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Role of enteropathogenic *Escherichia coli* in childhood diarrhoea in Norway

Thesis for the degree philosophiae doctor

Trondheim, December 2007

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Faculty of Medicine
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Children's and Women's Health
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Trondheim, December 2007

Jan Egil Afset

Abbreviations

A/E	Attaching and effacing
A/EEC	Attaching and effacing *E. coli*
AIEC	Adherent-invasive *E. coli*
Bfp	Bundle-forming pilus
CDEC	Cell-detaching *E.coli*
CFU	Colony-forming units
CNF	Cytotoxic necrotizing factor
Cy	Cyanine
DAEC	Diffusely enteroadherent *E. coli*
DEC	Diarrheagenic *E. coli*
eae	*E. coli* attaching and effacing
EAEC	Enteroaggregative *E. coli*
EAF	Enteropathogenic *E. coli* adherence plasmid
efa	EHEC factor for adherence
EHEC	Enterohaemorhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
esc	*E. coli* secretion system
esp	*E. coli* secreted protein
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
FAS	Fluorescent-actin staining test
HC	Haemorrhagic colitis
HUS	Haemorrhagic uremic syndrome
LEE	Locus of enterocyte effacement
lif	Lymphostatin inhibitory factor
lpf	Long polar flagella
LT	Heat-labile enterotoxin
MLEE	Multi locus enzyme electrophoresis
MLST	Multilocus sequence electrophoresis
nle	Non-LEE effector
NTEC	Necrotoxic *E. coli*
OI	Genomic O island, unique segments present in E. coli strain EDL933
OR	Odds Ratio
PEPEC	Pork pathogenic *E. coli*
PFGE	Pulsed-field gel electrophoresis
pINV	Invasion related plasmid
pAA	Plasmid AA, associated with aggregative adherence in EAEC
paa	Porcine attaching and effacing
REPEC	Rabbit pathogenic *E. coli*
SMAC	Sorbitol MacConkey
ST	Sequence type
STa/STb	Heat-stable enterotoxin A/B
STEC	Shiga toxin-producing *E. coli*
stx	Shiga toxin
tir	Translocated intimin receptor
TTSS	Type III secretion system
UPGMA	Unweighted pair group method with arithmetic mean
VTEC	Vero toxin-producing *E. coli*

List of papers

This thesis is based on the following papers, which are referred to by their Roman numerals in the text (I-IV):

I Afset JE, Bergh K, Bevanger L. High prevalence of atypical enteropathogenic Escherichia coli (EPEC) in Norwegian children with diarrhoea. J Med Microbiol. 2003;52:1015-9.

II Afset JE, Bevanger L, Romundstad P, Bergh K. Association of atypical enteropathogenic Escherichia coli (EPEC) with prolonged diarrhoea. J Med Microbiol. 2004;53:1137-44.

III Afset JE, Bruant G, Brousseau R, Harel J, Anderssen E, Bevanger L, Bergh K. Identification of virulence genes linked with diarrhea due to atypical enteropathogenic *Escherichia coli* by DNA microarray analysis and PCR. J Clin Microbiol. 2006;44:3703-11.

IV Afset JE, Anderssen E, Bruant G, Harel J, Wieler L, and Bergh K. Phylogenetic background and virulence profile of atypical enteropathogenic *Escherichia coli* from a case-control study using multilocus sequence typing and DNA microarray. (manuscript submitted)

Summary

Background

Diarrhoeal diseases are among the leading causes of illness and death among children in developing countries, and do also cause considerable morbidity in industrialized countries. Enteropathogenic *E. coli* (EPEC), characterized by its ability to induce "attaching and effacing" (A/E) lesions the intestinal epithelium, is recognized as an important diarrheagenic agent in developing countries. Recently, EPEC has also been reported to be prevalent in the industrialized part of the world. Two main classes of EPEC have been recognized: typical EPEC has the ability to adhere to epithelial cells in discrete microcolonies, named "localized adherence" (LA). This trait is encoded by genes on a plasmid, called the EPEC adherence factor (EAF). Atypical EPEC does not contain the EAF and is not able to produce LA.

Aims

The aim of the study was to investigate the prevalence of EPEC and its epidemiological association with childhood diarrhoea in Norway. We also wanted to characterize the EPEC strains identified in the study by phenotypic and genotypic methods, and to search for bacterial factors statistically linked with diarrhoeal disease.

Materials and methods

The study was conducted in the County of Sør-Trøndelag, Norway. The prevalence of EPEC was first investigated in a retrospective laboratory based study of the aetiology of diarrhoea in children less than two years of age. Next a case-control study was carried out in children less than five years old. Cases were recruited as in the previous study, and healthy controls were recruited through Maternal and Child Health Centres. EPEC was identified by PCR, and the bacterial strains were characterized by DNA microarray, multilocus sequence typing, pulsed-field gel electrophoresis (PFGE) and serotyping.

Results

EPEC was the most frequently identified enteropathogenic agent in the retrospective study, and was isolated from 38 (15.1%) of 251 children less than five years of age with diarrhoea in the case-control study. Strains of the EPEC pathotype were also common in healthy children where they were isolated from 21 (10%) of 210 subjects. There was no overall statistical

association between EPEC and diarrhoea (*P*=0.3). EPEC strains were rarely diagnosed in children with severe diarrhoea, and were less common in children less than 12 months of age.

The majority of strains, 56/58 strains in the case-control study, were classified as atypical EPEC. The atypical EPEC strains showed extensive heterogeneity in sequence types and PFGE profiles. The strains were separated in three clusters based on all the virulence genes identified: one large cluster included all phylogenetic group A, B1 and D strains, and two smaller clusters consisted exclusively of phylogenetic group B2 strains. There was also considerable variation in serotypes, and almost half the strains were O serogroup non-typable.

Among a total of 95 putative virulence genes detected, seven genes were positively statistically associated with diarrhoea. Among these, the strongest statistical association was observed for the pathogenicity island OI-122 gene *efa1/lifA* (*P*=0.0002). The phylogenetic marker gene *yjaA* was strongly negatively associated with diarrhoea (*P*=0.0004).

The atypical EPEC strains could be classified in two virulence groups based on their content of virulence genes positively and negatively associated with diarrhoea. Strains belonging to the group which was not associated with diarrhoeal disease should probably be considered as colonizers which do not cause disease. The frequent isolation of such strains may be due to their apparent propensity for protracted colonization. Strains belonging to the other virulence group, which was significantly associated with diarrhoea, were isolated both from patients with acute and protracted disease.

Conclusions

EPEC was frequently isolated from Norwegian children both with and without diarrhoea, but was rarely associated with severe diarrhoea. The majority of EPEC strains were characterized as atypical EPEC. Genetic characterization showed extensive heterogeneity among the atypical EPEC strains in the study. Several virulence genes were positively and negatively statistically associated with diarrhoea, and the strains could be classified in two virulence groups based on their content of these genes: one group was not associated with diarrhoea, but appeared to be associated with protracted colonization. The other group was associated with both acute and protracted diarrhoea.

1 Introduction

1.1 Diarrhoea – a global perspective

Infections of the gastrointestinal tract are among the world's leading causes of illness and death among children. Recently such infections were reported to cause more than 3.2 disease episodes per year in children under the age of five in developing countries (125). They have also been estimated to be the third most common cause of death by infectious diseases, only preceded by lower respiratory tract infections and HIV/AIDS (138). Globally 21% of all deaths in children under five years of age are estimated to be due to diarrhoeal infections (125). In recent years studies from several developing countries have shown that diarrhoeal diseases also cause considerable lasting disabilities both in physical growth and fitness, and in cognitive skills and school performance (85). Disabilities linked to diarrhoeal diseases in middle and low income countries have been estimated to account for 58.7 million years of lost healthy life (termed Disability Adjusted Life Years, DALYs) (138).

The incidence of diarrhoeal diseases does not appear to have changed much in recent years in spite of considerable efforts invested in control measures in this period (117). However, there has been a steady decline in diarrhoeal mortality during the last thirty years. Based on data from longitudinal studies using active surveillance, a reduction in diarrhoeal mortality was reported from 4.6 million deaths per year before 1980 (214), to 3.3 million in the period 1980 to 1990 (14), and to about 2.6 million deaths per year toward the end of the 1990s (125). Although there is some uncertainty in the figures, as evidenced by alternative estimates as low as 2.1 and 1.6 million deaths per year for the period 1990 to 2000 (117,174), these data show that a definite trend of reduced diarrhoeal mortality took place towards the end of the 20th century. This reduction is mainly attributed to oral rehydration therapy which was introduced during this period, and is now the mainstay of diarrhoea treatment in most developing countries (235).

In high-income countries children rarely die due to diarrhoeal diseases. Still, diarrhoeal episodes are common also in this part of the world. Recently an incidence of 740-900 episodes per 1000 person years for children less than 5 years old was observed in the Netherlands (45), and even higher incidence data have been reported from Norway (129) and other high-in come countries in studies including all age groups (91,203,223). Diarrhoeal diseases therefore lead to many visits to physicians, hospital admissions and lost work time for parents as well as to considerable expenses both for families and the community (12,89).

Diarrhoea has been defined as passage of unusually loose or watery stools, usually at least three times in a 24 hour period (244). A change in consistency and character of the stools is recognized as more important than the number of stools. The above definition does not apply to breastfed babies where loose stools are normal. Therefore it is usually agreed that the assessment whether a child fed on breastmilk has diarrhoea or not preferably should be made by the mother or another person who knows the child. The term diarrhoea is actually a description of a symptom, which may be caused by infections of the gastrointestinal tract (also termed infectious gastroenteritis), by systemic infections and by non-infectious conditions (86). However, it is also commonly used, as in this paper, as a term for the disease gastrointestinal infection leading to diarrhoea, and not only the symptom (117).

There are three main clinical types of diarrhoea; acute watery, persistent and bloody diarrhoea. Acute and persistent diarrhoea are not distinct diseases, but represent two ends of a continuum. Most episodes of acute diarrhoea resolve within seven days, but some persist beyond two, three and four weeks (140,207). The World Health Organization (WHO) and most investigators use a definition of persistent diarrhoea as diarrhoea lasting 14 days or longer (10). Although the 14 days limit is arbitrary, it is supported by an increased case fatality rate in children with diarrhoea of longer duration (5). Bloody diarrhoea is defined as diarrhoea with visible or microscopic blood in the stools, due to local mucosal damage and intestinal haemorrhage (117). A special form of bloody diarrhoea is the dysentery syndrome characterized by small-volume, bloody stools, abdominal cramps, and tenesmus, which is a severe pain in relation to straining to pass stools.

Of these categories, acute watery diarrhoea is most common and accounts for 80% of all cases of childhood diarrhoea, and for 50% of the diarrhoea associated mortality (243,244). Persistent diarrhoea is less common and contributes to only 10% of all diarrhoeal episodes, but is associated with a disproportionately increased risk of death due to severe malnutrition. Worldwide, persistent diarrhoea has been estimated to be responsible for 35% all diarrhoeal deaths (243), but has in some studies from South Asia been found to be responsible for more than half the diarrhoeal deaths (16,118). Dysentery is seen in 10% of all cases of diarrhoea and causes 15% of the deaths (243).

The intestinal physiology is altered by enteric infection principally in one of three ways: 1) changing water and electrolyte fluxes in the upper small bowel resulting in watery diarrhoea, 2) by inflammatory or cytotoxic destruction of the intestinal mucosa characterized by the presence of faecal leukocytes and dysentery, or 3) by microbial penetration through an intact mucosa to the reticuloendothelial system leading to enteric fever sometimes with only

mild diarrhoea [reviewed in (86)]. Most enteric agents alter the intestine in mainly one of the three mentioned ways. However, combinations of different mechanisms are not uncommon.

A number of microorganisms, including a variety of viral, bacterial, and protozoan agents may cause diarrhoeal disease. The four most common viruses associated with acute gastroenteritis are rotavirus, norovirus, enteric adenovirus, and astrovirus (36). Bacterial diarrheagenic agents include *Salmonella spp.*, *Shigella spp.*, *Yersinia enterocolitica*, *Campylobacter spp.,* and *Vibrio spp.,* including *Vibrio cholera*, and different variants of *Escherichia coli* (5,86). In addition, enterotoxin producing variants of *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus* cause gastroenteritis. *Clostridium difficile* associated diarrhoea is commonly seen in relation to prolonged antibiotic therapy. Other species like *Aeromonas spp.* and *Plesiomonas spp.* may more rarely be responsible for diarrhoea symptoms. The two best known protozoan agents that may cause diarrhoea are *Giardia lamblia*, and *Entamoeba histolytica*, but *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Isospora belli* and microsporidia may also cause such disease. In addition, malabsorption, inflammatory bowel disease, irritable bowel syndrome and a wide range of other non-infectious conditions may result in diarrhoeal symptoms.

