their variants, and restriction profile type in pulsed-field gel electrophoresis (PFGE). In addition, simultaneous molecular biological detection of several characteristics, such as *stx*, *eae*, *ehly*, *saa*, have enabled fast early phase epidemiological comparison of EHEC strains (Paton and Paton 2002).

## 6.1 Phenotypic methods

Among EHEC bacteria, phenotypic characteristics, such as sorbitol-fermentation ability, β-glucuronidase activity, production of enterohemolysin or Stx of the isolate, have been crucial determinants. Subsequent serotyping of the EHEC strains and phage typing of the EHEC O157 isolates have allowed fast epidemiological, internationally comparable, first step phenotypic categorization of these organisms. Moreover, antimicrobial susceptibility testing and the resulting resistance patterns of the EHEC O157 and non-O157 strains have been offered informative data as an epidemiological marker of these isolates.

**Serotyping** offers a valuable means for classification of the *E. coli* strains forming a basis for epidemiologic analysis. Since the first evidence that certain serological types of *E. coli* were associated with epidemics of enteritis during 1945–1950 and following pioneering work (Kauffmann 1947, Ørskov and Ørskov 1984), it was established that *E. coli* express three principal antigens: O, K, and H antigens. Identification of these *E. coli* surface structures relate to components of somatic, lipopolysaccharide (LPS)-associated O-polysaccharide (O-antigen), capsular polysaccharide (K), and flagellar (H)-antigen. Especially the O grouping can be performed in many clinical laboratories, while the H antigen can be determined in a limited number of laboratories, and the 80 types of K antigens (Wain et al. 2001) to an even less extent (Scheutz et al. 2004).

Currently, 176 distinct *E. coli* O antisera differentiating *E. coli* O groups are available (Scheutz et al. 2004, Olesen et al. 2005, www.ssi.dk). The O antigen is the highly polymorphic outer part of LPS, which comprises repeats of an O unit of generally two to seven sugars. Each strain express only a particular O-antigen form (Thomson-Carter 2001, Feng et al. 2005) but the surface structure of living *E. coli* bacterium might form unspecific crossreactions with different O antisera. However, boiling the bacteria eliminates other surface antigens leaving O chains intact, thus allowing final determination and confirmation of the O group. For this reason, international standard procedure for O-typing is requesting always boiling and agglutination test in microtiter plates as well as dilution of respective antisera. However, some commercial O antisera for *E. coli* O-grouping are also available, which do not require the use of boiled bacterial cultures for determination of the final O group. In determining of the O group, if no full panel of the O antisera is available in the typing laboratory, *E. coli* strains not reacting with any sera used have been designated O Nontypeable (ONT). In addition, strains typically having a rough phenotypic appearance and lacking the O antigen, and crossreacting with the panel of the sera are interpreted as O Rough. Two main differentiating EHEC O group categories, O157 and non-O157, have been designated among EHEC. Among the non-O157 strains, over 450 O:H types have been reported (Blanco et al. 2004), with over 1,000 reports of isolations of non-O157 strains (Blanco et al. 2004, www.microbionet.com.au).

For phenotypic determination of the flagellar H antigen, active motility of the strain is required. The motility is provided by the rotating flagellum which projects beyond the surface of *E. coli* cell (Wang et al. 2003). Flagellin is the protein subunit of the flagellum that carries H-antigenic specificity (Wang et al. 2003). For *E. coli*, 56 flagellar antigen groups (Ørskov and Ørskov 1984) that can be identified with H-specific antisera (Ratiner 1991) have been described. The mechanism of motility in *E. coli* is complex, under intricate regulatory control and affected by environmental factors (Feng et al. 1996, Pallen et al. 2005). However, the presence of flagella is not indicative that these strains are motile (Feng et al 1996). The nonmotility (NM) of the strains might be due to carriage cryptic flagellum genes (Prager et al. 2003, Coimbra et al. 2000, Machado et al. 2000, Ren et al. 2005) or they could be defective in other mechanisms of flagellum assembly or motility (Feng et al. 1996). Nonmotile strains are usually induced extensively for motility before being designated HNM. Of EHEC strains, both motile and nonmotile O:H serotypes have been found.

**Phage typing** has been another important phenotypic method in the differentation of pathogenic enteric bacteria. In phage typing, bacterial viruses (i.e. bacteriophages) are exploited to initiate an infection on a layer of bacterial cells. A zone of lysis may occur that results in a clear area in the lawn of growing host cells. Being based on

variable capabilities of different bacteriophages to lyse bacterial isolates, phage typing has enabled the rapid differentation between isolates during outbreaks and epidemiological surveillance. For EHEC O157, a standardized phage-typing scheme using 16 different phages was developed in Canada in 1987 (Ahmed et al. 1987). At present, more than 80 EHEC O157 phage types (PTs) can be recognized and compared internationally (Willshaw et al. 1997). Of the EHEC non-O157 strains, phage typing has preliminarily been used only for strains of O group O103 (Prager et al. 2002).

**Enterohemolysin** production has been exploited as a blood-based detection method in characterization of EHEC strains. The ability of certain *E. coli* strains to lyse erythrocytes was first described by Kayser in 1903 (Kayser 1903, Schmidt and Benz 2003). This ability was termed hemolysis and the proteins involved were termed hemolysins. One of the best characterized *E. coli* hemolysin is α-hemolysin producing large clear zones of hemolysis after 3-6 h of incubation at +37°C (Schmidt and Benz 2003). However, in 1988, Beutin et al. (1988) found a new type of hemolysin, Ehly, which was produced by some strains of EPEC. Soon, they noticed that there was a nearly 90% association also between certain strains of EHEC and production of Ehly (Beutin et al. 1989). In contrast to α-hemolysin, which produces hemolytic reactions after a few hours, enterohemolytic appearance is observed only after overnight incubation on blood plates containing washed sheep erythrocytes (Beutin et al. 1989). Currently, commercial agar plates detecting the Ehly production are also available (Table 2). This phenotypic appearance has been helpful not only in colony search of EHEC isolates in stool culture but also in epidemiological comparison.

## 6.2 Genotypic methods

Genotypic identification methods that utilize molecular biology-based techniques usually offer several potential advantages over conventional phenotypic methods. PCR or hybridization methods like colony hybridization are very suitable for both screening purposes and the isolation of the strains possessing the target genes. However, although PCR methods offer a distinct advantage for the detection of fastidious and noncultivable organisms (Vora et al. 2004), in clinical microbiology, isolation of the causative strain should not be neglected, as epidemiological research and microbiological confirmation requires the isolation of the pathogen.

**PCR** method was described first by Saiki et al. (1985), and Mullis and Faloona (1987). Soon, a first PCR assay to detect *stx* genes in *E. coli* was executed (Pollard et al. 1990), and today PCR has become a versatile, rapid, and sensitive molecular genetic typing method of pathogenic microbes. During the procedure, a known DNA

sequence is amplified by the use of a unique primer pair hybridized to the complementery target DNA-sequence. Polymerase enzyme and deoxynucleotide-triphospahate (dNTP) enable the exponential amplification of the target DNA in specific reaction conditions and stringent temperature providing a high sequence-specificity. Theoretically, each PCR-cycle doubles the amount of the target DNA, resulting in a billion-fold amplification of the target DNA.

PCR methods have been used for both overall detection of *stx* and differentiatiation of $stx_1$ and $stx_2$. Particularly the PCR methods combined with restriction fragment length polymorphism analysis (PCR-RFLP) allow further characterization of the $stx_1$ and $stx_2$ variants, which improves epidemiological discrimination of the strains and allows international comparison of subtypes of *stx* genes (Bastian et al. 1998). In addition, the subtyping of *stx* might have pathogenetic importance (Friedrich et al. 2002). PCR-related applications like real-time PCR (i.e. quantitive PCR), or multiplex PCRs detecting several genes simultaneously have been widely used recently.

For rapid detection of EHEC non-O157 from clinical samples, PCR has proven to be of widely used method for the detection of *stx* genes (Blanco et al. 2004). In addition, in epidemiological typing of EHEC non-O157, recently described PCR methods have enabled the more efficient and rapid detection of the most common O groups (Perelle et al. 2004, Beutin et al. 2005, Feng et al. 2005). Furthermore, the flagellar *fliC* genotyping by PCR-RFLP has enabled the detection of the certain H-antigen gene possession (Prager et al. 2003). PCR has enabled also the detection of genes determining metabolic characteristics, such as *uidA*, or virulence determinants, such as EHEC-*hlyA* or *eae*. With the use of multiplex-PCR, for example, detection of five different genes occurring in EHEC ($stx_1$, $stx_2$, *eae*, *ehly*, *saa*) is possible (Paton and Paton 2002).

**Pulsed-field gel electrophoresis** is one of the most important discriminatory methods in genetic characterization of bacterial strains and commonly used approach to assess the relatedness of clinical isolates (Goering 2000). PFGE is recommended worldwide as a gold standard molecular typing method, that provides a chromosomal overview scanning >90% of the chromosome (i.e. the sum of the restriction fragment sizes). The use of restriction enzyme(s) enables detection of chromosomal differences between isolates, and the macro-restriction patterns of large DNA fragments reflect the distances between restriction sites around the chromosome (Goering 2000, Tenover et al. 1995). Although minor genetic changes may go undetected (Goering 2000), PFGE has enabled discriminative determination in the molecular comparison of EHEC strains independent of the serotype, and in the investigation of EHEC O157 and non-O157 infections such as O26, O103, O111, and O118, PFGE has been widely used (Thomson-Carter 2001).

Isolates with indistinguishable macrorestriction patterns are generally considered to be associated with each other. Currently, however, no specific interpretative rules exist for EHEC PFGE data, only general guidelines for certain bacterial species (Thomson-Carter 2001). For example, in clonal analyses and in the long-term epidemiological surveillance, strains with up to four-band difference or with over 75% similarity indexes of PFGE dendrograms, have been considered related if other geno- and phenotypic markers have also supported the relatedness (Tenover et al. 1995, Bielaszewska et al. 2000, Liesegang et al. 2000, Beutin et al. 2002, Prager et al. 2002, Duffy et al. 2005). Minor variation (1-2 fragment(s) absent or present) in macrorestriction patterns has been observed among strains of EHEC, including isolates considered to be epidemiologically linked, although previously, certain fragment differences have been shown to be insignificant (Thomson-Carter 2001). In addition, the effect of chromosome evolution on EHEC macrorestriction patterns obtained under experimental conditions, has been suspected to be minimal (Thomson-Carter 2001).

Standardization of the PFGE protocols and electronic submission of gel images have created a genetic database where macrorestriction patterns for unknown isolates could be compared for epidemiological purposes, outbreak investigation and definition of clonal relationships. These harmonization acts have allowed very exact national epidemiological comparison of human and non-human isolates of EHEC in Finland (Rantala et al. 2000), and the USA (Swaminathan et al. 2001, PulseNet 2002) and international comparison of EHEC human isolates within European countries (PulseNet 2002, Fisher and Threlfall 2005, www.hpa.org.uk/inter/enter-net_menu.htm).