Although some microbial agents are more common causes of diarrhoea than others, assessment of the relative contribution of each agent is difficult. This is partly due to considerable variation in the isolation of enteropathogenic agents with the population and the geographical area studied, but also to the fact that in the majority of studies the investigation is restricted to a limited group of microbial agents (75). In addition, no infectious causative agent is identified in up to half the cases even when an extended search for microbial agents is done, either due to the presence of unrecognized enteropathogens, or a non-infectious cause of the diarrhoea (20).

1.2 *Escherichia coli*

Escherichia coli was first described as *Bacterium coli commune* by the German paediatrician Theodore Escherich in 1885. This name was used until the genus *Escherichia* with the type species *E. coli* was defined by Castellani and Chalmers in 1919 [reviewed by Cheasty and Smith in (33)]. The *E. coli* species is a member of the family *Enterobacteriaceae* within the phylum Proteobacteria. It is a rod-shaped bacterium 2-6 μm long and 1.1-1.5 μm wide, which has rounded ends and is gram-negative. Strains belonging to this species can be cultured both aerobically and under anaerobic conditions, have simple nutritional requirements and do not form spores. They can be grown at temperatures up to 44°C, and have a generation time of 20

min. under optimal conditions. Most strains are motile. When tested biochemically *E. coli* are oxidase negative, catalase positive, ferment glucose, reduce nitrate, give a positive *o*-nitrophenyl-β-galactopyranoside (ONPG) reaction, produce indole and fail to produce urea or H_2S. They do also normally ferment lactose. Other species within the genus are *E. blattae*, *E. fergusonii*, *E. hermannii*, *E. vulneris,* and the recently proposed *E. albertii*.

E. coli is a commensal of the intestines of humans, mammals and birds where it constitutes the most prevalent facultative anaerobic species with 10^7 colony forming units (CFU) per gram faecal content (153). However, this species is present in much smaller numbers than anaerobic bacteria which make up more than 99% of the 10^{10}-10^{11} bacteria/gram in faeces (149,233). *E. coli* bacteria are excreted with the stools and may survive for some time outside the body, but do not normally have any independent existence in the environment (33). Accordingly, detection of *E. coli* is extensively used as an indicator of faecal contamination of water and food.

In addition to its role as commensal, *E. coli* may cause various types of intestinal and extraintestinal infections. Of extraintestinal infections, urinary tract infections (UTIs), where *E. coli* is the single most prevalent pathogen, are among the most common infections both in the community and in hospitals (94,216). *E. coli* strains which cause UTIs often have specific virulence factors, while strains without such virulence factors, termed commensal strains, more rarely cause this type of infection. *E. coli* is also one of the most common infectious agents isolated from patients with bacteraemia and sepsis (63), and one of the leading causes of neonatal meningitis (43,120). In addition, commensal *E. coli* may cause infections in nearly any body site due to an aggravating factor such as a foreign body or host compromise (193).

Virulence factors which makes certain strains of *E. coli* pathogenic may be classified in two groups according to function (109). One of these consists of colonization and fitness factors making the bacterium able to colonize and survive on the mucosal surface or, more rarely, to invade the host. The second group consists of toxins and effectors which may induce damage to the host. Some of the virulence factors do also help the bacterium evade the host immune defences. Depending on their virulence profile *E. coli* strains are classified in a number of extraintestinal (ExPEC) and diarrheagenic (DEC) pathotypes.

Serotyping was for many years the only tool available to identify pathogenetic variants of *E. coli*. This method, established by Kauffmann in the 1940s, is based on heat-stable lipopolysaccharide O antigens and heat-labile flagellar H antigens. Kauffmann also described other heat-labile capsular K antigens [reviewed by Cheasty and Smith in (33)]. So far 174 O

serogroups and 53 H antigens have been described (84,205). Clinical microbiology laboratories have mainly used serogroup determination with the most common O antigens in the diagnosis of diarrheagenic *E. coli*. O:H serotyping requires a complete set of antisera as well as extensive experience, and is therefore done only in few international reference and research laboratories.

Based on multilocus enzyme electrophoresis (MLEE), *E. coli* was classified in phylogenetic groups named A, B1, B2, D and E (208). Group E has later been used rarely due to the inconsistent clustering of this group (242). Based on the early MLEE studies it was believed that the *E. coli* population was largely clonal and that recombination was infrequent in this species (208). However, recently this statement has been questioned when homologous recombination was found to be common among commensal and pathogenetic *E. coli* strains analysed with multilocus sequence typing (MLST) (103,242). Wirth *et al.* did also show that that one third of the all strains in their study contained significant ancestry from multiple sources, and did therefore assign such strains to hybrid groups, named ADB and AxB1 (242). It was not possible to deduce a definite ancestral relationship among the major phylogenetic groups in that study.

1.2.1 *E. coli* as diarrhoeal agent -historical perspective

The capability of certain *E. coli* strains to cause diarrhoea was reported as early as 1887 [reviewed by Clarke (37)]. However, only after Bray reported the isolation of *E. coli* from cases of summer diarrhoea in 1945 was more widespread interest in this organism as enteric pathogen evoked (27). Over the next three decades *E. coli* strains of certain serotypes (O111, O55 and O127) were frequently diagnosed as cause of childhood diarrhoea in industrialized countries [reviewed by Levine and Edelman (134)]. The term enteropathogenic *E. coli* (EPEC) was first introduced by Neter *et al.* in 1955 to describe *E. coli* strains that were epidemiologically implicated in infant diarrhoea (161). During this period mortality rates >50% were reported in diarrhoeal outbreaks among children attributed to EPEC. From around 1960, for unknown reasons, both the incidence and lethality of EPEC declined in industrialized countries (157). In Norway, during this period EPEC was found to be common in children admitted to hospital, but was in most cases associated only with mild symptoms (72,130).

The pathogenic potential of the *E. coli* serotypes most frequently isolated from children with diarrhoea was confirmed in several volunteer studies during the first half of the 1950s [reviewed in (134)]. In addition, such strains were usually present in pure culture in

children with diarrhoea, compared to only in small numbers in healthy children. Infants with diarrhoea were also shown to develop a serologic response to the EPEC strain isolated from their stool (83,161,247). In addition to the most common EPEC serogroups mentioned above, several other O-serogroups and O:H serotypes were associated with infant diarrhoea, and were accordingly classified as classical EPEC serogroups and serotypes (134).

During the 1960-70s, strains belonging to the classical EPEC serotypes were found to contain specific virulence factors (53,55,82,146,209). Strains with heat-labile and/or heat-stable enterotoxins were named enterotoxigenic *E. coli* (ETEC), and invasive strains were named enteroinvasive *E. coli* (EIEC). When it was shown that these new virulence factors were present in only some of the strains belonging to the classical EPEC serogroups, the use of serotyping to identify diarrheagenic *E. coli* was seriously questioned. However, the pathogenicity of EPEC strains without any of these virulence factors was definitely verified by Levine *et al.* in human volunteer studies in 1978 (133). This finding brought about an intensive search for the pathogenetic mechanism of *E. coli*-induced diarrhoea. Diagnostic methods based on diarrhoea-causing traits seemed to be better than identification based solely on serotype.

From the late 1970s rapid progress in the revelation of different phenotypic and genetic mechanisms linked to diarrhoeal disease lead to the identification of at least six pathotypes of diarrheagenic *E. coli*. In addition to the already known ETEC and EIEC pathotypes, enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), diffusely enteroadherent *E. coli* (DAEC) and enteropathogenic *E. coli* (EPEC) were shown to have distinct phenotypic and/or genetic characteristics (109,157) (Fig. 1). Through these revelations the use of the name EPEC changed from meaning all *E. coli* associated with diarrhoea to the new specific EPEC pathotype.

ETEC is primarily a pathogen of developing countries where it is a leading cause of weaning diarrhoea in infants [reviewed in (182)]. In addition, it is known as one of the major causes of traveller's diarrhoea. ETEC bacteria colonize the surface of the small intestines by one or more different variants of fimbrial or fibrillar colonization factors (CFs) (230). Diarrhoea is induced by two classes of toxins, the heat-labile (LTs) and heat-stable (STs) enterotoxins which cause secretory diarrhoea through increased Cl^--secretion (238). Although there are two variants of each enterotoxin, only ETEC strains with the LTI and STa variants are recognized human pathogens, while strains containing the variants LTII and STb are found mainly in animals.

Fig.1 is not included in the online version of this thesis due to copyright.

Fig. 1. Simplified presentation of the pathogenic mechanism for each of the six recognized categories diarrheagenic *E. coli* pathotypes. Reprinted from reference (109) with permission from the publisher.

EIEC has mainly been reported from outbreaks (157). This pathotype is closely related to *Shigella spp.* genetically (132), but may diagnostically be differentiated from the other species by biochemical tests (157). It usually causes watery diarrhoea, but inflammatory colitis and dysentery may also be seen. A capacity to invade the intestinal epithelium is an essential characteristic of this pathotype (as well as for *Shigella spp.*) (175,212). The genes necessary for invasiveness are carried on a large virulence plasmid named pINV. As with *Shigella spp.*, EIEC normally only invade the epithelial cell layer, and is rarely recovered from blood.

The EHEC pathotype characteristically causes haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (109). HUS is defined by the presence of microangiopathic haemolytic anaemia, thrombocytopenia and acute nephropathy (210). This pathotype was first recognized in the early 1980s when *E. coli* strains of serotype O157:H7 isolated from patients with HC and HUS were shown to be cytotoxic in Vero cell culture

(113,189). EHEC infections usually start as watery diarrhoea, and may progress to HC and HUS in some subjects. Diarrhoea-associated HUS is one of the most common causes of acute renal failure in otherwise healthy children (224). This syndrome is seen most often in children < 10 years of age. Case fatality rates up to 5% is seen in children with diarrhoea-associated HUS (210). The toxins responsible for cytotoxicity in Vero cell culture is closely related to the Shiga toxin of *Shigella dysenteriae* serotype 1 (169), and are divided in two major toxin families stx1 and stx2, with several variants in each group (168,195). All *E. coli* strains containing stx genes are termed verotoxigenic *E. coli* (VTEC), based on their Vero cell toxicity, or shiga toxin producing *E. coli* (STEC), based on the toxin type, while the term EHEC has been reserved for the subset of strains which are associated with human disease (157). Although the serotype O157:H7 has been associated with the most severe disease, shiga toxins have been detected in more than 200 *E. coli* serotypes (33). The majority of these serotypes are not associated with diarrhoeal disease in humans. EHEC strains usually contain a variety of virulence factors in addition to the stx. Some of these have been reported to be associated with severe EHEC disease (111,239). Among such virulence factors are the chromosomal pathogenicity island named the locus of enterocyte effacement (LEE), which enables intimate attachment of the bacterium to the intestinal epithelium, a large plasmid named pO157 containing several putative virulence genes including the haemolysin gene *ehxA*, and a variety of other virulence factors (109).

EAEC has in recent years increasingly been recognized as a cause of acute and persistent diarrhoea in children and adults, and has also been reported to be one of the most frequent causes of traveller's diarrhoea (95). Recognition of this pathotype was primarily based on the ability of EAEC bacteria to adhere to epithelial cells in cell culture in a characteristic "stacked-brick" pattern called aggregative adherence (158). The pathogenesis of EAEC infection is only partially revealed, but such bacteria have been shown to express enterotoxins and cytotoxins, and to elicit mild mucosal inflammation (155). EAEC strains appear to be heterogeneous and probably include both pathogenic and non-pathogenic clones (155). The pAA plasmid with the transcriptional regulator gene *aggR* have been associated with diarrhoea (102,196). Strains with this gene have therefore been termed typical EAEC, while strains lacking this gene have been classified as atypical EAEC (88). An interesting additional feature of the EAEC pathotype is the importance of host factors for the development of symptomatic disease. Jiang *et al.* showed that a certain nucleotide polymorphism in the IL-8 promoter was significantly associated with diarrhoea in US residents who were infected with EAEC during a travel to Mexico (102).

The DAEC pathotype is defined by the presence of a characteristic diffuse adherence pattern in cell culture (158). The pathogenicity of the DAEC pathotype is controversial as such strains are frequently isolated from subjects without diarrhoea. However, they have been associated with diarrhoeal disease in some studies, especially in children > 12 months of age (157,199). While the fimbrial adhesin F1845 is commonly present in DAEC strains (19), little is known about a pathogenetic mechanism for this pathotype.

Other potential pathotypes have been suggested. One such pathotype is known as adherent-invasive *E. coli* (AIEC) which has been reported to be associated with Crohn's disease (42). Strains belonging to this pathotype can invade and replicate within macrophages without inducing host cell death, and are able to induce release of high amounts of Tumor Necrosis Factor-alpha, possibly leading to intestinal inflammation. Two other putative pathotypes are the necrotoxic *E. coli* (NTEC) and the cell-detaching *E.coli* (CDEC). Both these pathotypes, defined by the presence of cytotoxic necrotizing factors (CNF1-2) and cytolethal distending toxins (CDT1-4), respectively, may infrequently be seen in subjects with diarrhoea (44,56).

1.2.2 Enteropathogenic *E. coli* (EPEC)

1.2.2.1 Pathogenetic mechanism

EPEC is characterized by its ability to cause a characteristic intimate attachment to the intestinal epithelium (148). This type of attachment, termed an attaching and effacing (A/E) lesion, is similar to the adherence seen with many EHEC strains. It is, in addition to the characteristic intimate attachment, characterized by effacement of intestinal microvilli, formation of pedestal-like structures, and aggregation and concentration of actin filaments in the intestinal cell directly beneath the adherent bacteria (122) (Fig. 2). The genes necessary for attaching and effacing lesion formation are located on the chromosomal pathogenicity island called locus of enterocyte effacement (LEE) (142). The LEE of the prototype EPEC strain 2348/69 contains 41 open reading frames organized into five polycistronic operons (gene clusters transcribed into one mRNA), named LEE1, LEE2, LEE3, TIR and LEE4 (Fig. 3) (57,145). Based on analyses of phylogenetic relationships and the site of insertion in the *E. coli* chromosome, there are three main types of the LEE region (104). However, the LEE may also be seen as a mosaic of genetic elements where the elements are differently affected by recombination and mutation (32).

Fig.2 is not included in the online version of this thesis due to copyright.

Fig. 2. A/E lesions with characteristic intimate attachment, loss of microvilli and pedestal formation. Reprinted from reference (13) with permission of the publisher.

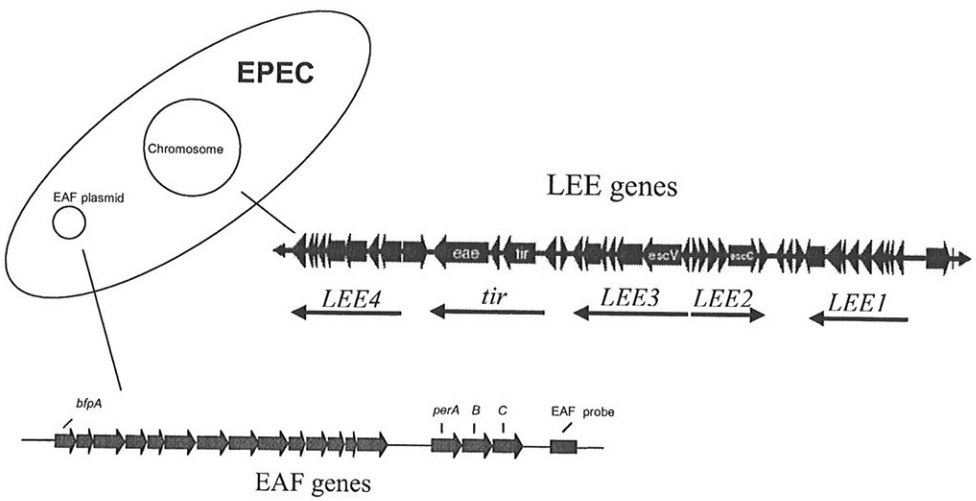

Fig. 3. Genetic organization of the locus of enterocyte effacement (LEE) and the EPEC adherence factor (EAF) plasmid. The chromosomal LEE region essential for A/E lesion formation is organized into five polycistronic operons termed *LEE1-3*, tir and *LEE4*. The EAF plasmid encodes bundle forming pili necessary for localized adherence (LA).

In addition to the above classifications, the LEE may be separated in three domains based on the function of genes (57,145); genes encoding intimate adherence, genes encoding proteins which are secreted from the bacterium, and genes encoding a type III secretion system. The first domain contains two genes essential for the ability of the bacterium to adhere intimately to the intestinal epithelial cell surface. Among these, the *eae* gene encodes a transmembrane protein, intimin, which functions as a ligand for epithelial cell adhesion (49,101). It also stimulates a systemic immune response, as demonstrated in a volunteer study (135), and intimin of the *Citrobacter rodentium* has been shown to induce mucosal Th1 immune response and intestinal crypt hyperplasia in mice (93). The intimin protein consists of an N-terminal part inserted in the bacterial outer membrane, and an extracellular C-terminal domain involved in receptor recognition (114,137). Whereas the N-terminal part is conserved, considerable variation is seen in the C-terminal domain between different EPEC strains (66,67).

At least five different antigenic variants of the intimin protein have been identified by serological methods (2,3,173). However, 21 different allelic variants of the *eae* gene encoding the intimin protein have been identified based on comparison of nucleotide sequences (21) (Fig. 4). The three most common intimin types alpha, beta and gamma have been reported to influence host specificity and tissue tropism using different animal *in vivo* and *in vitro* models, and human intestinal *in vitro* organ culture (62,76,152,179,231). These studies revealed that exchange by genetic manipulation of the intimin types alpha and gamma between EPEC and EHEC strains alters the tissue tropism according to intimin type. In contrast, substitution of EPEC intimin alpha and EHEC intimin gamma with the beta variant resulted in different tropism between the two strains indicating that other strain characteristics than intimin type are also of importance. However, since the results from the different experimental models are not necessarily exchangeable, comparison and interpretation of the results is difficult.

The translocated intimin receptor (Tir), another LEE encoded protein, is transferred from the bacterium into the intestinal epithelial cell where it is inserted into the outer membrane and acts as a receptor for intimin (116). In addition, Tir has been reported to have signalling functions within the epithelial cell implicated in pedestal formation (107).

The second functional domain of the LEE encodes several proteins which are secreted from the bacterium. Some of these proteins become part of the secretion apparatus (espA, espB and espD), while other (espF, espG, espH, Map and sepZ/espZ) are effector proteins

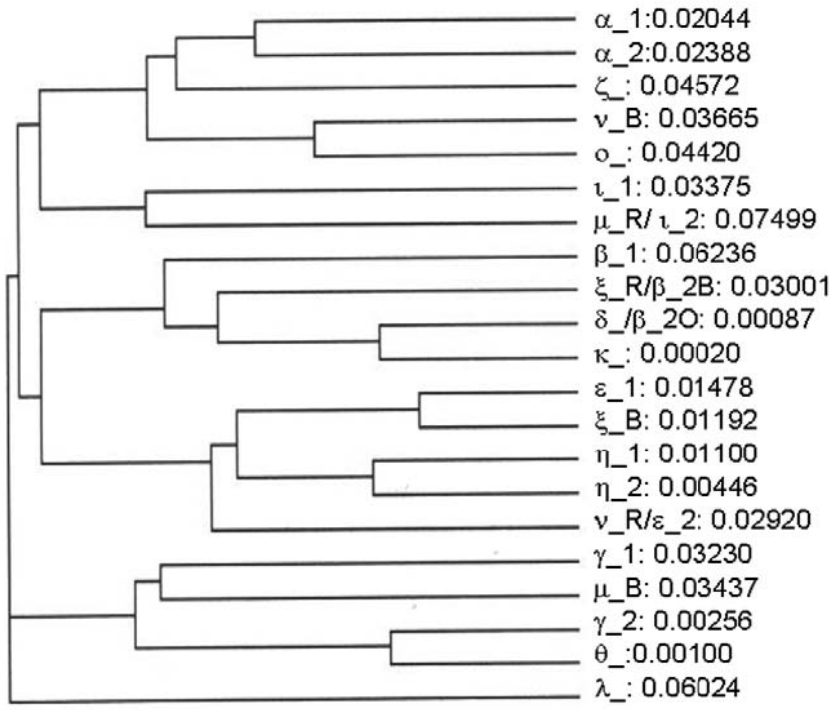

Fig. 4. Dendrogram showing phylogenetic relationships between 21 different intimin variants (intimin variants are denoted with greek letters).The tree was contructed using the Clustal W program. Phylogenetic analysis revealed six groups of closely related intimin genes. Numbers on branches denotes genetic distance. Reprinted from reference (21) with permission from the publisher.

which are delivered into the epithelial cell where they interfere with a variety of cellular processes (46).

The third and largest functional domain consists of about 20 highly conserved genes encoding the type III secretion system (TTSS). The TTSS forms a hollow needle structure which adheres (38,54) and establishes a transient link to the epithelial cell surface (123), through which effector proteins may be translocated to the interior of the host cell. After effector proteins have been transferred, the TTSS complex is removed to make possible intimate attachment between the bacterium and the epithelial cell surface through interaction between intimin and Tir (68,123).

Another important characteristic of EPEC is its ability to adhere to epithelial cells in discrete three-dimensional microcolonies, named localized adherence (LA) (202). This phenotypic trait is mediated by type IV fimbriae called bundle-forming pili (Bfp) (78). These are encoded by a cluster of genes, including *bfpA* which encodes the major structural subunit, located on the 50-70 MDa plasmid *E. coli* adherence plasmid (EAF) (225). This plasmid also

contains transcriptional activator (*per*) genes shown to be involved in the regulation of the expression of the *bfp* genes as well as the chromosomal LEE genes (226,248).

A number of toxins, adhesins and factors with other functions encoded by genes located outside the LEE have also recently been linked with virulence in EPEC. Effector proteins encoded by such genes may be secreted through the LEE encoded the TTSS or via some other mechanism. A list of virulence factors with a description of their function is presented in Table 1.

In addition to EPEC, the majority of EHEC strains [reviewed in reference (111)] and *E. coli* of several animal species contain the LEE and are capable of inducing A/E lesions. Animal pathogenic *E. coli* containing the LEE include the rabbit pathogenic *E. coli* (REPEC) (31) and the pork pathogenic *E. coli* (PEPEC) (249). Other bacterial species like the *E. albertii* (formerly subtype of *Hafnia alvei*) (98), and the mouse pathogen *C. rodentium* may also contain this virulence factor (204).

Soon after the discovery of the pathogenetic mechanisms of EPEC it became clear that not all EPEC strains contained the EAF plasmid crucial for ability of the bacterium to cause localized adherence, and it was questioned whether such bacteria were true pathogens (157). At the Second International Symposium on EPEC in 1995 a consensus definition was reached (108). The EPEC pathotype was then divided in two types; strains which induced both attaching and effacing lesions and contained the EAF plasmid, were classified as typical EPEC, while attaching and effacing strains not containing the EAF plasmid were classified as atypical EPEC. Serotype characteristics were not part of the new definition, but many researchers still included in the new pathotype only strains belonging of the known EPEC serogroups (229). *E. coli* strains which contained the LEE, but did not belong to any of the EPEC serogroups, were sometimes referred to as attaching and effacing *E. coli* (A/E *E. coli*). Considerable heterogeneity has been reported among non-typical EPEC strains (80,229,236).

EPEC is differentiated from the EHEC pathotype, which also usually contain the LEE and induce attaching and effacing lesions, by the presence of *stx* genes in strains of the latter pathotype (108).

1.2.2.2 Mechanism of diarrhoea

Although the pathogenetic mechanisms of EPEC infection have been unravelled to a great extent, the mechanism by which this organism induces diarrhoea is still unclear. This may be because several different mechanisms are involved. One such mechanism may be a dramatic loss of microvilli resulting in brush border enzyme deficiency and malabsorption (34). This

Table 1. Recognized or putative virulence factors in EPEC.