**Other genotypic methods** such as multilocus enzyme electrophoresis (MLE), plasmid analysis, DNA sequencing, and multilocuc sequence typing (MLST), have also been used, although MLST has not been succesful in discrimination of *E. coli* O157:H7 isolates (Noller et al. 2003). However, the use of the variable-number tandem repeats (VNTRs) technique has been exploited in clonality research of *E. coli* O157:H7 bacteria. In particular, multi-locus VNTR analysis (MLVA) that examines multiple VNTR loci has been utilized along with PFGE in epidemiological comparisons and to distinguish outbreak and sporadic *E. coli* O157:H7 isolates (Noller et al. 2003, Lindstedt et al. 2004). Moreover, microarray-based pathogen detection targeting *eae*, $stx_1$, $stx_2$, and *fliC* has recently been developed (Vora et al. 2004).

# AIMS OF THE STUDY

The aims of this study were:

- To set up pheno- and genotypic methods to gain epidemiological knowledge on the characteristics of human EHEC O157 and non-O157 strains isolated in Finland.

- To study the potential association of certain characteristics of human EHEC O157 and non-O157 strains with a clinical picture of a patient.

- To compare the molecular and phenotypic profiles of nonmotile SF O157 human strains isolated in Finland to those occurring in Germany.

- To help in epidemiological surveillance and trace back human EHEC O157 and non-O157 infections.

- To determine the geographical prevalence of EHEC in Finland and explore the putative sources of human EHEC infections by in-depth interviews of the infected people.

# MATERIALS AND METHODS

## 1. Bacterial strains and patients (I–V)

### 1.1 EHEC strains isolated from patients (I–V)

All EHEC strains of *E. coli* O157 (n=127) and non-O157 (n=73) isolated from Finnish subjects (n=200) with EHEC infection from 1990 through 2002 in Finland were studied (Table 3). Also, three additional EHEC non-O157 strains isolated from the patients with double infection, and three O157 strains being negative for *stx* were analysed. The strains originated from *E. coli* cultures or primary stool cultures sent from the clinical hospital laboratories to the Enteric Bacteria Laboratory (EBL) of the National Public Health Institute (KTL) for microbiological identification and verification of the presence of EHEC bacteria.

Table 3. Number of subjects and *E. coli* O157 and non-O157 strains isolated during 1990–2002 and used in this study.

| | | | Isolates of serogroup | | Time period |
|---|---|---|---|---|---|
| **Study** | **Subjects** | **Isolates** | **O157** | **Non-O157** | **(mo / year)** |
| I | 105 | 105 | 105 | 0 | 1/1990–12/1999 |
| II | 55 | 56 | 0 | 56 | 1/1990– 8/2000 |
| III | 170 | 173 | 111 | 62 | 1/1990–12/2000 |
| IV | 99 | 99 | 52 | 47 | 1/1998–12/2002 |
| V | 9 | 9 | 9 | 0 | 1/1997– 3/2002 |

### 1.2 Reference strains (I–V)

Reference strains used at EBL originated from the strain collections of the American Type Culture Collection (ATCC, USA), the Belgium Coordinated Collections of Microorganisms (BCCM, University of Ghent, Belgium), the National Veterinary and Food Research Institute (EELA, Helsinki, Finland), the Laboratory of Enteric Pathogens, Health Protection Agency (HPA, London, England), the International *Escherichia* and *Klebsiella* Centre (WHO), Statens Serum Institute (SSI, Denmark), and the Robert Koch Institute (RKI, Berlin, Germany) (Table 4). In addition, for O grouping, O-specific strains (n=40) originating from SSI or EBL were used.

Table 4. Reference strains used as positive or negative controls.

| Species and Strain No. | Characteristic needed | Strain reference and origin | | Study |
|---|---|---|---|---|
| *E. coli* | | | | |
| RH 1484 | Negative control | 25922 | ATCC | I-V |
| RH 4270 | $stx_1$, $stx_2$, *eae*, *ehlyA* | 43895 | ATCC | I-V |
| RH 4872 | $stx_{2d\text{-}Ount}$ | LMG 18459 | BCCM | I, III, IV |
| RH 4827 | O157:H7 PFGE pattern | G5244 | EELA | I, II, IV, V |
| RH 1741 | Phage type PT2 | TS2 | HPA | I, III, IV, V |
| RH 1760 | Phage type PT8 | TS4 | HPA | I, III, IV, V |
| RH 1743 | Phage type PT14 | TS8 | HPA | I, III, IV, V |
| RH 1744 | Phage type PT32 | TS14 | HPA | I, III, IV, V |
| RH 1745 | Phage type PT49 | TS32 | HPA | I, III, IV, V |
| RH 1746 | Phage type PT54 | TS54 | HPA | I, III, IV, V |
| IH 57022 | H-antigen H2[1] | IH 57022 | EBL | I-V |
| IH 57105 | H-antigen H16[1] | IH 57105 | EBL | I-V |
| IH 57074 | H-antigen H49[1] | IH 57074 | EBL | I-V |
| IH 53472 | Enterohemolysis | CB 4120 | RKI | II, III, V |
| IH 53471 | Non-hemolysis | KK7/1 | RKI | II, III, V |
| IH 53470 | α-Hemolysis | U4/41 | RKI | II, III, V |
| RH 6015 | *eae*-α | RD 267 | EELA | V |
| RH 6017 | *eae*-β | RD 396 | EELA | V |
| RH 6019 | *eae*-γ | RD 609 | EELA | V |
| RH 6021 | *eae*-ε | C 622 | EELA | V |
| *Salmonella* Braenderup | | | | |
| RH 6184 | International PFGE control | H9812 | SSI | V |

[1] Verified by SSI.

## 1.3 Data collection of the patients with EHEC infection (I–V)

The data on the age, gender, symptoms, a recent travel abroad, and home locality of the subject were collected on a special form accompanying the isolate or were asked by telephone from the hospital. In addition, during 1998–2002, a subset of patients (n=68) was interviewed in-depth by telephone by a trained person at KTL.

Interviewees were asked about consumption of cooked, undercooked, or unpasteurized food items, contact with cattle farm or other animals, contact with persons having diarrhea, or environmental exposure.

## 2. Investigation of the stool- and pure *E. coli* cultures (I–V)

The presence of EHEC bacteria in primary stool cultures or pure cultures was investigated by PCR detecting the $stx_1$ and $stx_2$ genes (Table 5). In addition, the possession of *eaeA* was studied (Heuvelink et al. 1995, Keskimäki et al. 1996). The primers for the $stx_1$ and $stx_2$ genes were according to Olsvik and Strockbine 1993, Jackson et al. 1987a and Jackson et al. 1987b. The protocol was executed as previously described (Olsvik and Strockbine 1993, Keskimäki et al. 1998) except that the PCRs were carried out separately and with minor modifications (Eklund et al. 2001). A loopful of the bacterial growth on agar plates suspended in 500 µl of sterile water was boiled for 10 minutes, and the amount of template was 1.5 µl. The PCR run of $stx_1$ was executed as described (Olsvik and Strockbine 1993) except that the amplification steps were repeated 35 times. All PCR runs included positive (RH 4270) and negative (RH 1484) control strains. PCR reactions were also internally controlled with one colony of the positive control strain spiked into the suspensions of the primary cultures to reveal the possible inhibitory factors for the PCR reaction. All PCR products were electrophoresed through 1% agarose gel (SeaKem ME, FCM BioProducts, Mich.), the gel was stained with ethidiumbromide, and visualized and photographed by AlphaImager system (Alpha Innotech, CA) under UV transillumination. The amplified fragments were identified according to their specific size. After confirmation of the carriage of the *stx* gene(s), the specific colony was subcultured as pure culture and stored in skim milk tubes at -70°C for further use.

## 3. Identification of the EHEC isolates (I–V)

All isolates were identified biochemically as *E. coli* species by API 20E (BioMérieux SA, Marcy l'Etoile, France). In addition, sorbitol fermentation of the isolates was detected on SMAC agar plates and in tubes containing 0.5% sorbitol after overnight incubation at 37°C (Wells et al. 1983). The β-glucuronidase (PGUA) activity of the isolates was investigated with Rosco diagnostic tablets (A/S Rosco, Taastrup, Denmark) after four hours to overnight incubation at 37°C.

## 4. O:H serotyping (I–V)

The isolates were O grouped as previously described (Ørskov and Ørskov 1984, Siitonen 1992). The agglutination of the heated bacterial suspension with O-specific antisera indicated the O type. The strains giving clumping with 4% saline indicated the loss of the O antigen, and were defined as O Rough. In addition, the O157 antigen was tested with the *E. coli* O157 antigen kit (Oxoid, Basingstoke, England). Of the specific O-sera used, 35 (O1, O2, O4, O6, O7, O8, O9, O11, O15, O16, O18, O22, O25, O26, O44, O50, O55, O75, O77, O83, O85, O86, O91, O100, O111, O112, O114, O119, O124, O125, O126, O127, O128, O142, O157) originated from KTL and five from SSI (O5, O103, O121, O145, O174). The strains nontypeable with the sera available at EBL were sent to SSI for further typing. The H type of the isolates was indicated by the agglutination of the bacterial suspension of motile isolates with specific H antisera detecting all known flagellar antigens. Strains, which did not move through a semisolid agar tube after cultivation of five days, were indicated as nonmotile (HNM or $H^-$) (Ratiner 1991). The H antisera were obtained from Dr. Yuli Ratiner, the Mechnikov Research Institute for Vaccines and Sera, Russia.

## 5. Phage typing of O157 strains (I, III–V)

The bacteriophages specific for *E. coli* O157 phage typing were kindly provided as 100 x Routine Test Dilutions (RTDs) from HPA. The bacterial layer grown on double strength nutrient agar plates were subsequently exposed to the phages using the scheme originally described by Ahmed et al. (1987) and extended by Khakhria et al. (1990). The results were interpreted according to a phage type list that reveals 66 confirmed and 14 provisional phage types. A strain that reacted with the typing phages, but did not conform to a defined phage-typing pattern, was defined as Reacts but Does Not Conform (RDNC). The result of a RDNC strain was confirmed by phage typing five additional colonies of the strain.

## 6. Detection of Shiga toxin (Stx) production (III, V)

The production of Stx1 and Stx2 toxin of the isolates were determined by a commercial reversed passive latex agglutination (VTEC-RPLA) test kit (Denka Seiken Co, Ltd., Tokyo, Japan). The bacterial cells were grown shaken in Casamino Acids-Yeast extract broth overnight at +37°C, lysed with polymyxin (5000 U/ml) (Beutin et al. 1996b) during 1 hour incubation in shaking at +37°C, and centrifuged

for 30 min at 3,000 rpm (BiofugeA instrument, Heraeus, Sepatech, West Germany). The titer of the supernatant was determined according to the manufacturer's instructions. The quantification of Stx production was detected up to a dilution of 1:128.

## 7. Detection of enterohemolytic (Ehly) activity (II, III, V)

The ability of the isolates to produce enterohemolysin was detected on tryptose blood agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 10 mM $CaCl_2$ and 5% defibrinated sheep blood cells, and washed three times with phosphate buffered saline, pH 7.2 (Beutin et al. 1989). The hemolytic reactions of the colonies growing on the plates were investigated after three hours (hemolysis around the colonies indicated production of α-hemolysin, negative reaction indicated production of enterohemolysin or indicated nonhemolysis), and after overnight incubations at 37°C (positive reaction indicated production of enterohemolysin and negative reaction indicated nonhemolysis).

## 8. Antimicrobial susceptibility testing (II)

The susceptibility of the isolates for antimicrobial agents were tested by the agar diffusion method (National Committee for Clinical Microbiology Standards, 1997) on Iso-Sensitest agar (Oxoid, Hampshire, England) for the following 12 agents (Oxoid, Hampshire; England): ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline, ciprofloxacin, trimethoprim, gentamicin, nalidixic acid, cefotaxime, mecillinam, and imipenem.

## 9. Pulsed-field gel electrophoresis (PFGE) (I, II, IV, V)

For studies I, II, and IV, bacterial growth of a pure isolate on nutrient agar was suspended in TEN-buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.1 M EDTA, pH 7.5) to obtain an absorbance of 0.280-0.310 at 600 nm (UV/VIS Spectrophotometer Lambda Bio 10, Perkin Elmer, Überlingen, Germany). A sample plug containing equally (500 µl) bacterial suspension and 1.8% of low-melting-point agarose (InCert agarose; FMC BioProducts, Rockland, Maine) was digested overnight with 0.18 mg/ml of proteinase K (Boehringer Mannheim, Indianapolis, USA) at 50°C in 3 ml of ES-buffer (0.5 M EDTA, 1% N-lauroylsarcosine). The plugs were washed as previously described (Keskimäki et al. 1998), digested with 10 U of *Xba*I restriction

enzyme (New England Biolabs Inc., Mass., USA) (Table 7) and were electrophoresed through 1% SeaKem Gold agar (Cambrex Bio Science Rockland Inc., Rockland, ME, USA) using nationally standardized parameters: 5 to 40 s, 21 h, 6 V/cm, 120°, 14°C, and including the internal reference strain RH 4827 (Rantala et al. 2000). After staining in ethidiumbromide, the gel was documented by AlphaImager System (Alpha Innotech Corporation, CA., USA) and analysed by BioNumerics (versions 1.01, 2.0 or 3.0 software, Applied Maths bvba, Belgium). One fragment difference was defined as significant. The O157 PFGE types were coded from 1.1 to 1.76. The PFGE types the human non-O157 strains were coded according to the O group (for example O26a, O26b etc.). In addition, for study V, an internationally standardized PFGE method was used (PulseNet 2002).

## 10. PCR-restriction fragment length polymorphism (RFLP) (I, III–V)

Possession of the *stx* genes and their variants ($stx_1$, $stx_2$, $stx_{2c}$) were detected by PCR-RFLP according to Bastian et al. 1998 and Lin et al. 1993. In addition, carriages of $stx_{2d\text{-}Ount}$ and $stx_{2d\text{-}OX3a}$ were detected according to Pierard et al. 1998 (Table 5, Table 6). The genes were investigated with minor modifications (Eklund et al. 2002). A loopful of bacterial growth on the SMAC agar plates was suspended in 500 µl of sterile water, boiled for 10 min, and centrifuged at 10 000 rpm for 15 sec. Of the suspensions, 1 µl was used as a template, and *AmpliTaq* Gold (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, N. J.) was used as a polymerase. The cycling steps of a PCR run were executed as published, except the final elongation step was 10 in at 72°C. Strains RH 4270, RH 4872 were used as positive control strains and strain RH 1484 was used as a negative control strain. The amplified DNA was analysed by gel electrophoresis through a 1% gel (SeaKem ME; FCM BioProducts; Mich, USA) and photographed by AlphaImager system (Alpha Innotech Corporation, CA., USA) under UV transillumination after staining with ethidiumbromide. The PCR products were restricted with 20 U of the restriction endonucleases *Hinc*II and *Acc*I (Lin et al. 1993, Bastian et al. 1998) and *Pvu*II and *Hae*III (Pierard et al. 1998) (Table 7). The restricted products were analysed by gel electrophoresis by using 2% agarose gel (SeaKem ME agarose, BioWhittaker Molecular Applications, Rockland, USA), and photographed as mentioned above.

## 11. Intimin typing (V)

The intimin typing of the *eae*-α, *eae*-β, *eae*-γ, *eae*-ε genes was executed by multiplex-PCR as described earlier (Reid et al. 1999, Zhang et al. 2002) (Table 5, Table 6), except that the common reverse primer eaeP2 was replaced by the primer

SK2 (Karch et al. 1993). In addition, the *eae*-allele specific forward primer EaeF2 of this study was complementary to the reverse primer eaeP2 published by Reid et al. 1999. In addition, bacterial suspensions in 500 µl of sterile water were boiled for 10 minutes and centrifuged briefly. For the amplification, a 1.5 µl sample was added in 50 µl reaction mixture (Table 6), and the PCR amplification (Peltier Thermal Cycler, PTC 200, MJ Research, Mass., USA) was executed. The amplified products were electrophoresed through 1.2% agarose gel (SeaKem ME agarose, BioWhittaker Molecular Applications, Rockland, USA), and stained with ethidium bromide.

## 12. Additional methods (V)

For the nine SF *E.coli* O157:H$^-$ strains, a carriage of *stx*$_1$, *stx*$_2$, *stx*$_{2c}$, *eae*-α, *eae*-β, *eae*-γ, *eae*-ε, *efa1*, *cdt-V A*, *cdt-V B*, *cdt-V C*, EHEC-*hlyA*, *etpD*, *katP*, *espP* and *sfpA* were determined by PCR and carriage of *fliC* by PCR-RFLP (Table 5) at the Institute of Hygiene and the National Consulting Laboratory on Hemolytic Uremic Syndrome, University Hospital Münster, Münster, Germany. In addition, the production of CDT toxin was investigated there, with the use of Chinese hamster ovary (CHO) cell assay as previously described (Janka et al. 2003, Bielaszewska et al. 2004).

## 13. Statistical methods (II, III, IV)

Epi-Info 6 software (Dean et al. 1994) were used for statistical analyzes. In addition, Epi-Info 2000 version 1.12 was used for Fisher's exact two-tailed test to determine statistical significance. $P<0.05$ indicated statistical significance.

Table 5. PCR methods and conditions used.

| **Primer orientation and sequences: Forward Reverse 5' → 3'** | **Target** | **Size (bp)** | **PCR conditions [1]** | **Study** | **References** |
|---|---|---|---|---|---|
| stx1-1: CAGTTAATGTGGTGGCGAAG<br>stx1-2: CTGCTAATAGTTCTGCGCATC | *stx*$_1$ | 894 | 95º, 94º, 60º, 72º, 72º<br>6', 1', 1', 1', 10' | I-V | Olsvik, Strockbine 1993, Keskimäki et al. 1998, Eklund et al. 2001 |
| KS7: CCCGGATCCATGAAAAAAACA-TTATTAATAGC<br>KS8: CCCGAATTCAGCTATTCTGAGT-CAACG | *stx$_1$B*[5] | 285 | 94º, 94º, 52º, 72º, 72º<br>5', 30'', 1', 1', 5' | V | Friedrich et al. 2002 [2] |
| stx2-1: CTTCGGTATCCTATTCCCGG<br>stx2-2: CGATGCATCTCTGGTCATTG | *stx*$_2$ | 478 | 95º, 95º, 62º, 72º, 72º<br>4,5', 1', 1', 1', 5' | I-V | Olsvik, Strockbine 1993, Keskimäki et al. 1998 |
| GK3: ATGAAGAAGAAGATGTTTATG<br>GK4: TCAGTCATTATTAAACTG | *stx*$_2$B[5], *stx*$_{2c}$ | 260 | 94º, 94º, 52º, 72º, 72º<br>5', 30'', 1' , 1' , 5' | V | Friedrich et al. 2002[2] |
| VT2-cm: AAGAAGATATTTGTAGCGG<br>VT2-f: TAAACTGCACTTCAGCAAAT | *stx*$_{2d\text{-}Ount}$, s*tx*$_{2d\text{-}OX3a}$ | 256 | 94º, 94º, 55º, 72º, 72º<br>5', 30'', 1' , 1' , 5' | V | Friedrich et al. 2002[2] |
| VT2-cm: AAGAAGATATTTGTAGCGG<br>VT2-f: TAAACTGCACTTCAGCAAAT | *stx*$_{2d\text{-}Ount}$, s*tx*$_{2d\text{-}OX3a}$ | 256 | 95º, 94º, 55º, 72º , 72º<br>6', 25'',50'', 26'',10' | I, III, IV | Pierard et al. 1998, Eklund et al. 2002 |
| VT2-e: AATACATTATGGGAAAGTA-ATA<br>VT2-f: TAAACTGCACTTCAGCAAAT | *stx*$_{2d\text{-}Ount}$, *stx*$_{2d\text{-}OX3a}$ | 348 | 95º, 94º, 45º, 72º , 72º<br>6', 25'',50'', 26'',10' | I, III, IV | Pierard et al. 1998, Eklund et al. 2002 |