Factor	Description	Function	References
Bfp	Bundle forming pilus	Causes localized adesion, Type IV pilus	(79)
Cif	Cycle-inhibiting factor	Blocks mitosis in cell cycle g2/M phase	(141)
EAST	Heat stable enterotoxin	Activates guanylate cyclase resulting in ion secretion	(53)
Efa1/lifA	E. coli factor of adherence/ lymphycyte inhibiting factor	Adhesin, inhibits lymphocyte activation	(1,122,171)
EspA	*E. coli* secreted protein A	Hollow tube structure essential for transferal of TTSS dependent effector proteins	(74,126)
EspB	*E. coli* secreted protein B	TTSS component involved in pore formation in the epithelial cell plasma membrane	(74,100,246)
EspC	*E. coli* secreted protein C	Serine protease, induces damage to host cell cytoskeleton	(150,167)
EspD	*E. coli* secreted protein D	TTSS component involved in posre formation in the epithelial cell plasma membrane	(74,100,131)
EspF	*E. coli* secreted protein F	Opens tight junctions, induces apoptosis	(39,149)
EspG	*E. coli* secreted protein G	Disrupts the microtubule network, alter epithelial cell permeability	(143,212)
EspG2	*E. coli* secreted protein G2	Disrupts the microtubule network, alter epithelial cell permeability	(143,212)
EspH	*E. coli* secreted protein H	Modulates filopodia and pedestal formation	(211,231)
EspI/nleA	*E. coli* secreted protein I	Effector protein, unknown function	(87,157)
EspJ	*E. coli* secreted protein J	Influences dynamics of clearence from the intestinal tract	(41)
EspZ/sepZ	*E. coli* secreted protein Z	Effector protein, unknown function	(109)
Flagellin	Flagellin	Induces cytokine expression through toll-like receptor type 5 (TLR5), may act as adhesin	(46,80,253)
Intimin	Intimin	Intimate adhesion, induces TH1 response	(50,96,103,138)
Lpf	Long polar fimbriae	Adhesin	(169,229)
LPS	Lipopolysaccharide	Induces cytokine expression through toll-like receptor 4 (TLR4)	(111)
Map	Mitochondrial-associated protein	Disrupts mitochondrial membrane potential	(74,119)
Paa	Porcine attaching and effacing.associated factor	Adhesin	(10)
NleB	Non-LEE encoded effector protein B	Unknown function, associated with virulence	(47,117)
NleC	Non-LEE encoded effector protein C	Unknown function	(47)
NleD	Non-LEE encoded effector protein D	Unknown function	(47)
NleE	Non-LEE encoded effector protein E	Unknown function	(47)
NleF	Non-LEE encoded effector protein F	Unknown function	(47)
Tccp/espFu	Tir cytoskeleton-coupling protein	Effector protein that couples Tir to the actin cytoskeleton	(74)
Tccp2	Tir cytoskeleton-coupling protein 2	Effector protein that couples Tir to the actin cytoskeleton	(177)
Tir	Translocated intimin receptor	Nucleation of cytoskeletal proteins, loss of microvilli, GTPase-activating protein like activity	(108,111,118)

mechanism cannot, however, explain the rapid onset of diarrhoea less than three hours after the ingestion of EPEC bacteria seen in volunteer studies (49). Another possible mechanism may be a direct effect of EPEC on the alteration of electrolyte transport across the epithelial cell membrane observed in vitro studies (41,217). A third mechanism may be an increase in epithelial permeability through disruption of tight junctions as the result from an intracellular signalling cascade within the host cell mediated by the EPEC effector protein EspF (143,144). Lastly, flagella from EPEC bacteria have also been shown to induce an inflammatory host reaction through increased IL-8 production which may cause tissue damage (197). However, whether this mechanism actually contributes to an inflammatory response in EPEC infections is controversial. EPEC bacteria have also been shown to inhibit IL-8 expression (46).

1.2.2.3 Epidemiology

Historically studies on epidemiology and clinical aspects of EPEC infections were based on the identification of EPEC serogroups (134), or, after 1980, on the detection of the EAF plasmid or localized adherence (157). Differentiation between typical and atypical EPEC is therefore usually not possible in studies performed before the 1990s when the distinction between the two EPEC subtypes was accepted in the scientific community (108). However, most of the EPEC strains detected in these studies were probably typical EPEC, and therefore epidemiological data from these studies are most likely representative for this EPEC subtype.

As previously mentioned, EPEC used to cause frequent outbreaks with high case fatality rates in industrialized countries until the 1970s (134). From then on EPEC was rarely identified both in outbreaks and sporadic cases of diarrhoea (157). The latter may partly have been due to the fact that many laboratories stopped including EPEC in their test panel. In contrast, EPEC is still an important cause of childhood diarrhoea in low-income countries where it also in recent years has been a major cause of infant diarrhoea. The prevalence of typical and atypical EPEC among children with diarrhoea reported in studies from many different countries are presented in Table 2. Interestingly, there are considerable differences in the frequency of isolation of EPEC from children with diarrhoea between different countries, and even within the same country. This may be caused by differences in the patient selection and the diagnostic methods used, but is probably also due to actual geographic differences in EPEC epidemiology. Interestingly, a decline in the role of EPEC similar to that seen in industrialized countries some decades ago was recently reported from Brazil (191).

Case-control studies from many countries have shown that EPEC primarily causes disease in children less than two years of age [reviewed in (134,157)]. The correlation

Table 2. Prevalence of typical and atypical EPEC in children with diarrhoea from different countries reported in recent years. Data compiled from published reports by the author.

Author	Year	Country	No.	Typical EPEC no. (%)[a]	Atypical EPEC no. (%)[a]
Escheverria *et al.* (55)	1991	Thailand	509	30 (5.9)	5 (1.0)
Gomez *et al.* (81)	1991	Brazil	500	137 (27.4)	32 (6.4)
Morelli *et al.* (150)	1994	Italy	112	2 (1.8)	-
Albert *et al.* (7)	1995	Bangladesh	451	70 (15.5)	0
Forestier *et al.* (64)	1996	France	220	3 (1.0)	12 (5.5)
Bokete *et al.* (23)	1997	USA	445	3 (0.7)	17 (3.8)
Scaletsky *et al.* (201)	1999	Brazil	40	9 (22.5)	7 (17.5)
Gascon *et al.* (74)	2000	Tanzania	103	4 (3.9) [c]	-
Okeke *et al.* (170)	2000	Nigeria	187	4 (2.1) [c]	-
Galane *et al.* (70)	2001	South Africa	151	6 (4.0)	48 (31.7)
Knutton *et al.* (124)	2001	UK	1496	6 (0.4)	112 (7.5)
Scaletsky *et al.* (198)	2002	Brazil	237	21 (8.9)	13 (5.5)
Scaletsky *et al.* (200)	2002	Brazil	100	17 (17.0)	6 (6.0)
Dulguer *et al.* (52)	2003	Brazil	438	45 (10.3)	38 (8.7)
Nunes *et al.* (167)	2003	Brazil	125	14 (11.2)	11 (8.8)
Presterl *et al.* (181)	2003	Gabon	150	0	0
Ratchtrachenchai *et al.* (185)	2004	Thailand	2100	61 (2.9)	24 (1.1)
Regua-Mangia *et al.* (186)	2004	Brazil	199	5 (2.5)	11 (5.5)
Robbins-Browne *et al.* (190)	2004	Australia	696	2 (0.3)	89 (12.8)
Nguyen *et al.* (164)	2005	Vietnam	587	0	39 (6.6)
Rappelli *et al.* (184)	2005	Mozambique	548	7 (1.3)[d]	9 (1.6)[d]
Cohen *et al.* (40)	2005	USA	684	-(0.4)	- (6.5)
Olesen *et al.* (171)	2005	Denmark	396	7 (2.0)	44 (11.0)
Orlandi *et al.* (172)	2006	Brazil	470	10 (2.1)	19 (4.0)
Nataro *et al.* (159)	2006	USA	317	2 (0.2)[e]	20 (6.3)
Prere *et al.* (180)	2006	France	280	30 (10.7) [c]	-
Nguyen *et al.* (163)	2006	Australia	134	0	30 (22.4)
Vernacchio *et al.* (234)	2006	USA	442	0	57 (12.9)
Alikhani *et al.* (8)	2006	Iran	247	35 (14.2)	23 (9.3)
Hien *et al.* (92)	2007	Vietnam	111	2 (0.9)	5 (4.5)

[a] Numbers presented in the table are calculated from available information if not explicitly given in the referred study.
[b] Only methods used for the identification of, and differentiation between, typical and atypical EPEC are listed
[c] Includes both typical and atypical EPEC
[d] Three of 16 EPEC strains were isolated from healthy children
[e] Typical EPEC strains isolated from patients of all ages

between EPEC infection and diarrhoea has been shown to be strongest for infants < 6 months old, while EPEC is frequently present without causing symptoms among children older than 6 months to 2 years (134).

The incubation period and infectious dose necessary to cause naturally transmitted EPEC diarrhoea is not known. In adult volunteer studies incubation periods of 7-16 hrs have been most common (47). However, this may not be representative for the incubation period for infants due both to differences in the intestine as well as different inoculum sizes in experimental compared to natural infections. Only high inocula (10^8-10^9 CFU) causes diarrhoea in adults volunteers (17,133,135) while probably considerably lower doses is needed to cause disease in infants.

Transmission of EPEC is predominantly through person to person spread via the faecal-oral route (134). However, food and waterborne transmission have been identified in EPEC outbreaks in adults (157,194,246), and even airborne transmission was suggested as a potential route of EPEC infection since it was isolated from dust and aerosols in one study (192). Many studies have documented spread of infection from index cases in hospitals, nurseries and day care centres (25,134,245).

The primary reservoir of EPEC belonging to classical serogroups is believed to be children with EPEC diarrhoea, as well as asymptomatic children and adults (134). Attaching and effacing *E. coli* not belonging to EPEC serogroups, however, have been detected in many animal and bird species (126).

1.2.2.4 Clinical features of EPEC infection

EPEC most commonly causes acute diarrhoea (134,157). It is often accompanied by vomiting and low-grade fever. Many investigators have reported EPEC to cause severe diarrhoea with a high case fatality rate (47,134). In one study from Brazil 51% of the children infected with EPEC were dehydrated (81). However, this organism may also cause persistent diarrhoea (47,134). Fagundes–Neto *et al.* found that more than one fourth of the children admitted to hospital with EPEC infection had diarrhoea that lasted more than 14 days (60). EPEC infection does not cause bloody diarrhoea, but faecal leucocytes may occasionally be seen.

1.2.2.5 Diagnosis

For many years the diagnosis of EPEC was based solely on the identification of O serogroups, or O:H serotypes (134). Slide agglutination with polyvalent O antisera has been used for the diagnosis of EPEC by many laboratories. However, since EPEC O serogroups also contain

commensal *E. coli* strains, it has been estimated that only 10-25% of strains agglutinated by EPEC O antisera actually do belong to a recognized EPEC O:H serotype (134). Positive results may also be due to cross reactions between several of the O antigens. Complete serotyping for all 174 O serogroups and 53 H types would improve the diagnostic accuracy, but would be very laborious and is therefore done only for research purposes in few international reference laboratories. After the pathogenetic mechanism of EPEC diarrhoea was revealed, new phenotypic and genotypic diagnostic tests were developed.

The diagnosis of EPEC should be based on the characteristics that define this pathotype (108): 1) bacterial strain confirmed as *E. coli* species, 2) the ability of the strains to cause attaching and effacing lesions, and 3) localized adherence, and 4) the absence of shiga toxins. This may be achieved by phenotypic and/ or genotypic tests.

Phenotypic tests. Identification of the bacterial strain as *E. coli* may be done by a variety of biochemical methods. The attaching and effacing phenotype may be demonstrated by the fluorescent actin staining (FAS) test originally described by Knutton *et al.* (121). This test is designed to visualize aggregated filamentous actin in the epithelial cell immediately below intimately adherent EPEC bacteria. The bacterial strain is incubated in a HEp-2 or HeLa cell culture for 3 to 6 hours. After permeabilization and repeated washing, the cells are stained using the mushroom toxin phalloidin conjugated to fluorescein isothiocyanate (FITC). The specimens are then examined under a fluorescence microscope as well as in phase-contrast microscopy. Spots of fluorescence corresponding to the location of bacterial cells visualize actin aggregation and are recorded as a positive FAS test. The attaching and effacing phenotype may also be visualized by electron microscopy.

Localized adherence, which is used to differentiate between typical and atypical EPEC, is demonstrated in HeLa or HEp-2 cell culture performed as described above, but strained with Giemsa and examined by light microscopy (48). An ELISA test has also been developed which was shown to be specific for EAF containing EPEC strains (6).

Phenotypic tests to differentiate EPEC from the shiga toxin-containing EHEC pathotype, includes detection of cytotoxicity in Vero cell culture and immunoassays (176,213).

Genotypic methods. Genotypic methods can be used to identify the presence of genes essential for the attaching and effacing phenotype (usually the *eae* gene) and localized adherence (EAF plasmid or bundle forming pilus genes), and the absence of genes encoding shiga toxins (stx_1 and stx_2). Both DNA probes and a variety of PCR primers and protocols have been developed for the diagnosis and characterization of genes important for each of

these traits. Whereas hybridization methods generally are cheaper than PCR, the latter method enables a rapid identification of EPEC from clinical specimens.

The *eae* gene is usually chosen as target for the primary diagnosis of EPEC (157). The first genotypic *eae* test was based on a 1-kb fragment probe used in a hybridization assay developed by Jerse *et al.* (101). Later a variety of PCRs, both conventional (71,110,136,240) and real-time PCR (141,166,188), have been developed for the conserved 5' end of the *eae* gene. Several multiplex PCRs have also been designed with a combination of primers for the *eae* gene and other genes specific for other diarrheagenic *E. coli* pathotypes (26,178). Recently it was suggested to use the gene *escV*, encoding the *E. coli* secreted protein V, instead of the *eae* for the detection of the LEE since this gene has been shown to be the most conserved gene on the LEE pathogenicity island (151).