*continues on the following page*

Table 5. continued

| Primer orientation and sequences: Forward Reverse 5' → 3' | Target | Size (bp) | PCR conditions | Study | References |
|---|---|---|---|---|---|
| FK1:CCCGGATCCAAGAAGATGTTTATAG<br>FK2:CCCGAATTCTCAGTTAAACTTCACC | *stx*$_{2e}$*B*[5] | 280 | 94º, 94º, 55º, 72º, 72º<br>5', 30'', 1', 40'', 5' | V | Friedrich et al. 2002 [2] |
| LP43: ATCCTATTCCCGGGAGTTTACG<br>LP44: GCGTCATCGTATACACAGGAGC | *stx*$_2$ and variant | 584 | 94º, 94º, 57º, 72º, 72º<br>5', 30'', 1', 1', 5' | V | Friedrich et al. 2002 [2] |
| LINU: GAACGAAATAATTTATATGT<br>LIND: TTTGATTGTTACAGTCAT | *stx*$_1$, *stx*$_2$ variants | ~900 | 95º, 94º, 43º, 72º , 72º<br>6', 1' , 1,5', 1,5' 10' | I, III, IV | Lin et al. 1993, Bastian et al. 1998, Eklund et al. 2002 |
| eae1: TGCGGCACAACAGGCGGCGA<br>eae2: CGGTCGCCGCACCAGGATTC | *eaeA* | 629 | 95º, 95º, 72º, 72º<br>5', 1', 1', 5' | I-V | Heuvelink et al. 1995, Keskimäki et al. 1996 |
| EaeP1: CTGAACGGCGATTACGCGAA | *eae*[3] | 360 | | V | Reid et al. 1999 |
| SK2: CCGGATCCGTCTCGCCAGTA-TTCG | | | | | Karch et al., 1993 Eklund et al. 2005a |
| EaeF2: CTGGATCGTATCGTCTGG | *eae*[4] | | 95º, 94°, 54°, 72°, 72° | | Reid et al. 1999 |
| Ecoeaeα: CTGGAGTTGTCGATGTT | *eae*-α | 739 | 3 ', 1', 2', 45 '',10' | | Reid et al. 1999 |
| Ecoeaeβ: GTAATTGTGGCACTCC | *eae*-β | 1,022 | | | Reid et al. 1999 |
| Ecoeaeγ: GCCTCTGACATTGTTAC | *eae*-γ | 870 | | | Zhang et al. 2002 |
| LP5: AGCTCACTCGTAGATGAC-GGCAAGCG | *eae*-ε | 1,189 | | | |
| E643f: TATCAGGCCAATCAAAACAG<br>E1598r: AGACACTGGTAAATTTCGC | *efa1* | 974 | 94º, 94º, 50º, 72º, 72º<br>5', 30'', 1', 1', 5' | V | Janka et al. 2002 [2] |

*continues on the following page*

Table 5. continued

| Primer orientation and sequences: Forward Reverse 5' → 3' | Target | Size (bp) | PCR conditions | Study | References |
|---|---|---|---|---|---|
| E5242f: TAAGCGAGCCCTGATAAGCA<br>E5854r: CGTGTTGCTTGCCTTTGC | *efa2* | 630 | 94º, 94º, 55º, 72º, 72º<br>5', 30'', 1', 1', 5' | V | Janka et al. 2002 [2] |
| E7044f: TGTCTAACTGGATTGTATGGC<br>E7710r: ATGTTGTTCCCGGCCCAG T | *efa3* | 685 | 94º, 94º, 56º, 72º, 72º<br>5', 30'', 1', 1', 5' | V | Janka et al. 2002 [2] |
| c338f: AGCATTAAATAAAAGCACGA<br>c2135r: TACTTGCTGTGGTCTGCTAT | *cdt-VA* | 1,329 | 94º, 94º, 52º, 72º, 72º<br>5', 30'', 1', 1', 5' | V | Janka et al. 2003 [2] |
| c1309f: AGCACCCGCAGTATCTTTGA<br>c2166r: AGCCTCTTTTATCGTCTGGA | *cdt-VB* | 1,363 | 94º, 94º , 52º, 72º, 72º<br>5', 30'', 1', 1', 5' | V | Janka et al. 2003 [2] |
| P105: GTCAACGAACATTAGATTAT<br>c2767r: ATGGTCATGCTTTGTTATAT | *cdt-VC* | 748 | 94º, 94º, 49º, 72º, 72º<br>5', 30'', 1', 1', 5' | V | Janka et al. 2003 [2] |
| HlyA1: GGTGCAGCAGAAAAAGTTG-TAG<br>HlyA4:TCTCGCCTGATAGTGTTTGGTA | EHEC-*hlyA* | 1,550 | 94º, 94º, 57º, 72º, 72º<br>5', 30'', 1', 90'', 5' | V | Schmidt et al. 1995 [2] |
| D1: CGTCAGGAGGATGTTCAG<br>D13R: CGACTGCACCTGTTCCTGATTA | *etpD* | 1,062 | 94º, 94º, 56º, 72º, 72º<br>5', 30'', 1', 70'', 5' | V | Friedrich et al. 2003 [2] |
| wkat-B:CTTCCTGTTCTGATTCTTCTGG<br>wkat-F: AACTTATTTCTCGCATCATCC | *katP* | 2,125 | 94º, 94º, 56º, 72º, 72º<br>5', 30'', 1', 150'', 5' | V | Zhang et al. 2000 [2] |

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Table 5. continued

| Primer orientation and sequences: Forward Reverse 5' → 3' | Target | Size (bp) | PCR conditions | Study | References |
|---|---|---|---|---|---|
| esp-A: AAACAGCAGGCACTTGAACG<br>esp-B: GGAGTCGTCAGTCAGTAGAT | *espP* | 1,830 | 94º, 94º, 56º, 72º, 72º 5', 30'', 1', 150'', 5' | V | Friedrich et al. 2003 [2] |
| sfpA-U: AGCCAAGGCCAAGGGAT-TATTA<br>sfpA-L: TTAGCAACAGCAGTGAAG -TCTC | *sfpA* | 440 | 94º, 94º, 59º, 72º, 72º 5', 30'', 1', 1', 5' | V | Friedrich et al. 2004 [2] |
| FSa1:CAAGTCATTAATAC(A/C)AA-CAGCC<br>rFSa1: GACAT(A/G)TT(A/G)GA(G/A/C)-ACTTC(G/C)GT | *fliC* | 1,600 | 94º, 94º, 55º, 72º, 72º 5', 30'', 1', 2', 10' | V | Sonntag et al. 2004 [2] |

[1] PCR conditions contained 30 cycles of denaturing, annealing and extension, except PCR targeting $stx_1$ (35 cycles) , $stx_1$ , $stx_2$ variants (39 cycles), *eae*-α, *eae*-β, *eae*-γ, *eae*-ε (26 cycles).

[2] Methods performed at the Institute of Hygiene and the National Consulting Laboratory on Hemolytic Uremic Syndrome, University Hospital Münster, Münster, Germany.

[3] Universal primers targeting the *eae*.

[4] Allele specific primers targeting the subtypes of the *eae*. The primer EaeF2 is complementary to reverse primer eaeP2 (Reid et al. 1999).

[5] *stx* gene locating in the B subunit.

Table 6. PCR reaction mixtures used at EBL.

| Gene(s) to be detected | Master-Mix / µl | | | | | Study | References |
|---|---|---|---|---|---|---|---|
| | $H_2O$ | Buffer | dNTP, 5 mM | Primers (C in reaction) | Enzyme[a] | | |
| *stx*$_1$ | 39.7 | 5.0 | 2.0 | forw. 0.6 (9 µM)<br>rev. 0.7 (9 µM) | 0.50 | I-V | Olsvik, Strockbine 1993,<br>Keskimäki et al. 1998, Eklund et al. 2001 |
| *stx*$_2$ | 26.2 | 3.5 | 0.98 | forw. 1.0 (10 µM)<br>rev. 1.0 (10 µM) | 0.70 | I-V | Olsvik, Strockbine 1993,<br>Keskimäki et al. 1998 |
| *eae* | 27.0 | 3.5 | 0.98 | forw. 0.6 (10 µM)<br>rev. 0.6 (10 µM) | 0.60 | I-V | Heuvelink et al. 1995, Keskimäki et al. 1996 |
| *stx* or<br>*stx* variant | 32.0 | 5.0 | 2.0 | forw. 5.0 (6 µM)<br>rev. 5.0 (6 µM) | 0.25 | I, III, IV | Lin et al. 1993, Bastian et al. 1998,<br>Eklund et al. 2002 |
| *stx*$_{2\text{-dOunt}}$<br>s*tx*$_{2d\text{-}OX3a}$ | 40.7 | 5.0 | 2.0 | forw. 0.9 (6 µM)<br>rev. 0.9 (6 µM) | 0.50 | I, III, IV | Pierard et al. 1998, Eklund et al. 2002 |
| *eae*-α or<br>*eae*-β or<br>*eae*-γ or<br>*eae*-ε | 33.8 | 5.0 | 2.0 | a:forw. 0.52 (200ng)<br>a:rev. 0.34 (200ng)<br>b:forw: 0.51(200ng)<br>b:rev. 0.49 (200ng)<br>b:rev. 0.76 (200ng)<br>b:rev. 0.70 (200ng)<br>b:rev. 0.41 (200ng) | 0.50 | V | Karch et al., 1993, Reid et al. 1999,<br>Zhang et al. 2002, Eklund et al. 2005a |

[a] Enzyme used for the detection of the *stx*$_1$, *stx*$_{2\text{-dOunt}}$: AmpliTaq Gold (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, N. J.), *stx*$_2$ and *eae*: Dynazyme II (Finnzymes, Espoo, Finland), subtypes of *eae*: Taq-polymerase (Fermentas Life Sciences, Lithuania), with an addition of 4 µl of $MgCl_2$ (25mM) per reaction.

Table 7. Restriction enzymes used at EBL.

| Method | Enzyme[1] | Recognition site | Study |
|---|---|---|---|
| PCR-RFLP | *Hinc*II | 5'…GTY-RAC…3'<br>3'…CAR-YTG…5' | I, III, IV |
| | *Acc*I | 5'…GT-MKAC…3'<br>3'…CAKM-TG…5' | I, III, IV |
| | *Hae*III | 5'…GG-CC…3'<br>3'…CC-GG…5' | I, III, IV |
| | *Pvu*II | 5'…CAG-CTG…3'<br>3'…GTC-GAC…5' | I, III, IV |
| PFGE | *Xba*I | 5'…T-CTAGA…3'<br>3'…AGATC-T…5' | I, II, IV, V |

[1] New England Biolabs Inc., Mass., USA.

# RESULTS

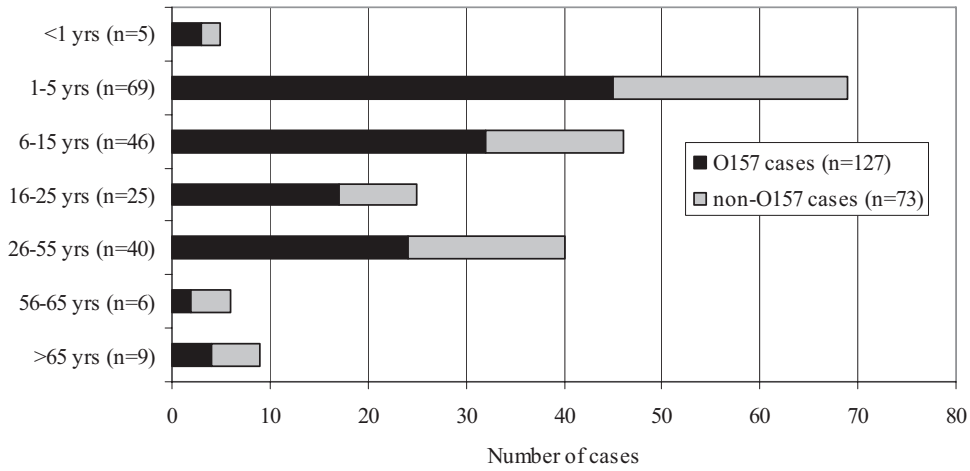
## 1. Occurrence of EHEC infections (I – V)

During 1990–2002, EHEC infections were detected in 200 patients. In addition, a double infection caused by two different strains occurred in three patients. Also, *stx* negative strains belonging to the typical EHEC O group O157 caused three infections. Of the 200 EHEC infections, strains of O157 caused 127 (64%), and in 84% of the cases, the infection was indigenously acquired. The remaining 73 (36%) infections were caused by strains of EHEC non-O157, also most (78%) being of domestic origin. Of all the infections, the majority (187 cases; 94%) occurred after 1996 with the following findings: 61 cases in 1997, 43 in 1998, 36 in 1999, 17 in 2000, 15 in 2001, and 15 in 2002. In addition, during the study period were detected 24 stool samples or mixed cultures being Stx positive in EIA test in clinical hospital laboratories (National Public Health Institute, EBL). However, the respective cultures of these samples lacked *stx* genes, did not show detectable Stx production in the RPLA test and EHEC isolate could not be found. These cases were excluded from further analysis for this thesis.