The first genotypic method for the detection of the EAF plasmid was a 1-kb fragment probe described by Nataro *et al.* (156). A specific oligonucleotide probe (100) and PCR (65), for the same probe sequence were also developed. Later a DNA probe (77) and a PCR targeting the bundle forming pilus gene *bfpA* (87) were developed and used either in stead of, or together with, methods targeting the initial EAF probe sequence. In general, there is excellent correlation between the genotypic methods and localized adherence in cell culture (198). Rarely, however, EAF probe negative strains may show localized adherence (23), possibly due to the presence of other virulence factors, and certain EAF positive strains may fail to induce localized adherence, due to a deletion in the *bfpA* operon (24).

Genotyping for the differentiation between the EPEC and EHEC pathotypes was first based on the detection of the stx_1 and stx_2 toxin genes by DNA probes (241). Later various variants of the PCR method have been developed and used for the same purpose (157). Due to sequence variation between the different variants of the stx_2 gene, not all primer pairs designed for stx_2 will detect all variants of this gene (183). In recent years real-time PCR methods (188), and multiplex PCR methods designed to detect all diarrheagenic pathotypes, have been developed, as mentioned above.

PCR detection of EPEC directly in stool samples is possible, but may be problematic due to inhibitory factors resulting in poor sensitivity (157). These problems are avoided when PCR is done on bacterial culture either on solid medium or in broth. However, an overnight culture step causes a delay of the result which is a disadvantage of this method.

1.2.2.6 Treatment

Most children recover completely from EPEC diarrhoea if dehydration and electrolyte balance are corrected in time (37). Early feeding is recommended to avoid weight loss (244). In some cases, especially with persistent diarrhoea, parenteral nutrition is necessary due to extensive malabsorption (47). Antibiotics are not generally recommended by the WHO in the treatment of diarrhoea (244), but are sometimes used in severe protracted EPEC diarrhoea. Few studies have investigated the role of antimicrobial therapy for EPEC diarrhoea. Agents that have been reported to reduce the duration of diarrhoea are trimethoprim-sulfamethoxazole and mecillinam (47). Rifaximin, a rifampin-like antimicrobial agent, was also recently reported to limit the course of bacterial diarrhoea, often caused by EPEC, to 1-2 days (58). No effect on the course of diarrhoeal disease was observed in a recent study where oral polymyxin was compared with placebo in infants with severe diarrhoea (219). Differences in outcome between various studies may be due to the antimicrobial agent used, antimicrobial susceptibility of the causative bacterial strain, and differences between patient groups and in disease severity. Other therapies like bismuth subsalicylate and specific bovine anti-*E. coli* milk immunoglobulin have also proven useful in isolated studies (61,147). So far, there are no EPEC specific vaccines available although extensive research has been carried out in this field.

2 Aims of the studies

Principal objective:

The main aim of the study was to investigate the role of EPEC in diarrhoea among children in Norway.

Specific aims:

1. To investigate the prevalence of EPEC in children with infectious diarrhoea
2. To investigate whether EPEC is epidemiologically associated with diarrhoea
3. To compare EPEC strains with phenotypic and genotypic methods
4. To search for genetic characteristics in EPEC strains epidemiologically linked with diarrhoea

3 Materials and Methods

3.1 Study area

The study was conducted in the County of Sør-Trøndelag situated in the central part of Norway with a total population of 268 000 inhabitants as of January 2003 (11), among whom 6.5% were less than five years old (9). More than half the population (152 700 inhabitants) live in the city of Trondheim (11). The Department of Medical Microbiology, St. Olavs Hospital, Trondheim, is responsible for all microbiological analyses of human clinical specimens from Sør-Trøndelag.

3.2 Study population

The first study (Paper I) was a retrospective analysis of etiological agents in children less than 2 years of age with diarrhoea from whom the laboratory had received a faecal specimen during the year 2001. In the subsequent case-control study (Paper II) the age range was extended to children less than five years old. In that study cases were children with suspected infectious gastroenteritis from whom the laboratory received a stool specimen, while controls were healthy children recruited through Maternal and Child Health Centres.

3.3 Clinical information

In the retrospective study information on the duration of diarrhoea was collected from hospital records and the referral forms (Paper I). In the case-control study demographic data and information on possible risk factors for gastrointestinal infection, medical history, and duration of disease was collected in a questionnaire as well as from the physician's referral form. Information about hospital admission and discharge diagnosis was collected from hospital records (Paper I), referral forms and a questionnaire (Papers II-IV).

3.4 Identification of EPEC

EPEC was primarily diagnosed by PCR analysis of the *eae* gene on a streak from solid agar culture of faecal specimens. The PCR was done using published primers designed for the conserved part of the gene (71), with reagents as described below (see 3.7 Genotypic characterization of EPEC strains), and the following cycling conditions: 94ºC for 15 min, then denaturation at 94ºC (1 min), annealing at 60ºC (1 min), and extension at 72ºC (2 min) for 35 cycles. Thereafter the mixture was held at 72ºC for seven min until cooling, and analysed with

2% agarose gel electrophoresis after staining with ethidium bromide. PCR for the stx_1/stx_2 genes to differentiate EPEC from EHEC was done as previously described (28). *eae* and/ or *stx* positive isolates were identified by subculture and retesting of four (Paper I) or ten distinct colonies (Paper II) from primary PCR-positive cultures. Bacterial isolates that were *eae* positive, *stx* negative, and were confirmed biochemically to be *E. coli* (Api10S/ 20E, BioMerieux, France), were classified as EPEC. EPEC isolates were further analysed for the presence of the EAF plasmid gene *bfpA*. Based on the results of *bfpA* PCR (87), the isolates were classified as typical (*bfpA* positive) or atypical (*bfpA* negative) EPEC.

Follow-up specimens were analysed by PCR for the *eae* gene on primary mixed culture without further subculture of positive specimens (Paper II).

3.5 Diagnosis of other enteropathogenic agents - microbiological methods

All specimens from children with diarrhoea were analysed for the bacterial enteropathogens *Salmonella spp.*, *Shigella spp.*, *Yersinia spp.*, *Areomonas spp.*, and *Plesiomonas spp.* using lycine saccharose-urea agar (106), SSI- enteric medium (22), and selenite broth (Difco, Maryland, USA). For the isolation of *Campylobacter spp.*, specimens were cultured on charcoal cefoperazone desoxycholate agar (Mast Diagnostics, Merseyside, UK) (Paper I and II). In addition, specimens from patients with clinical information of bloody diarrhoea were cultured on Sorbitol-MacConkey (SMAC) agar. Identification of bacterial pathogens was done by standard microbiological methods.

The specimens were examined for rotavirus with enzyme immunoassay (DakoCytomation, UK), and for adenovirus by enzyme immunoassay (DakoCytomation), PCR (127) and viral cell culture. Stool specimens containing *eae* positive isolates from children with diarrhoea in the case-control study were additionally tested for the presence of other diarrheagenic *E. coli* pathotypes (ETEC, EIEC and EAEC) by PCR (Paper II). These specimens were also examined with enzyme immunoassay for astrovirus and norovirus (DakoCytomation), *Giardia lamblia* (Remel), and Cryptosporidium (Cellabs). Stool specimens from control subjects were tested only for EPEC (Paper II).

3.6 Phenotypic characterization of EPEC strains

Serotyping. Bacterial growth of stool specimens on MacConkey agar was primarily tested for EPEC serogroups with the polyspecific O-antisera Anti-Coli I and Anti-Coli II (Sifin,). *E. coli* strains which agglutinated with polyspecific antisera were further tested using monospecific O:K antisera at the Norwegian Institute of Public Health, Oslo (Sifin and in-

house antisera). Later, all atypical EPEC strains were analysed for somatic (O) antigens (serogroups O1-O177) and flaggelar (H) antigens using standard methods (84,205) at the *Escherichia, Shigella, Yersinia & Vibrio* Reference Unit, Laboratory for Enteric Pathogens at the Health Protection Agency, London, UK.

EHEC immunoassay. Phenotypic analysis for shiga toxin I/II production was done using immunoassay (Premier EHEC, Meridan Bioscience, Cincinnati, USA) (Paper IV).

3.7 Genotypic characterization of EPEC strains

PCR. In addition to the use in the primary diagnosis of EPEC described above, PCR was used for the analysis of putative virulence genes not included in the microarray panel (Paper III and IV), to control the results of the microarray experiments for selected genes or gene variants (Paper III), and to differentiate between complete and truncated genes (Paper III). The PCR method was also used for amplification of housekeeping gene loci in the MLST analysis (see later, Paper IV).

All analyses were done by conventional PCR using primers and amplification conditions as described by other investigators, except for the genes *nleB*, *nleD*, *nleC*, *nleE* and *nleF*. For each of these genes a PCR method was developed for this study (Paper III). PCR amplification was then performed in a total volume of 50 µl, containing 50 µM (each) dATP, dCTP, dGTP, and dTTP, 0.5 µM each primer, 10x PCR buffer (Applied Biosystems, Branchburg, N.J.), 1.5 mM $MgCl_2$, 1 U AmpliTaq Gold polymerase (Applied Biosystems), and 2 µl bacterial DNA extract as template. After the polymerase enzyme was activated by heating at 94°C for 15 min, amplification was carried out with the following conditions: denaturation at 94°C (1 min.), annealing at 53°C (1 min.), and extension at 72°C (1 min.) for 35 cycles. Thereafter the mixture was held at 72°C for 7 min. before cooling to 10°C. Amplified products were analysed by agarose gel electrophoresis as described earlier.

PFGE. Macrorestriction analysis (PFGE) was done to rule out that the EPEC strains were part of any unrecognized outbreak. Bacterial chromosomal DNA was digested with *Xba*I before separation by electrophoresis using the CHEF XA Mapper (Bio-Rad. Richmond, CA, USA) with the following electrophoretic conditions: 14 °C, linear ramp of 5–60 s over 24 h, 120° switch angle and a gradient of 6.0 V cm^{-1}(Paper I and IV). DNA fragments were stained by ethidium bromide and photographed under ultraviolet illumination. Image capturing was done by Gel Doc 2000 (Bio-Rad).

DNA oligonucleotide microarray. DNA microarray analysis was done to search for virulence genes possibly associated with diarrhoea among a broad range of known and putative *E. coli* virulence genes (Paper III). For this purpose a DNA oligonucleotide microarray developed by Bruant *et al*. was used (29). The version of the microarray used in our study was composed of 242 70-mer oligonucleotide probes specific for 182 virulence genes, or gene variants, from all known pathotypes of *E. coli*. Genomic DNA was fluorescently labeled with Cy5 with a random-priming protocol derived from the Invitrogen's Bioprime® DNA Labeling System (Invitrogen life technologies, Burlington, Ontario) before hybridization was carried out at 50ºC, and scanned with a ScanArray® Lite fluorescent microarray analysis system (Canberra-Packard Canada, Montreal, Quebec).

MLST. This method was applied to characterize the phylogenetic relationship between the atypical EPEC strains from the case-control study (Paper IV). For each strain the seven housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* were amplified and sequenced according to the protocol of the *Escherichia coli* MLST database (http://web.mpiib-berlin.mpg.de). PCR amplification of the seven gene loci was carried out with primers as previously published (242), sequenced with the PCR primer set, primers published by Tartof *et al.* (220), or with primers designed in this study: gyrB(Trh) F, icd(Trh) F, icd(Trh) R, and purA(Trh) R (Paper IV). The latter primers were designed due to unsatisfactory results of sequencing using available primers. Sequencing was performed using either the CEQ DTCS-Quick Star Kit (Beckman Coulter, Fullerton, California, USA) or the Big Dye terminator Cycle Sequencing Kit v 3.1 (Applied Biosystems) with subsequent capillary electrophoresis, respectively, on a Beckman Coulter CEQ 8800 or an ABI 3130x Genetic Analyzer. The sequence traces for each of the seven gene loci were assigned an allele number, and each strain was assigned a sequence type (ST) according to allelic profile, by submission of the sequences to the *E. coli* MLST database.

Methods for genetic comparison of EPEC strains. PFGE fragments were compared both by manual inspection (222) and by cluster analysis. Similarities of fragments between strains were compared using the Dice coefficient, and a dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) clustering method using either the Fingerprinting II software (Bio-Rad)(Paper I) or the Bionumerics software (version 4.6, Applied Maths, Sint-Martens-Latem, Belgium). Significant clusters were determined by the point-bisectional correlation method (Bionumerics manual, version 4.6) (Paper IV). The EPEC strains were characterized in virulence clusters (Paper IV) using principal component analysis (Bionumerics) of all the detected virulence genes, gene variants and markers

identified in the microarray study. EPEC strains were assigned to a phylogenetic group according to its content of the three genes *chuA*, *yjaA* and *tspE4C2* as proposed by Clermont *et al.* (39). Concatenated DNA sequences from the seven gene loci used for MLST analysis were aligned using the ClustalW algorithm of the MEGA3 software (128). A rooted Neighbour-joining tree was constructed using the Kimura 2-parameter model of nucleotide substitution. Phylogenetic network analysis was performed using the Neighbour-net algorithm and untransformed distances (p distance) of SplitsTree 4 (97). Evidence of recombination was assessed using the SplitsTree ϕ_w recombination test of the SplitsTree software. Assignations of STs in ST complexes were done by the curator of the *E. coli* MLST database using the MSTree application of Bionumerics. Finally, the Simpson's index of diversity (96,211) was used to compare phylogenetic diversity between EPEC strains.

3.8 Ethical issues

The study was reported to the Norwegian Social Science Data Services, and was approved by the Regional Committee for Medical Research Ethics, and the Norwegian Data Inspectorate.

3.9 Statistical analyses

The Chi-square or Fischer's exact tests were used for comparison of differences between groups of nominal data. In the case-control study multiple logistic regression was used to study the potential association between EPEC and diarrhoea. The analyses were adjusted for matching factors (sex, age group, and time of specimen collection), and were controlled for potential confounding from other risk factors. The data were also analysed by conditional logistic regression. The Mann-Whitney U test was employed for testing of ordinal variables and quantitative data which were not normally distributed. A *P*-value < 0.05 was considered significant.

The statistical analyses were mostly performed using SPSS (SPSS Inc, Illinois, USA). In addition, Stata version 8.0 (StataCorp 2003, Texas, USA) was used for the conditional logistic regression analyses (Paper II), and the R software package (version R2.1.1, http://www.r-project.org/) was used for the analyses of association between virulence genes and diarrhoea (Paper III).

4 Results

4.1 Paper I

Potential enteric pathogens were identified in 124 (28.2 %) of 440 children < 2 years of age with diarrhoea. EPEC was the most frequently identified agent in the study, and was isolated from 44 (10%) of the 440 patients. One of the *eae*-positive *E. coli* isolates was classified as typical EPEC (*bfpA* positive), while 43 isolates were classified as atypical EPEC (*bfpA* negative). Eight (18.6%) of 43 atypical EPEC isolates belonged to EPEC serogroups, while 35 strains were not agglutinated by EPEC antisera. EPEC was detected in four (3.0%) of 135 children admitted to hospital. None of these had severe acute gastroenteritis. Protracted diarrhoea was recorded for 12 (31.6%) of 38 patients with atypical EPEC infection. There was a considerable genetic diversity in PFGE patterns among the EPEC isolates, and each isolate displayed a unique pattern. EPEC was more common in children 12-23 months old (33/206 patients, 16%) than in children < 12 months of age (11/234 patients, 4.7%; $P<0.001$).

4.2 Paper II

In this case-control study EPEC was isolated from 38 (15.1%) of 251 children < 5 years old with diarrhoea. EPEC was detected in 21 (10%) of 210 healthy children < 5 years old. One isolate was classified as typical EPEC while 58 isolates were classified as atypical. EPEC was a less frequent finding in children < 1 year of age (6 isolates/148 children, 4.1%) than in older children (52 isolates/313 children, 17.6%). Four isolates, all from patients, belonged to EPEC serogroups. EPEC was found in 3 (5.1%) of 59 children who were admitted to hospital. Atypical EPEC was more commonly diagnosed in patients (37/251, 14.7%) than in controls (21/210, 10%), but the association with diarrhoea was not significant (OR=1.4, *P*=0.3). The prevalence of atypical EPEC was higher in children with prolonged diarrhoea (20/89, 22.5%) than in healthy children (21/210, 10%, OR= 2.1, *P*=0.04). Atypical EPEC was found in pure culture in 17 of 37 (45.9%) children with diarrhoea and in 5 of 21 (23.8%) controls (*P*=0.3). Ten of 30 (33.3%) patients and 4 of 20 (20%) healthy controls who submitted a follow-up specimen, did still have EPEC in their stools after a median of 38 and 59 days, respectively.

4.3 Paper III

A total of 95 putative virulence genes or gene variants were detected in the 57 atypical EPEC strains included in this study. Seven genes were positively statistically associated with diarrhoea (Table 5). These were the OI-122 genes *efa1/lifA* (*P*=0.0002), *set/ent*, *nleB* and *nleE* (all with *P*=0.0006), the *paa* (*P*=0.01), the $lpfA_{O113}$ (*P*=0.02), *ehxA* (*P*=0.04) and *ureD* (*P*=0.05). In addition the *lpfA* was significantly associated with diarrhoeal disease when all three variants of the gene were analysed together (*P*=0.0008). A significant negative association with diarrhoea was shown for the phylogenetic marker gene *yjaA* (*P*=0.0004), as well as for the *astA* (*P*=0.02), *b1121* (*P*=0.02) and *ibeA* (*P*=0.05) genes. All except one of the atypical EPEC strains could be classified in one of two main virulence groups based on their content of OI-122 genes, and *lpfA* and *yjaA* genes: group I strains were defined by the presence of OI-122 genes and/ or *lpfA* genes, as well as the absence of the *yjaA* gene, while group II strains were defined by the presence of the *yjaA* gene and the absence of OI-122 and *lpfA* genes. Twenty four (64.9%) of 37 atypical EPEC isolates from children with diarrhoea belonged to virulence group I, while only 3 (15.8) of 19 isolates from healthy children belonged to the same group (*P*<0.001). In contrast, virulence group II strains were more commonly detected in healthy children (16/19 subjects, 84.2%) than in those with diarrhoea (13/37 subjects, 35.1%). Among children with diarrhoea, virulence group I strains were present both in those with acute (8 subjects) and prolonged diarrhoea (10 subjects) (Table 6). In contrast, virulence group II strains were mainly isolated from patients with protracted diarrhoea (10 subjects), and rarely from children with diarrhoea of short duration. Among children who submitted follow-up specimens, 12 (66.7%) of 18 subjects with virulence group II strains still had EPEC on follow-up, compared 3 (18.8%) of 16 subjects infected with group I strains (*P*=0.005).

4.4 Paper IV

Among the 56 atypical EPEC strains included in this study 10 strains belonged to phylogenetic group A, 16 to group B1, 24 to group B2, and 6 strains to group D. Twenty six different STs and 20 different clonal groups were represented in the study. Phylogenetic analysis revealed evidence of phylogenetic incompatibility in the divergence of the atypical EPEC clones, and significant evidence of recombinational events (P=6.8x10^{-6}). The strains were separated in three clusters by overall virulence gene profile: one large cluster included all phylogenetic group A, B1 and D strains, and two clusters consisted exclusively of group B2 strains (labeled B2-A and B2-B). Thirty one of the genes or gene variants observed in at least five bacterial strains were restricted to strains of one virulence cluster or phylogenetic group. Eleven different variants of the pathogenicity island LEE was present among the atypical EPEC strains when they were classified by their content of different variants of the *espA*, *espB*, *tir* and *eae* genes. Pathogenicity island OI-122 genes were present in strains belonging to 12 different STs, and the *lpfA* gene variants in 14 different STs, but only within the three phylogenetic groups A, B1 and D. The presence of the OI-122 genes was linked with LEE type. Genes related to the EHEC pathotype were detected in 14 (25%) of the atypical EPEC strains, all within the phylogenetic groups A, B1 and D. There was considerable heterogeneity in PFGE profiles and serotypes, and almost half the strains were O non-typable. The two phylogenetic groups B1 and D were weakly associated with diarrhoea (P=0.06 and P=0.09, respectively), while group B2 was isolated most frequently from healthy controls (P=0.05).

5 General discussion

Having demonstrated that EPEC was a frequent finding in children with diarrhoea, a case-control study was conducted to investigate the role of EPEC as a diarrheagenic agent (Paper II). To further clarify this issue, isolates collected in the case-control study were characterized with phenotypic and genetic methods (Papers III and IV).

5.1 Identification and classification of EPEC strains

It is difficult to design PCR primers which detect all variants of the *eae* gene due to sequence heterogeneity between different variants of the gene. The variability is most extensive in the 3´ region of the gene, but there is also some degree of heterogeneity between different variants of the conserved 5´ region (157). In this study EPEC strains were primarily identified by a PCR with primers designed for the conserved region of the gene (71). The demonstration of eight different variants of the *eae* gene in the EPEC strains in this study shows that the PCR used was able to amplify many different variants of the gene, including the variants most frequently occurring in EPEC strains (21). However, sequence comparison with all published variants of the *eae* gene reveals polymorphisms in the target sequence for the PCR primers in some variants of the gene (BLAST searches, data not shown). It is therefore possible that the PCR may give a false negative result with EPEC strains with certain variants of the *eae* gene. This is, however, unlikely to represent a major issue since the PCR method covers all the six main variants of the *eae* gene (21)(Fig. 4).

At the Second International EPEC Symposium in 1995 it was agreed that the classification of EPEC as typical or atypical should be based on the newly discovered pathogenetic mechanisms (108). EPEC strains were classified as typical EPEC if they contained the EAF plasmid encoding localized adherence and atypical EPEC if they did not contain this plasmid. Data regarding the serogroup or serotype of the EPEC strain was not included in the classification agreed at the meeting, although it was recognized that the majority of EPEC strains belonged to well-recognized O:H serotypes. Despite the agreed classification, however, many authors have restricted the label atypical EPEC to strains belonging to recognized EPEC serogroups, and have placed attaching and effacing strains not belonging to EPEC serogroups in a separate group, termed attaching and effacing *E. coli* (A/EEC) (229). The reason for this differentiation based on serogroups is not obvious, and may be due mainly to historical reasons. In the present study we did not use serogroup data in

the differentiation between typical and atypical EPEC, and classified all *eae* positive *E. coli* strains which were *stx* and *bfpA* negative as atypical EPEC.

One bacterial strain was reclassified from atypical to typical EPEC during the study. This was done after it was shown to hybridize with the *bfpA* probe in the microarray experiment (Paper III). Primarily it had been recorded as *bfpA* negative, since only a weak amplicon of a different size than expected had been observed in the *bfpA* PCR analysis (Paper II). The EAF plasmid encoding Bfp, essential for the localized adherence characteristic of typical EPEC, has been shown to contain sequence variations and deletions in some EPEC strains (24,131). Thus, such strains may be classified either as typical or atypical in different studies depending on the specific methods used. As in this study, however, they usually constitute a minor proportion of the isolates. Therefore variable classification of the strains is unlikely to have any substantial influence on the results in most studies (79,218).

One strain isolated in the case-control study hybridized with probes for the genes $stxA_{2f}$ and $stxB_{2f}$ in the microarray experiment. This strain, which had produced a negative result for the *stx* gene in the primary PCR analysis, was reclassified from atypical EPEC (Paper II and III) to STEC and was therefore not in included in Paper IV. The stx_{2f} variant of the *stx* gene was originally isolated from feral pigeons (206), and has not been related to human disease (69). The false negative PCR result with the stx_{2f} variant may be explained by a significant sequence difference between this gene compared to the other *stx* gene variants.

For fifteen strains, which hybridized with the $stxB_1$ probe in the microarray analysis (Paper III), the classification as atypical EPEC was maintained (Paper IV) on the following basis: they did not hybridize with the corresponding $stxA_1$ probe, were negative in PCR analysis with $stxB_1$ sequence specific primers, and did not produce shiga toxins (Premier EHEC, Meridian Bioscience, US) (unpublished results). We therefore concluded that the gene sequences detected by the $stxB_1$ hybridization probe did not represent a complete *stxB* gene.

5.2 EPEC prevalence

EPEC was found to be highly prevalent in Norwegian children with diarrhoea, with a prevalence of 10.0% and 15.1% in the age groups < 2 years old (Paper I) and < 5 years old (Paper II), respectively. However, EPEC was also detected in 10% of healthy children (Paper II).

Atypical EPEC was the predominant type of EPEC detected, accounting for 42/43 strains in the retrospective study (Paper I) and for 57/59 strains in the case-control study

Table 3. Prevalence of atypical EPEC in case control-studies among children reported in recent years. Data compiled from published reports by the author.