## 2. Demographic data of the patients (I - V)

The median age of the 200 patients was 9.5 years (range 0.2 to 85.3 years). Most (35%) of these patients belonged to the age group of 1 to 5 years (Figure 3). About half (n=101) of all patients were males.

Figure 3. Age distribution (years) of all patients with EHEC infection and serogroups of the infecting strains.



## 3. Pheno- and genotypic characteristics of strains (I - V)

### 3.1 O157 strains (I, III–V)

In all, 127 EHEC O157 strains were isolated the study period 1990–2002. Of these, 110 isolates (87%) carried *stx*$_2$ only, and 17 isolates possessed both *stx*$_1$ and *stx*$_2$. None of the strains carried the *stx*$_1$ only. Of all O157 strains, the majority (114 strains; 90%) were sorbitol-and β-glucuronidase negative, and expressed the H7 antigen. All these strains produced Stx1 and/or Stx2 with titers ranging from 1:2 to 1:128, and were enterohemolytic. In addition 13 SF, β-glucuronidase positive, EHEC O157:H$^-$ strains were detected.

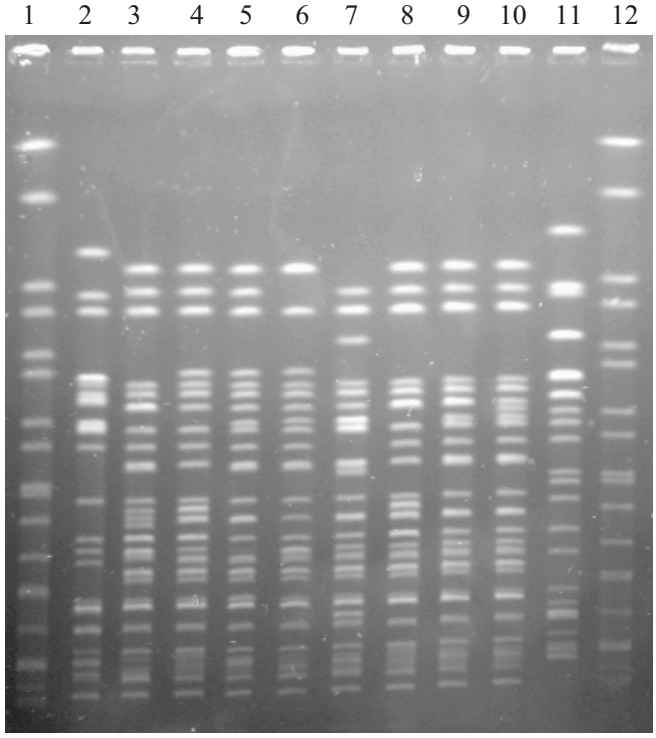
Of all EHEC O157 strains isolated from 105 Finnish patients during 1990–1999, the main phage types of the strains were PT2 (56%), PT49 (11%), PT4 (10%) and PT88 (6%). Among the 105 infections, 93 O157 strains of domestic origin were detected, which were distributed into 24 PFGE types. The remaining 12 strains were associated with a recent trip abroad, which were distributed into 10 PFGE types. Of the PFGE types of foreign origin, two occurred also indigenously. The main PT/PFGE combination was PT2/1.1 representing 46% followed by PT49/1.12 (4%) and PT4/1.47 (4%). Of all 32 PFGE types, 30 (94%) associated only with a certain PT. The remaining two PFGE types (1.1 and 1.3) included PTs 2, 14, and 2, 50, RDNC, respectively.

By PCR-RFLP, 70% of the 105 strains possessed *stx*$_2$ with *stx*$_{2c}$. Most (71/74 strains) of the strains carrying both of these two genes were of domestic origin. The strains possessing *stx*$_2$ only (21 strains) belonged to six PTs (PT2, 4, 14, 50, 88, and RDNC) and 12 PFGE subtypes. Of the *stx*$_2$ positive strains 18 (86%) were of domestic origin, only three strains (PT4/1.47) being linked to a cruise between Finland and Sweden. The strains carrying *stx*$_{2c}$ only were all of foreign origin (Turkey [PT8/1.9], Spain [RDNC 3/1.11], and Greece [RDNC3/1.59]. The five strains positive for *stx*$_1$ and *stx*$_{2c}$ associated mostly with a recent trip to Spain (PT8/1.52), Turkey (PT8/1.29), and the Czech Republic (PT8/1.30).

Of the domestically acquired *E. coli* O157 infections during 1997–2001, nine SF, β-glucuronidase-positive O157:H$^-$ strains isolated from patients with no epidemiological link to each other were investigated further. Within these strains, several phenotypic (sorbitol fermentation, β-glucuronidase-activity, PT88 or PT88 variant, production of CDT, and lack of Ehly-production) and genotypic characteristics (possession of *stx*$_2$, *eae*-γ, *efa1*, *cdt-V*, EHEC-*hlyA*, *etpD*, *sfpA*, or lack of *katP*, *espP*) were very similar. However, among these isolates strains negative for *stx* also occurred. Seven strains clustered in the PFGE profiling with

highly similar SIs (92% to 98%), banding patterns differing with one to seven bands. Within two remaining strains, (i.e. first [IH 53440] and last [IH 57201] strain isolated), PFGE profiles with 14 bands difference were seen (Figure 4).

Figure 4. PFGE fingerprint patterns of the sorbitol-fermenting *E. coli* O157 human strains isolated in Finland. Lanes 1, 12: PFGE standard *Salmonella* Braenderup (H9812); 2, IH 53440; 3, IH 56776; 4, IH 56909; 5, IH 56969; 6, IH 57086; 7, IH 57201; 8, IH 56905; 9, IH 57075; 10, IH 57084; 11, *E. coli* O157:H7 control strain (G 5244).



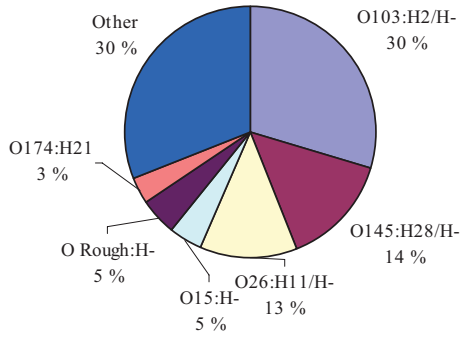
## 3.2 Non-O157 strains (II, III, IV)

During 1990–2002, 73 human EHEC non-O157 infections of domestic (59 cases; 81%) and foreign (14 cases; 19%) origin were detected. The causative strains belonged to 22 O groups, 16 H antigens, and 34 O:H serotypes. In addition, strains of serogroup O157:H7/H$^-$ caused over 120 infections (Table 8). The most common non-O157 O groups of the strains were O103 (n=19), O145 (n=9), and O26 (n=8) (Figure 5). In addition, two new serotypes of EHEC strains were found: O102:H7 and O181:H49. Almost all isolates (71 strains; 97%) fermented sorbitol, and were positive for β-glucoronidase production (67 strains; 92%). Only two strains of the serotypes O76:H19 and O145:H$^-$ were sorbitol-negative. Of the non-O157 strains, approximately half (39 strains, 53%) carried *stx*$_2$, 30 strains (41%) possessed *stx*$_1$ only, and four strains of serotypes O43:H2, O111:H$^-$, and ONT:H19 were positive for both *stx*$_2$ and *stx*$_1$. The *stx*$_1$ gene was common among strains of O26 (8/8 isolates), O103 (16/19), and O15 (2/3). The majority (48/73 strains; 66%) possessed *eae*, and 53 strains (73%) produced Ehly.

Table 8. Serotypes and corresponding number of EHEC strains of domestic and foreign origin isolated during 1990–2002.

| **Serotype** | **N** | **Serotype** | **N** | **Serotype** | **N** |
|---|---|---|---|---|---|
| O2:H27 | 1 | O102:H7 | 1 | O157:H$^-$ | 13 |
| O2:H29 | 1 | O103:H2 | 18 | O165:H25 | 1 |
| O8:H9 | 1 | O103:H$^-$ | 1 | O174:H2 | 1 |
| O15:H$^-$ | 3 | O107:H27 | 1 | O174:H21 | 2 |
| O20:H7 | 2 | O111:H8 | 1 | O178:H19 | 2 |
| O26:H11 | 7 | O111:H$^-$ | 1 | O181:H49 | 1 |
| O26:H$^-$ | 1 | O116:H21 | 1 | R:H2 | 1 |
| O43:H2 | 1 | O145:H28 | 4 | R:H4 | 3 |
| O76:H19 | 1 | O145:H$^-$ | 5 | R:H18 | 1 |
| O91:H21 | 1 | O153:H33 | 1 | R:H21 | 1 |
| O91:H40 | 1 | O156:H25 | 1 | R:H49 | 1 |
| O101:H$^-$ | 1 | O157:H7 | 114 | R:H$^-$ | 3 |

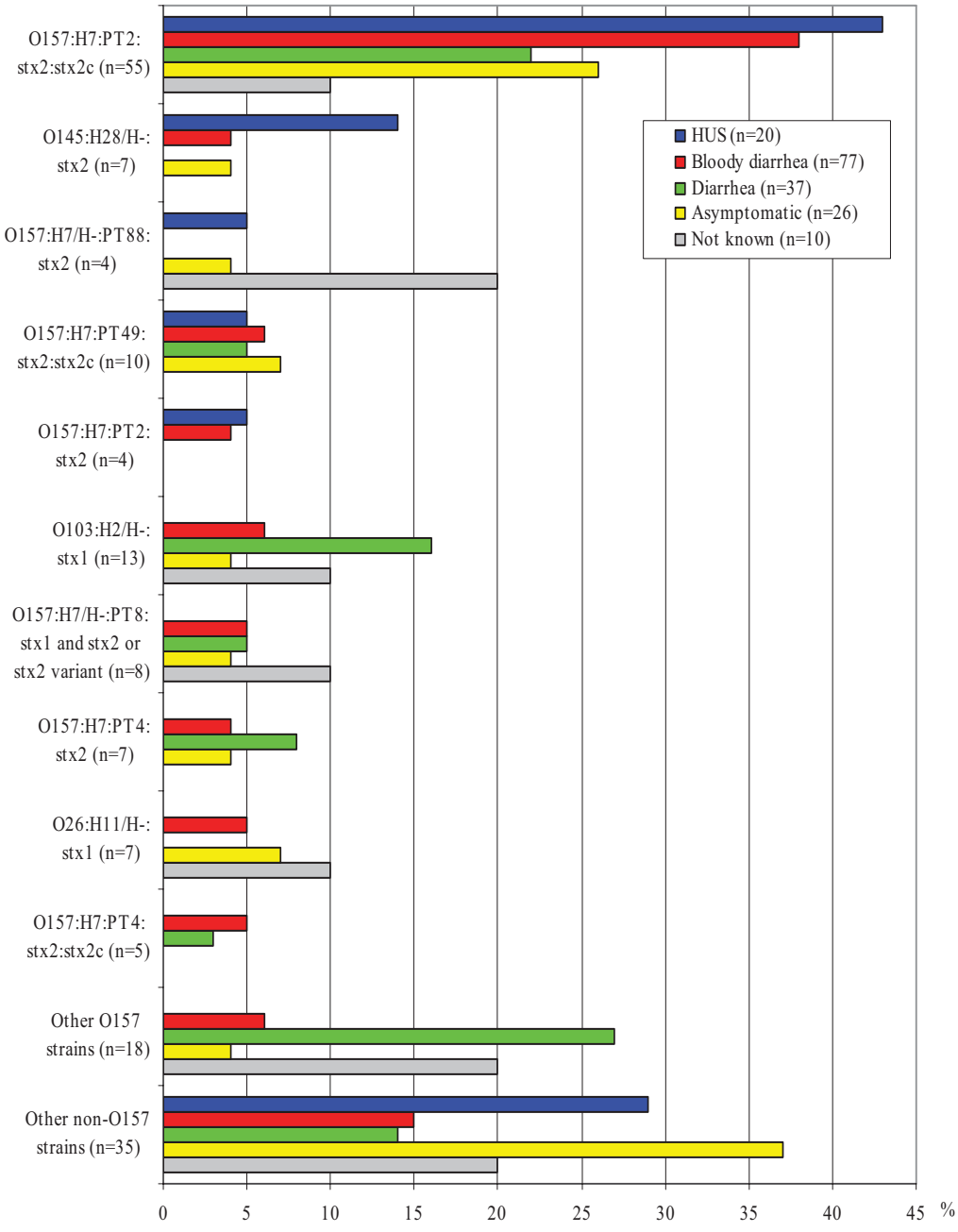
Figure 5. Distribution of serotypes of EHEC non-O157 strains (n=73) isolated from Finnish patients during 1990–2002.




## 4. Virulence profiles of EHEC strains (III)

In a comparison of characteristics of EHEC O157 or non-O157 strains with severe clinical picture (bloody diarrhea, HUS, TTP) of the patients (n=173), 10 *stx*-positive virulene profiles were associated with 71% of all infections (Figure 6). Of these, a virulence profile of the O157 group (O157:H7:PT2:$stx_2$:$stx_{2c}$:*eae*:Ehly) was significantly ($P$=0.02) more frequently associated with a severe clinical picture than were other profiles. In addition, the risk factor for severe symptoms was an age of less than five years and infection by this O157:H7 strain type. Strains of this virulence profile also caused the deaths of two children. However, among the non-O157 group, seven pheno- and genotypic virulence profiles of EHEC non-O157 strains were associated with patients suffering from HUS or TTP, but none of these infections was fatal. In particular, strains possessing $stx_2$ (O145:H28/H$^-$:$stx_2$, O174:H21:$stx_{2c}$, O Rough:H4:$stx_{2c}$, O101:H$^-$:$stx_2$, O Rough:H49:$stx_{2c}$, O107:H27 lacking *stx* [probably due to a loss of the *stx* gene], and O2:H29: $stx_2$ undigestable) were associated with a severe clinical picture of the patients (HUS or TTP).

Figure 6. Virulence profiles of EHEC strains (n=173) and symptoms of patients (n=170).



## 5. Geographical distribution and incidence of domestic EHEC infections (IV)

During 1998–2002, 99 EHEC infections of domestic origin occurred in 14 of the 21 hospital districts of Finland. Fifty-two were of O157 and 47 non-O157 infections, occurring in 13 and nine hospital districts, respectively. Most EHEC infections were detected in the Helsinki Region (28%), Kymenlaakso (16%), Central Ostrobothnia (8%), North Ostrobothnia (8%), Southwest Finland (8%), South Ostrobothnia (6%) and Central Finland (6%). However, the incidence was highest (10.3 / $10^5$ population) in Central Ostrobothnia (Figure 7).

Strains of serogroup O157 were associated with three clusters (C1-C3). In C1, the strains of same subtypes (O157:H7:PT2:*stx*$_2$:*stx*$_{2c}$:*eae*:1.1) caused three of a total of four infections. The remaining strain (O157:H$^-$:PT88:*stx*$_2$:*eae*:1.13) was isolated from a child whose mother was infected with a strain of the former C1 type. In C2, strains of subtypes (O157:H7:PT4:*stx*$_2$:*eae*:1.57 [3 strains], O157:H7:PT4:*stx*$_2$:*eae*:1.58 [1 strain] caused the infections. Among these strains, a two-band difference was observed in the PFGE patterns of the isolates. In C3 with three cases, an identical strain of subtype O157:H7:PT14:*stx*$_{1,2}$:*eae*:1.67 caused all the infections. The clusters were detected in 1998 (C1, C2) and 2001 (C3). However, no outbreak clusters caused by strains of non-O157 occurred. Overall, the most common (11 isolates) and widely distributed (in five hospital districts) subtype was O157:H7:PT2:*stx*$_2$:*stx*$_{2c}$:*eae*:1.1 which also caused C1.

During this study period (1998–2002), the incidence of the EHEC infections was highest in 1998 (0.64). In this year alone, the incidence of O157 (0.47) cases was higher than that of the non-O157 (0.17) cases. Since 1999, the incidence of all EHEC infections declined (0.62) being lowest (0.17) in 2002. EHEC infections were more prevalent during the summer months (June–August) than in other seasons. However, between infections caused by strains of EHEC O157 or non-O157, no clear seasonal variation was seen. Of all 26 serotypes detected, only strains of O groups O157, O103, and O145 caused infections in 2002 (Figure 8).

## 6. Vehicles for and transmission of EHEC infection (IV)

Of the 99 domestically acquired EHEC infections, nearly half, 49%, were family-related cases detected in 26 families. Sporadic infections represented 39%, and the remaining 11% was associated with small clusters. The vehicles for these infections were analyzed using in-depth interviews. No statistical difference between the exposure factors for O157 or non-O157 infection was observed. However, of the 53 sporadic or index patients interviewed, cattle or other animal contact was reported

by 42 patients; of them 79% were children under 10 years old. In addition, of the 53 patients, 86% had consumed ground meat products (three had eaten raw ground meat), and 18% unpasteurized milk. It was highly suspected that the C1 cluster was due to person-to-person spread. Also, within families, person-to-person transimission was a putative infection route. The C2 was associated with possible consumtion of hamburgers. For the C3, a food-borne source was implicated as a vehicle.

Figure 7. Incidence and number of domestic EHEC O157 / non-O157 infections in 21 hospital districts of Finland during 1998–2002.

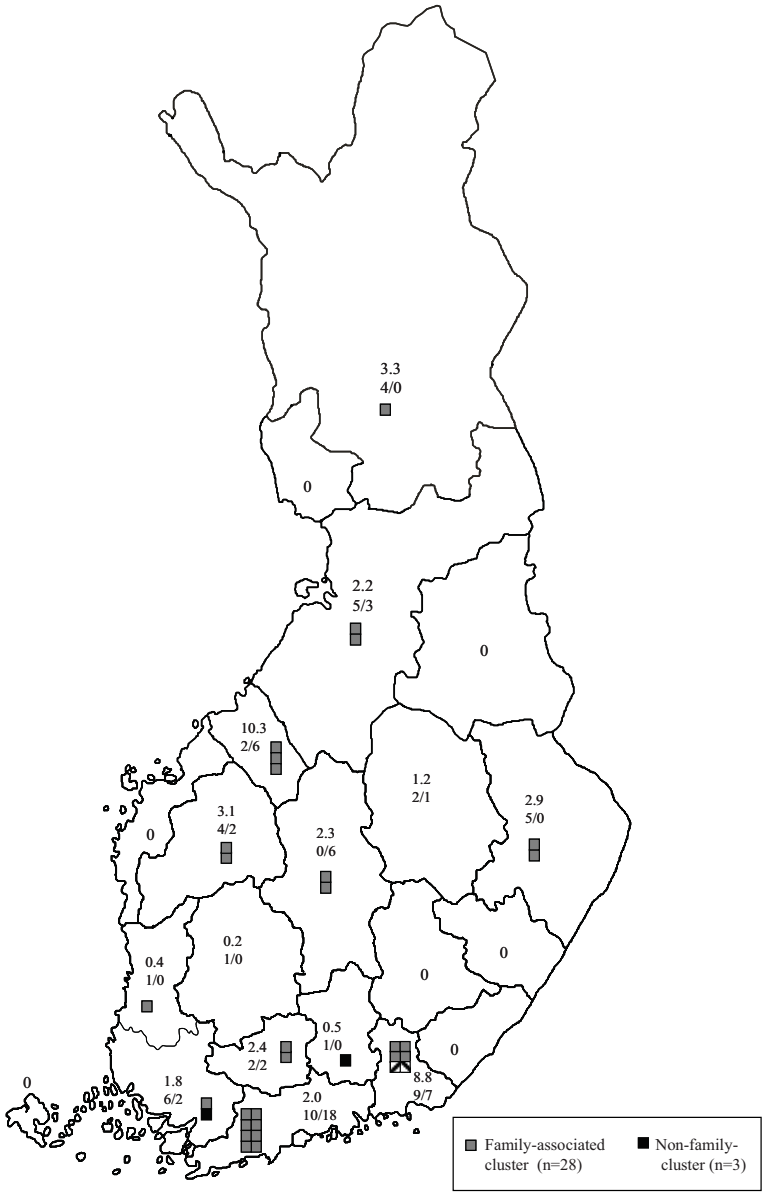
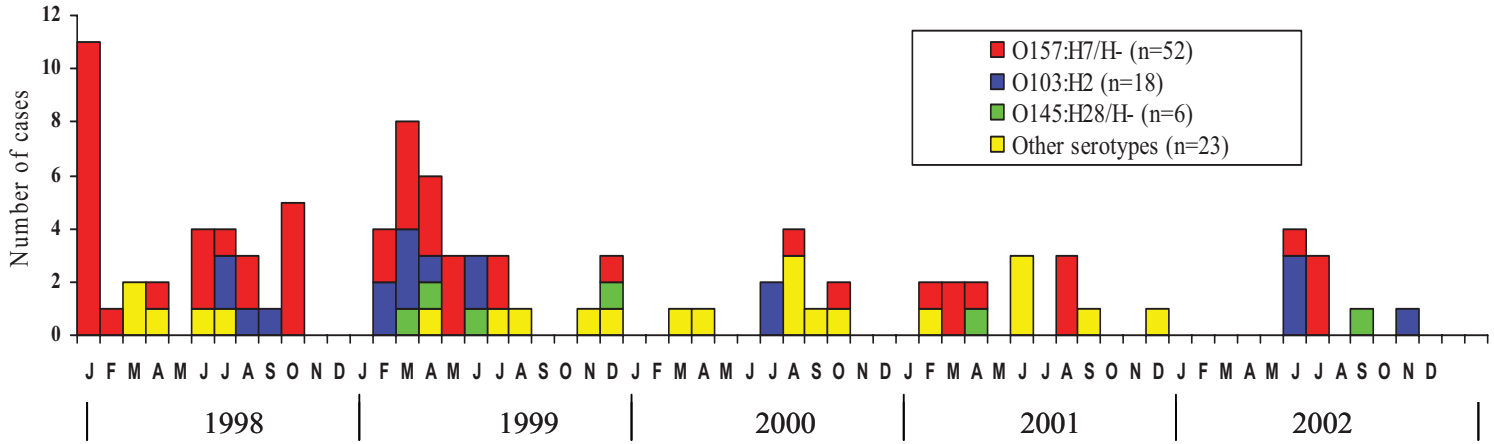