Author	Year	Country	Patients	Controls	*P*
			No. /total (%) [a]	No. /total (%) [a]	
Escheverria *et al.* (55)	1991	Thailand	5/509 (1.0)	6/509 (1.2)	Ns
Gomez *et al.* (81)	1991	Brazil	32/500 (6.4)	20/500 (4.0)	Ns
Morelli *et al.* (150)	1994	Italy	2/112 (1.8)	0/56 (0)	Ns
Albert *et al.* (7)	1995	Bangladesh	0/451	7/602 (1.2)	Ns
Forestier *et al.* (64)	1996	France	13/220 (5.9)	12/211 (5.7)	Ns
Scaletsky *et al.* (201)	1999	Brazil	7/40 (17.5)	1/40 (2.5)	0.028
Knutton *et al.* (124)	2001	UK	112/1496 (7.5)	32/546 (5.9)	Ns
Vieira *et al.* (236)	2001	Brazil	32/505 (6.3)	27/505 (5.3)	Ns
Scaletsky *et al.* (198)	2002	Brazil	13/237 (5.5)	13/231 (5.6)	Ns
Scaletsky *et al.* (200)	2002	Brazil	6/100 (6.0)	2/100 (2.0)	Ns
Dulguer *et al.* (52)	2003	Brazil	38/438 (8.7)	27/422 (6.4)	Ns
Nunes *et al.* (167)	2003	Brazil	11/125 (8.8)	5/98 (5.1)	Ns
Regua-Mangia *et al.* (186)	2004	Brazil	11/199 (5.5)	6/54 (11.1)	Ns
Robbins-Browne *et al.*(190)[b]	2004	Australia	89/696 (12.8)	11/489 (2.3)	<0.0001
Cohen *et al.* (40)	2005	USA	- [c] /684 (6.5)	- [c] /486 (3.9)	<0.05
Nguyen *et al.* (164)	2005	Vietnam	39/587 (6.6)	11/249 (4.4)	Ns
Orlandi *et al.* (172)	2006	Brazil	19/470 (4.0)	2/407 (0.5)	0.006
Olesen *et al.* (171)	2005	Denmark	44/396 (11)	91/714 (13)	Ns
Nataro *et al.* (159)	2006	USA	20/317 (6.3)	6/56 (10.7)	Ns
Vernacchio *et al.* (234)	2006	USA	59/482 (12.2)	57/442 (12.9)	Ns
Alikhani *et al.* (8)	2006	Iran	23/247 (9.3)	13/1108 (1.2)	<0.0001
Hien *et al.* (92)	2007	Vietnam	5/111 (4.5)	4/111 (3.6)	Ns

[a] Numbers presented are calculated from available information if not explicitly given in the referred study, and in most studies include all *eae* positive *E. coli* strains which are EAF and/ or *bfpA* and *stx* negative. In some of the studies classification was partly based on phenotypic methods.
[b] This study included families with at least two children 1-15 years of age. The mean age of subjects with diarrhoea was 3.4 years.
[c] Only percentages, but not actual figures, of subjects with atypical EPEC available from published study

(Paper II, paper III and reclassifications as detailed above). This finding is in line with similar reports from other high-income countries (Table 2), although the prevalence of atypical EPEC varies considerably (< 5 up to 22 %) between different studies (23,163,206). This variation could be due to the epidemiological situation in different geographic regions, but could also reflect the population of children studied, study design and diagnostic methods which may vary between different studies. One possible explanation for the high prevalence rates recorded in this study could be the inclusion criteria used. Research studies based on hospital admissions most likely would find different prevalence rates compared to studies of community acquired diarrhoea where the majority usually has less severe disease.

5.3 Atypical EPEC - role of in diarrhoea

Although atypical EPEC was more common in patients (14.7%) than in controls (10.0%), the association with diarrhoea was not statistically significant when all subjects with and without diarrhoea were compared (OR 1.4, *P*=0.3) (Paper II). This lack of a statistically significant association is consistent with results from numerous other studies (Table 3). However, there are also data indicating that atypical EPEC may have a role as a diarrheagenic agent: a) in most case control studies (Table 3) a higher prevalence of atypical EPEC has been observed in children with diarrhoea than in healthy children; b) in some studies the association between atypical EPEC and diarrhoea was significant, either for the group as a whole (8,40,172,190,201) or a subgroup (52,236); c) in addition, volunteer studies have shown that adult subjects who were given EAF-plasmid cured or *bfpA*-mutated EPEC strains developed diarrhoea, although to a lesser extent than subjects infected with the wild type typical EPEC strain (17,135); and d) finally, strains classified as atypical EPEC have been reported to cause diarrhoea outbreaks (90,99,237,242,246).

The microarray revealed that there are considerable differences in the content of virulence genes between the atypical EPEC strains in the study (Paper III). Interestingly, a number of putative virulence genes were significantly associated with diarrhoea (Table 4). These were the OI-122 pathogenicity island genes *efa1/lifA*, *nleB*, *nleE* and *set/ent*, $lpfA_{O113}$, the EHEC associated genes *ehxA* and *ureD*, and the porcine attaching and effacing gene *paa*. In addition, the association was highly significant (*P*=0.0008) for the three variants of the *lpfA* genes when they were analysed together.

In contrast to the above findings, a study from Brazil reported no difference in the prevalence of the genes *efa1/lifA*, $lpfA_{R141}$ and *nleA/espI* in atypical EPEC strains between children with and without diarrhoea (51). Likewise, in 22 randomly selected atypical EPEC

Table 4. Genes significantly associated with diarrhoea in atypical EPEC strains from Norwegian children less than five years old with and without diarrhoea.

Gene	Gene description	Total no. (%)	Patients (n=37)	Controls (n=20) [b]	*P*[c]	Type of association[f]
efa1/lifA[a]	EHEC factor for adherence (*efa1*)/ lymphocyte inhibitory factor A (*lifA*)	17 (28.8)	17	0	0.0002	+
nleB[a]	Non-LEE effector protein B encoding gene, located on the OI-122	23 (40.4)	21	2	0.0006	+
nleE[a]	Non-LEE effector protein E encoding gene, located on the OI-122	23 (40.4)	21	2	0.0006	+
set/ent[a]	Gene encoding putative enterotoxin, similar to ShET2 enterotoxin of *S. flexneri*, located on the OI-122	23 (40.4)	21	2	0.0006	+
$lfpA_{O113}$	Gene endcoding major fimbrial subunit of long polar fimbriae, first described in STEC O113	13 (22.8)	12	1	0.02[d]	+
ehxA	EHEC hemolysin gene, located on the EHEC plasmid	8 (14.0)	8	0	0.04[e]	+
ureD	Urease-associated protein, located on the EHEC plasmid	7 (12.3)	7	0	0.05[e]	+
paa	Porcine attaching and effacing associated protein	34 (59.6)	27	7	0.01	+
yjaA	Hypothetical protein gene, used as phylogenetic marker	30 (52.6)	13	17	0.0004	-
astA	Enteroaggregative *E. coli* heat-stable enterotoxin 1 gene	8 (14.0)	2	6	0.02	-
ibeA	Gene encoding an *E. coli* invasion protein (invasion of the blood-brain barrier)	23 (40.4)	11	12	0.05	-
b1121	Hypothetical protein gene, homologous to virulence factor	48 (84.2)	28	20	0.02[e]	-

[a] Genes belonging to a pathogenicity island, named OI-122 in the EDL933 genome.
[b] One strain initially identified as atypical EPEC by PCR was reclassified as EHEC when it was shown to contain the stx_{2f} gene by microarray analysis.
[c] Fisher's exact test.
[d] When the analysis was done for all *lpfA* variants together ($lpfA_{O113}$, *lpfA1*, and $lpfA_{R141}$), the association was significant with a *P*-value of 0.0008.
[e] Genes no longer significantly associated with diarrhea at the $P<0.05$ level when strains from patients with other pathogenic agents (n=8) were excluded from the analysis.
[f] Positive (+) or negative (-) statistical association with diarrhea.

strains from Australian children with and without diarrhoea only 3 (13.6%) strains contained the *efa1* gene, and 2 (9.0%) strains contained the *lpfD*$_{R141}$ gene variant (190). The inconsistency between the results of these two studies and our own, where 17 of 57 (28.9%) strains were *efa1/lifA* positive and 8 of 57 (14.0%) strains contained the *lpfA*$_{R141}$, gene is not known, but may be due differences in virulence profile of the atypical EPEC strains. However, this will need to be confirmed by comparison of strains from the different geographic regions. It is also possible that a subpopulation of *efa1/lifA* or *lpf* positive atypical EPEC strains could have been missed in the Australian study since only 22 of the 100 atypical EPEC strains in that study were tested.

In the present study virulence genes significantly associated with diarrhoea were concomitantly present in many bacterial strains (Paper III). This fact needs to be considered when assessing the results. It is possible that the significant result obtained for some of the genes may have been due to the concomitant presence of other virulence genes. Analysis of the relative contribution of each of the virulence genes was not possible in the present study due both to the limited number of strains available and to the concomitant presence of such genes in the many strains.

The importance of some of the virulence genes linked with diarrhoea in this study is supported by experimental studies. The OI-122 gene *efa1/lifA* encodes a protein, lymphostatin, which inhibits lymphocyte proliferation and the synthesis of proinflammatory cytokines (1,119), but which has also been shown to have adhesive properties (165). Another OI-122 gene, the *nleB*, was also recently reported to be involved in virulence, while no such effect was seen for the *nleE* gene (115). The function of the *set/ent* gene is not known, but due to sequence homology with the ShET2 enterotoxin of *Shigella flexneri*, a similar function has been suggested (112,160). From a study of EHEC it was shown that strains with a complete OI-122 were associated with increased epidemic potential and severity of disease (112). In another study an additive effect was observed between different OI-122 genes with respect to severity and outbreak potential of non-O157 EHEC strains (239). In the same study an *nleB* mutant *C. rodentium* strain had a decreased virulence potential compared with the wild type strain when tested in mice. Conflicting results have been reported regarding the function of the long polar fimbriae encoded by the *lpf* family of genes. Lpf have been reported to be involved in adherence in several studies (50,162,227,228). However, recently Tatsuno *et al.* could not find that it played any role in the pathogenesis for either of EPEC or *C. rodentium* (221). It is possible that the significant results observed for the *lpfA* genes in our study may

Table 5. Distribution of atypical EPEC strains of virulence group I and II between subjects with acute and protracted diarrhoea, and healthy controls.

Diarrhoea?		Virulence group			Total
		I	II	Non-classifiable	
Yes	Acute	8	1	-	9
	Protracted	10	10	-	20
	Unknown	6	2	-	8
No		3[a]	16	1	20
Sum		27	29	1	57

[a] One strain initially identified as atypical EPEC by PCR was reclassified as EHEC when it was shown to contain the stx_{2f} gene by microarray analysis.

have been due to other coexisting factors like the OI-122 genes which were present in most of the strains containing *lpfA* genes.

There were also genes which were negatively associated with diarrhoea (Table 4). Especially for the *yjaA* gene the association was strong. This gene, which has no known function, is closely linked to phylogenetic ancestry, and has therefore been used as a phylogenetic marker gene (39). The negative link with diarrhoea for the *yjaA* gene observed in this study may indicate the presence of a phylogenetic lineage of atypical EPEC strains lacking diarrheagenic potential. Another possibility could be that the protein encoded by the *yjaA* gene may inhibit other virulence factors present in the strain. Such a negative association between a gene and disease has been reported for other pathotypes of *E. coli* and other bacterial species (139,215). The *astA* gene, which previously was linked with diarrhoea in atypical EPEC strains from Brazil (52), was surprisingly found to be negatively associated with diarrhoeal disease in the present study (Paper III).

Based on their content of genes significantly linked with diarrhoea, all but one of the atypical EPEC strains could be classified in two virulence groups (Table 5) (Paper III). Virulence group I strains containing OI-122 and *lpfA* genes were mainly present in children with diarrhoea, whereas group II strains containing the *yjaA* gene were more common in healthy children than in those with diarrhoea ($P<0.001$).

5.4 Duration of diarrhoea

Virulence group I atypical EPEC strains, which were significantly associated with diarrhoea in this study, were about equally distributed between children with acute and protracted diarrhoea (Table 5). The association of atypical EPEC with protracted diarrhoea observed in

Paper II was based on analyses before the information of virulence groups became available (Paper III). Table 5 shows that atypical EPEC strains of both virulence group I and II were prevalent among children with protracted diarrhoea. However, since strains belonging to virulence group II were not statistically associated with diarrhoea, they should most likely be considered as colonisers which do not cause disease. The statistical association between atypical EPEC and protracted diarrhoea reported in Paper II may therefore have been an overestimation. Based on the analysis in virulence groups, virulence group I strains are associated with both acute and protracted diarrhoea, while virulence group II strains do not appear to have a diarrheagenic potential. On the other hand, we cannot exclude the possibility that strains belonging to the latter virulence group, which appear to have a propensity for protracted intestinal colonization, do have the potential to cause disease in susceptible subjects (154). Virulence group II strains were rarely isolated from patients with acute diarrhoea. The reason behind this finding is not clear, but a possible explanation might be that intestinal environmental changes during acute diarrhoea disfavour colonization with this type of atypical EPEC strains.