Figure 8. The temporal distribution of domestically acquired EHEC O157 (n=52) and non-O157 (n=47) infections during 1998–2002.



# DISCUSSION

## 1. Epidemiology of EHEC infections

In the epidemiological typing of EHEC O157:H7/H$^-$ strains, phage typing has been exploited as an internationally standardized epidemiological surveillance method (Ahmed et al. 1987, Khakhria et al. 1990, Willshaw et al. 1997). However, because within a PT there may exist genotypic variations of the bacterial strains, combined use of phage typing and molecular genetic methods, such as PFGE and subtyping of the *stx* genes, often provides advantages for epidemiological tracing over the use of one system alone (Liesegang et al. 2000). In Finland, in epidemiological surveillance it is possible to compare human and non-human EHEC–bacteria using the electronic, standardized PFGE network nationally (Rantala et al. 2000) and internationally among the Enter-net participants in Europe (PulseNet 2002, Fisher and Threlfall 2005, www.hpa.org.uk/inter/enter-net_menu.htm). For example, by using the pheno- and genotypic profiling of the O157 strains of Finnish human and non-human isolates, nine human EHEC O157 infections have been traced to five cattle farms (Lahti et al. 2002). In addition, geno- and phenotypic methods have also been exploited in the study of the sources of the EHEC non-O157 infections, where cattle were indicated as a source of EHEC O145 human infections (Heinikainen et al. 2004).

In our study of international phenotypic and genotypic comparison of the EHEC strains isolated during 1990–1999, the overall distribution of O157 PTs occurring in Finland was similar to that found in several other countries in Europe, Asia and America (Barrett et al. 1994, Smith et al. 1998, Cheasty et al. 1999, Mora et al. 2004). Just as this study points out that the PT2 type is the most prevalent, the study by Cheasty et al. (1999) showed that PT2 isolates were prevalent also in England and Wales, and in Germany, whereas PT4 prevailed in Sweden, PT14 in Denmark, and PT32 in Ireland. In Finland, some PT4 isolates were also associated with a cruise between Finland and Sweden suggesting that the infections of Finnish passengers acquired during the cruise may have originated from the same unknown sources as those in Sweden. In the study of 50 human isolates from cases of HUS in the Netherlands, Belgium, and Germany, altogether seven PTs: PT2 (44%), PT4 (33%), PT8 (7%), PT14 (4%), PT32 (4%), PT49 (4%), PT54 (4%) were identified (Heuvelink et al. 1995). Of these, five (PT2, 4, 8, 14, 49) corresponded to PTs found among human isolates in Finland. PT2, the most common PT in Finland, associated with the Finnish outbreak in 1997 (Keskimäki et al. 1998, Paunio et al. 1999), also predominated in the United Kingdom in previous years, accounting for 46% of all 1,266 human isolates during 1992–1994 (Thomas et al. 1996), and was the most common type also in 1996 (Smith et al. 1998). In addition, the PT2 strain with $stx_2$

and *stx*$_{2c}$ genes caused six of 11 outbreaks in England and Wales in 1995 (Thomas et al. 1996). The fact that these strains and the majority of the Finnish O157:H7:PT2 strains possessed the genes *stx*$_2$ and *stx*$_{2c}$, may suggest that they have originated from the same source. The other common phage types in Finland, PT4 and PT49, have also been common in England and Wales (Strockbine et al. 1998). The reason for the sudden emergence of Finnish EHEC strains of O157:H7:PT2, which were genotypically of PFGE type 1.1 and carried *stx*$_2$ and *stx*$_{2c}$, however, is not known for certain. This emergence occurred about two years after Finland had joined the European Union and animal and food trade barriers were removed. Also, the similarity of the strains that belong to different PTs in Finland and other European countries might be due to this event.

Among the O157 strains in Finland, approximately 10% have been nonmotile O157 variants of which 75% have fermented sorbitol (Eklund et al. 2003). In other European countries, strains of SF O157:H$^-$ have covered about 30% of all O157 strains (Karch and Bielaszewska 2001). Interestingly, in the epidemiological comparison of the SF O157:H$^-$ strains obtained in Finland, numerous pheno- and genotypic characteristics of the Finnish strains were similar with those strains occurring in Germany, suggesting a transmission of the strains at international level and supporting their relatedness with similar strains which have recently emerged in Europe.

In a nationwide geographical comparison of EHEC O157 and non-O157 infections, these infections occurred in 14 and nine of all hospital districts of Finland, respectively. Most of the infections were detected in the Helsinki Region, the population-based incidence being highest in northwest Finland. The high occurrence especially in northwest Finland might have been due to high cattle density. Also in other countries, an association between cattle density and human infections has been reported (Michel et al. 1999, Valcour et al. 2002, Kistemann et al. 2004). In general, in Europe, the prevalence of EHEC O157 and non-O157 bacteria in cattle has been even 70% in France (Pradel et al. 2000), 58% in Switzerland (Kuhnert et al. 2005), 49% Denmark (Møller-Nielsen and Frydendahl 2000), and 18% in Germany (Zschöck et al. 2000). The prevalence of EHEC O157 strains has been 6% in Belgium and Poland (Tutenel et al. 2002). In our country, the occurrence of EHEC O157 in cattle feces has been approximately 1 % (Lahti et al. 2001), and cattle during slaughter have excreted strains of serogroup O157 from 10 to 39% (Lahti et al. 2003), and EHEC non-O157 from 30 to even 90% (Pelkonen 2002). Of non-O157 serotypes detected in Finnish cattle, eight have also occurred among Finnish human isolates (Pelkonen 2002). However, no clear reason for the high geographical occurrence of the infections was seen. In addition, enhanced EHEC diagnostics based on not only the serological detection of O157, but also the Stx detection from stool cultures in those hospital districts might have also affected the high detection rate of the infections.

Stx positive non-O157 strains have also been of growing concern (Blanco et al. 2004). For example, in Spain EHEC strains have been the third most frequently isolated enteropathogenic bacteria, 78% of which have been of strains of EHEC non-O157 (Blanco et al. 2004). In Sweden, sporadic EHEC non-O157 infections have been as frequent as those of O157 during 1997-1998 (Welinder-Olsson et al. 2002). Strains of non-O157 have caused a large proportion of EHEC infections also in the Netherlands and in Denmark (Anon. 2003a). In Norway, in the past decade, approximately 100 EHEC cases have been reported, about one third being of non-O157 infections (Kløvstad et al. 2003). In addition, in the USA, EHEC strains of non-O157 group have accounted for up to 25% of HUS cases (Karmali 2004). In Oceania, EHEC non-O157 infections have been considered commoner than O157 infections (Tozzi et al. 2001). Of all EHEC infections studied, strains of EHEC non-O157 caused high proportion, 40%. The most common O groups detected belonged to O groups O26, O103, and O145, which have typically prevailed also in other European countries as causes of sporadic infections and outbreaks (Caprioli and Tozzi 1998, Zhang et al. 2000, Tozzi et al. 2001, Gerber et al. 2002, Prager et al. 2002, Jenkins et al. 2003). However, although EHEC non-O157 infections represented considerable share of the infections in Finland, no outbreaks caused by these pathogens occurred.

## 2. Microbiological properties of EHEC strains

Based on phenotypical properties of EHEC bacteria, recognition of sorbitol-negative O157:H7 strains has been well covered worldwide. However, sorbitol-positive strains of O157:H$^-$ and the majority of EHEC non-O157 bacteria are still most probably under-diagnosed in human infections (Scheutz et al. 2004), although techniques based on the detection of *stx* genes and Stx toxins, have proved to be crucial instruments in EHEC diagnostics. However, in addition to the mere toxin detection, the isolation of EHEC bacteria is of great importance. Namely, determining the relatedness of the bacterial strains by serotyping, Stx typing, phage typing, and genotyping quickly provides strict microbiological information to be used in conjunction with epidemiological data in order to prevent more infections, in surveillance of the sources of the infections, and in determining the virulence factors of the causative strain.

The strains of serotype O157:H7 have commonly carried the $stx_2$ virulence genes among strains originating in Great Britain (Willshaw et al. 2001) and Germany (Liesegang et al. 2000), although in Germany strains of this serogroup have also possessed both genes $stx_1$ and $stx_2$ (Beutin et al. 2004). Also in our study strains of serotypes O157:H7/H$^-$ typically carried $stx_2$. Instead, strains of serotype O157:H7/H$^-$ have typically carried both $stx_1$ and $stx_2$ especially among the isolates

originating from the USA, Australia (Kim et al. 2001), and Spain (Blanco et al. 2004), which suggests that strains occurring in Finland are similar to those occurring in Europe.

Among the SF O157:H⁻ strains occurring in continental Europe certain phenotypic (sorbitol fermentation, PT88, production of CDT, and lack of Ehly-production) and genotypic characteristics (possession of *stx*$_2$, *eae*-γ, *efa1*, *cdt-V*, EHEC-*hlyA*, *etpD*, *sfpA*, or lack of *katP*, *espP*) have been typical (Karch and Bielaszewska 2001). Also among the Finnish SF O157:H⁻ strains studied, these pheno- and genotypic characteristics were very similar, suggesting the pheno- and genotypic similarity and putative clonality of the strains occurring in continental Europe and Finland. In addition, in a study by Liesegang et al., 2002, the data supported the hypothesis that clonal turnover and genetic rearrangement of individual EHEC clones remain rare under environmental or epidemiological situations (Liesegang et al. 2000). These data also support the clonality of the SF O157 strains occurring in Finland and in Europe.

Strains negative for *stx* also occurred among the isolates studied. O157 strains lacking *stx* genes have also been found in other studies, where the loss of *stx* genes and the respective bacteriophages during laboratory storage, subcultivation, or by other environmental factors have been reported (Schmidt et al. 1999b, Willshaw et al. 2001).

During the study period, 73 human EHEC non-O157 infections were detected. The causative strains belonged to over 30 O:H serotypes. Among the EHEC non-O157 strains, only two strains of the serotypes O76:H19 and O145:H⁻ were sorbitol-negative, sorbitol-positive EHEC non-O157 strains representing the vast majority of the isolates. These data support the importance of detection methods not relying on sorbitol-negative appearance in the search of EHEC strains.

Of the non-O157 strains studied, approximately half carried either *stx*$_1$ or *stx*$_2$ only. The *stx*$_1$ gene was common among strains of O26, O103, and O15. Also in other studies, the carriage of the *stx*$_1$ gene has been common within the O26 and O103 group (Schmidt et al. 1999a, Willshaw et al. 2001, Prager et al. 2002). However, genetical shift of the carriage of sole *stx*$_1$ to *stx*$_2$ only, or acquization of *stx*$_2$ together with *stx*$_1$ has occurred recently among O26 strains (Zhang et al. 2000), which emphasizes the genetical diversity of the EHEC non-O157 bacteria. However, among the Finnish EHEC infections caused by strains of O26, no such phenomenon had occurred.

Because even approximately 80% of EHEC strains have been observed to produce Ehly (Beutin et al. 1989), this detail can be used in diagnostics in detection of putative EHEC strains on Ehly agar plates. Also, enterohemolytic activity might

affect the virulence of the strain (Paton and Paton 1998). Of the non-O157 strains in our study, the majority produced Ehly. In addition, among the non-O157 strains, the carriage of the *eae* gene was typical. However, although possession of the *eae* has been strong evidence for colonization ability of the strains, strains negative for *eae* might possess other aiding mechanisms for colonization of the strains in epithelial cells, such as Saa (Paton et al. 2001).

Recently, 30 new EHEC serotypes were associated with human infections in Spain (Blanco et al. 2004), and 31 serotypes in Germany (Beutin et al. 2004), strengthening the importance of detection of all EHEC non-O157 infections. Among the non-O157 strains in our study, two new EHEC serotypes (O102:H7 and O181:H49) were detected that emphasize the versatility of the non-O157 group. Of these types, only isolates of O181:H49 have recently occurred also in other countries, in Austria and Germany (Scheutz et al. 2004). However, their genotypic profile of *stx* gene possession ($stx_1$ and $stx_2$) differed from that of the Finnish isolate ($stx_2$ only), thus suggesting non-clonality of the strains.

## 3. Transmission and virulence of EHEC strains

The severity of the EHEC-infection and its easy transmission, even via hands, have had an impact on the decision to define this disease as a generally dangerous contagious disease in the Communicable Disease Act in Finland. As EHEC infection in humans has typically lead to bloody diarrhea, HUS or TTP, and even death, the virulent profile of the strain has become of great importance. For example, EHEC strains have caused 1 to 9% of diarrheal cases and 25 to 93% of HUS cases in Europe and the USA (Caprioli and Tozzi 1998, Tozzi et al. 2003, Karmali 2004, Scheutz et al 2004).

To study the virulence of EHEC O157 and non-O157 strains isolated in Finland, the virulence factors of the strains were explored by pheno- and genotypical methods to compare their virulence characteristics with the clinical picture of the patients. In all, 10 *stx*-positive virulene profiles were associated with 71% of all infections. Of these, a virulence profile O157:H7:PT2:$stx_2$:$stx_{2c}$:*eae*:Ehly was significantly more frequently associated with a severe clinical picture than were other profiles, especially among patients of under five years old. Strains of this virulence profile also caused the deaths of two children. Based on the literature, strains carrying $stx_2$ or $stx_{2c}$ have been associated more commonly with HUS than strains harboring $stx_1$ (Law 2000, Karch 2001, Friedrich et al. 2002). Similarly, the most virulent profile mentioned above possessed these virulence genes. Instead, among the $stx_2$ gene group, $stx_{2d\text{-}Ount}$, a recently found $stx_2$ variant among isolates particularly in Belgium, has been considered less virulent compared to that of $stx_2$ or $stx_{2c}$ (Pierard et al.

1998). Among the strains studied in Finland, only two isolates possessed $stx_{2d\text{-}Ount}$, and neither of the patients suffered from HUS or TTP.

However, although the serogroup O157 strains are very important, seven pheno- and genotypic virulence profiles of EHEC non-O157 strains of O groups O2, O101, O107, O145, O174, and O Rough were associated with patients suffering from HUS or TTP, but none of these infections was fatal. In particular, strains possessing $stx_2$ were associated with severe clinical picture of the patients (HUS or TTP). Also in other studies, irrespectively of EHEC O groups, strains carrying $stx_2$, $stx_{2c}$ or $stx_{2dac}$ genes have been associated with HUS patients (Schmidt et al. 1999a, Cornu et al. 1999, Friedrich et al. 2002), which emphasized the importance of all EHEC strains carrying these genes as severe human pathogens.

## 4. Sources of EHEC infections

A major reservoir of EHEC bacteria has been asymptomatic cattle and other ruminants (Caprioli et al 2005). A plethora of fecal-contaminated food items including ground meat, unpasteurized dairy products, unpasteurized refreshments, fruits and vegetables (such as sprouts, lettuce, coleslaw) have been well-known vehicles for EHEC infections (Karmali 2004, Schlundt et al. 2004, Caprioli et al. 2005). In addition, waterborne infections (Garcia-Aljaro et al. 2005), and infections associated with rural settings have been of growing importance (Karmali 2004). In particular, environment-related exposures have been associated with EHEC infections during summer and fall (Karmali 2004, Caprioli et al. 2005). Furthermore, through the oral-fecal route, person-to-person contact has been important in transmission of EHEC bacteria (Schlundt et al. 2004).

In Finland, most EHEC infections (80%) were of domestic origin, typically being either sporadic or small family-associated infections. However, until now, the source of the infections has been confirmed microbiologically in only about ten O157 cases (Tast et al. 1997, Lahti et al. 2002), and one EHEC non-O157 case (Heinikainen et al. 2004). In all these cases, either unpasteurized milk (Tast et al. 1997) or cattle (Lahti et al. 2002, Heinikainen et al. 2004) were implicated as the vehicle for the infections. In our study of domestic EHEC infections, according to the in-depth interviews, the vehicles for the infections were putative person-to-person contact with a diarrheal patient, or consuming ground meat or visiting cattle farms. Also in other studies, similar transmissions of EHEC infection have been reported (Caprioli et al. 2005, Schlundt et al. 2004). Also asymptomatic human carriers seem to be of particular concern in food-handling environments. For example, *stx* genes were detected in 3.5% of more than 5,500 stool samples taken from healthy employees in the Swiss meat processing industry (Stephan and Untermann 1999). Thus, healthy food handlers must also be regarded as a potential source of EHEC.

During 1998–2002, three small clusters caused by strains of serogroup O157 occurred. Person-to person spread was concluded to be a cause of the transmission of the subtype O157:H7:PT2:*stx*$_2$:*stx*$_{2c}$:*eae*:1.1 strain in 1998. In addition, this particular type was the commonest and most widely distributed in hospital districts. The emergence of this subtype was seen soon after the first EHEC O157 outbreak in Finland had occurred during summer 1997 (Keskimäki et al. 1998, Paunio et al., 1999). In addition, in 1998 and 2001 two other small EHEC O157 clusters occurred. EHEC-contaminated hamburgers were implicated as a putative source of infections in the cluster in 1998. The cluster in 2001 occurred in Kymenlaakso, and involved EHEC contaminated kebab meat imported from the Netherlands (Hatakka et al. 2002).

Among the EHEC infections of domestic origin during 1998–2002, the incidence of the infections was highest in 1998 but lowest in 2002. Interestingly, after 1999, nationwide hygienic counseling concerning the whole “from farm to fork” chain was intensified (Anon. 2003b). A similar decline in the number of EHEC infections was observed also in Germany in 2000, probably affected by the national hygiene legislation and directives of the European Union (Kothmann 1999). This counseling might have diminished the spread of EHEC bacteria in the food chain, resulting in fewer human EHEC infections.

# KEY FINDINGS AND CONCLUSIONS

In this thesis, epidemiological knowledge and data on microbiological pheno- and genotypic characteristics of human EHEC O157 and non-O157 strains isolated in Finland was gained. During 1990–2002, 200 microbiologically confirmed EHEC infections were diagnosed. Of these, 127 were EHEC O157 and 73 non-O157 infections. In addition, a double infection occurred in three patients.

As EHEC bacteria are pheno- and genotypically heterogeneous human pathogens causing severe infectious diseases, surveillance and early phase-detection of EHEC infections caused by EHEC O157 and non-O157 serogroups is continuously needed, thus allowing detailed early diagnosis of EHEC and prevention of new infections. In particular, pheno- and genotypic characterization of the human EHEC isolates both at a national and international level enables the sources the existing infections to be rapidly traced and allows detailed information for the microbiological and statistical epidemiological analysis of the sporadic or outbreak -related infections. Furthermore, the determination of several virulence associated genes in addition to the epidemiological meaning, helps to evaluate the putative virulence ability of the strain. Thus, the emergence of EHEC infections also in Finland requires constant epidemiological study and characterization of human EHEC isolates.

Of the infections studied, approximately half of the EHEC O157 and non-O157 infections were family-related cases. In addition, during the study period of indigenously acquired EHEC infections, three small outbreak clusters caused by strains of EHEC O157 occurred. These data suggest the easy spread of EHEC O157 strains. Moreover a particular virulence profile of EHEC O157 group (O157:H7:PT2:*stx*$_2$:*stx*$_{2c}$:*eae*:Ehly) was statistically more frequently associated with a severe clinical picture (bloody diarrhea, HUS, TTP) than were other profiles. Strains of this virulence profile also caused the deaths of two children. Overall, the risk factors for severe symptoms were concluded to be an age of less than five years and infection by this O157:H7 strain type, which emphasizes the early detection of EHEC infections especially in young children.

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