In a study comparing clinical characteristics with infectious aetiology in children attending hospital, Nguyen *et al.* found that the duration of diarrhoea was significantly longer in patients infected with atypical EPEC than in children with other enteropathogens or where no agents were identified (164). This finding seems to be in agreement with our primary observation in Paper II, but not with the analysis based on virulence groups (Paper III). Further virulence typing might show whether the atypical EPEC strains in that study also belonged to different virulence groups.

5.5 Severity of disease

In both the retrospective (Paper I) and the case-control study (Paper II) few patients infected with atypical EPEC were admitted to hospital due to severe gastroenteritis. This observation indicates that atypical EPEC usually does not lead to severe disease, at least not the type of atypical EPEC detected in our geographical area. This finding is in agreement with the previous observation that EAF-plasmid cured or *bfpA*-mutated EPEC strains induced milder symptoms than typical EPEC (17,135), and also with the observation that Australian patients infected with atypical EPEC had less severe symptoms than those infected with rotavirus- and Salmonella (163).

5.6 Age distribution

Atypical EPEC was more commonly detected in children older than one year than in infants (Paper II). This age distribution was surprising since EPEC diarrhoea usually has been considered to be as disease in children less than 6-12 months old (134,157). Since many of the infants in this study had older siblings with a high probability of atypical EPEC infection, they would actually be expected to have a high risk of atypical EPEC infection (Paper II). However, our observations of a higher age among children infected with atypical EPEC than reported for typical EPEC are in agreement with Australian studies were children with atypical EPEC gastroenteritis had a mean age of 3.4 years and 16.9 months in two different studies (163,190). In contrast, the rate of infection with *eae* positive *E. coli* in infants in Guinea Bisseau was very high (232). In a study where infants where followed with frequent stool samples from birth for two years, fifty per cent of the cohort were infected with *eae* positive *E. coli* within 4 months, and almost all had been infected by ten months of age. One possible explanation for the discrepancy between these studies may be that the age distribution of atypical EPEC infections is different in high and low income countries.

5.7 Quantity of atypical EPEC in stool specimens

Usually it has been assumed that EPEC bacteria causing diarrhoea will be the predominant *E. coli* strain in the patient's stools (135,157). In our case-control study there was no difference between patients and controls in the relative quantity of atypical EPEC in their stool specimens (Paper II), nor when analysed with respect to virulence group (Paper III). These results need to be interpreted cautiously, as it is generally difficult to pick colonies from a culture plate with mixed growth in a random fashion. Therefore, a subjective element might have influenced the results. Nevertheless, the results do indicate that virulence group I atypical EPEC may be the cause of diarrhoea even though they are present in low numbers at the time of diagnosis. If this finding is confirmed, the prevailing assumption (135,157) that atypical EPEC should be regarded as a causative agent only when detected as the predominant agent in the patient's stool, may need to be reconsidered.

5.8 Duration of carriage of atypical EPEC

In the case-control study persistent colonization (≥1 month) was observed in 14 (28%) of the 50 children who submitted follow-up specimens (Paper II). Three subjects were still colonized after three months and one child even after six months when follow-up was terminated. When compared with respect to the virulence group of the infecting atypical EPEC strains, subjects infected with virulence group II strains were significantly more often

colonized at the time of follow up than those with group I strains (*P*=0.005) (Paper III). This could not be explained by different intervals to the follow up test between the two groups, as this interval was actually longer for virulence group II (mean 38.0 days) than for virulence group I strains (mean 28.6 days, *P*= 0.07). This fact supports the finding in this study that virulence group II strains are persistent colonizers.

5.9 Genetic and phenotypic characterization of atypical EPEC strains

Extensive characterization with several methods showed considerable heterogeneity among the atypical EPEC strains in this study. Using PFGE analysis we confirmed that the high prevalence of atypical EPEC was not due to a clonal outbreak but to endemic infections with a high number of distinct atypical EPEC strains (Paper I and IV). Phylogenetic analysis of the atypical EPEC strains from the case-control study showed that all four phylogenetic groups (A, B1, B2 and D) were represented among the strains (Paper IV). The MLST analysis further showed that the atypical EPEC strains within each of the phylogenetic groups belonged to a high number of different STs as well as clonal lineages. More than half of the STs were detected in only one strain each (Paper IV). There was also evidence of phylogenetic incompatibility in the divergence of the atypical EPEC clones, explained at least in part by recombinational events.

The poor separation between strains belonging to different groups in the phylogenetic analysis might be explained by the presence of hybrid strains carrying ancestry from more than one source as reported from the *E. coli* MLST database (242) (Paper IV). Such hybrid strains were not identifiable by the PCR method used for the phylogenetic group analysis in this study. However, at least four of the STs identified in this study (ST 28, 32, 154, and 206) have previously been shown to contain phylogenetic ancestry from more than one source (242).

Comparison between phylogenetic background and virulence characteristics have usually been done only for a limited number of virulence factors (52,80,167,177,187,236) or for strains belonging to specific EPEC serotypes (15). The use of data from DNA microarray analysis (Paper IV) enabled an extensive characterization of the atypical EPEC strains with respect to overall virulence gene content as well as a comparison between virulence profile and phylogenetic ancestry. The main division between group B2 strains and the three other groups A, B1 and D observed in the phylogenetic analysis was supported by differences in virulence profiles. There was also agreement between the two different types of analyses in the separation of phylogenetic group B2 strains in two clusters. Strains belonging to the

Table 6. Comparison between virulence classifications based on specific genes significantly associated with diarrhoea and overall virulence gene content

Virulence cluster	No.	Virulence group		
		I	II	Non-classifiable
Cluster B2-A	9	0	9	-
Cluster B2-B	15	0	15	-
Cluster A-B1-D	33	27	5	1
Sum	57	27	29	1

phylogenetic groups A and B1 were not reliably differentiated neither by the phylogenetic analysis nor by their overall virulence gene profiles or specific gene content. Group D strains, on the other hand, were narrowly scattered at the periphery of this cluster, and was also shown to contain significantly more virulence genes that strains belonging to the other virulence clusters. The link observed in this study between phylogenetic ancestry and virulence profile, overall as well as for many genes separately, may be explained by the requirement of a specific genetic background for the acquisition of certain virulence factors (59).

In the present study two different classifications of virulence profile was used. The first ("virulence groups") was based on virulence genes significantly associated with diarrhoea (Paper III), while the second ("virulence clusters") was based on overall content of all the putative virulence genes tested in the microarray analysis (Paper IV). The main difference between these two classifications of the atypical EPEC strains was the differentiation of phylogenetic group B2 strains (all belonging to virulence group II) in two clusters, and the localization of all ten phylogenetic group A strains in one cluster (cluster A-B1-D) (Table 6). Among phylogenetic group A strains four belonged to virulence group I and five strains to virulence group II. In addition, the one strain which did not fit within any of the two virulence groups due to its content of both OI-122 genes and the *yjaA* gene, belonged to cluster A-B1-D.

The *efa1/lifA* gene most strongly associated with diarrhoea was present in strains belonging to the phylogenetic groups A, B1 and D, but not phylogenetic group B2 (Paper IV). The other genes statistically associated with diarrhoea were also present in the same three phylogenetic groups, except the *paa* gene which was found in all four groups. The selective distribution of these virulence genes, as well as the correlation between phylogenetic ancestry and virulence genes described above, seems to be consistent with the notion that a specific genetic background may be required for the acquisition of certain virulence factors (59).

Phylogenetic ancestry was less useful as an indicator of diarrheagenic potential than specific virulence genes in this collection of atypical EPEC strains. This observation is most likely explained by considerable heterogeneity in virulence factors within each of the phylogenetic groups. The *efa1/lifA* gene most strongly associated with diarrhoea in these strains (Paper III) was present in only some of the strains within phylogenetic group A and B1. On the other hand, the strong negative association with diarrhoea shown for the phylogenetic group B2 seems to indicate close association between phylogenetic descent and lack of diarrheagenic potential.

In this study the LEE region of each strain was classified by its composition of *espA*, *espB*, *tir* and *eae* variants. Typing of LEE genes is of importance in the characterization of A/E pathogens since different variants of these genes have been associated with host specificity (62,179). The number of variants detected (11 LEE types, Paper IV) was comparable with that reported from STEC and EPEC strains in Spain recently (73). However, the use of different hybridization probes (20-25-mer probes), as well as different type strains for some LEE variants, makes comparison of LEE types between the two studies difficult. In contrast to previous reports (35,73) several of the *espA*, *espB*, *tir* and *eae* variants could be observed in combination with more than just one variant of the other LEE genes. This finding is consistent, as recently suggested, with horizontal exchange between different strains not only of entire LEE sequences, but also of smaller gene elements within the LEE (32). The link between certain LEE types and OI-122 genes shown in this study (Paper IV) may be due to close proximity of the genomic islands in the chromosome of these atypical EPEC strains, similar to what has been shown for O103:H2 EHEC strains (105).

The finding of genes in a considerable proportion of the atypical EPEC strains usually linked to the EHEC pathotype (Paper III and IV) is consistent with evidence from epidemiological and experimental studies showing that atypical EPEC may convert to, or be a conversion from, the EHEC pathotype through acquisition or loss of *stx* genes (18,236,242). Such a relationship is also supported by the detection of STs belonging to the phylogenetic lineages EHEC1 and EHEC2 among the atypical EPEC strains in the study (Paper IV). It is also interesting to observe considerable variability in the content of plasmid genes between different strains (Paper IV). This observation is in agreement with reports of extensive heterogeneity of large plasmids in STEC (30) and in attaching and effacing (A/E) *E. coli* of animal origin (4). This heterogeneity makes reliable detection of such plasmids with DNA based methods difficult without testing for several or all the genes encoded on the plasmid.

Finally, serotyping showed that few strains belonged to classical EPEC serogroups (O55 and O125ac). The majority of strains belonged to non-EPEC serogroups, or were non-typable or rough (Paper IV). Similarly, many different H types were present among the 42 strains were an H type was identified. All the above results confirm the previously reported heterogeneity among atypical EPEC strains (52,80,190,236).

6 Main conclusions

- EPEC was frequently isolated from Norwegian children both with and without diarrhoea, but was rarely observed in association with severe diarrhoea.

- The majority of EPEC strains were characterized as atypical EPEC.

- Serotyping does no longer appear to be a useful method for the diagnosis of atypical EPEC

- Phylogenetic and genetic characterization showed extensive diversity between the atypical EPEC strains in the study.

- Several virulence genes in atypical EPEC were statistically associated with diarrhoea.

- The atypical EPEC strains could be classified in two virulence groups based on their content of the virulence genes statistically linked with diarrhoea.

- Virulence group II strains, which all belonged to phylogenetic group B2, were not associated with diarrhoea, but appeared to be associated with protracted intestinal colonization.

- Virulence group I strains, which belonged to the phylogenetic groups A, B1 and D, were significantly associated with diarrhoea, and were isolated both from children with acute and protracted diarrhoea.

7 Future perspectives

- Further verification of the pathogenicity of the virulence genes identified in this study in experimental volunteer studies.

- Clarification of the role of atypical EPEC belonging to virulence group I as diarrheagenic agent among children in developing countries.

- Further study of the role of atypical EPEC belonging to virulence group II; diarrheagenic potential depending on host factors, and study of specific factors enabling protracted intestinal colonization.

8 Errata

In Table 1 of both Papers I and II the nucleotide sequences for the PCR primers for the two variants of the *stx* gene are not correct. The correct primer sequences (5'-3') are as follows: SLT-I F: AAATCGCCATTCGTTGACTACTTCT, SLT-I R: TGCCATTCTGGCAACTCGCGATGCA, SLT-II F: CAGTCGTCACTCACTGGTTTCATCA, and SLT-II R: GGATATTCTCCCCACTCTGACACC.

In Paper III the *P*-value =0.0008 for the association between the *efa1/lifA* gene and diarrhoea given in the abstract in incorrect. The value *P*=0.0002 listed in Table 2 is correct.

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24. Dagfinn Berntzen: ANALYSIS AND MANAGEMENT OF EXPERIMENTAL AND CLINICAL PAIN.
25. Odd Arnold Kildahl-Andersen: PRODUCTION AND CHARACTERIZATION OF MONOCYTE-DERIVED CYTOTOXIN AND ITS ROLE IN MONOCYTE-MEDIATED CYTOTOXICITY.
26. Ola Dale: VOLATILE ANAESTHETICS.

1987

27. Per Martin Kleveland: STUDIES ON GASTRIN.
28. Audun N. Øksendal: THE CALCIUM PARADOX AND THE HEART.
29. Vilhjalmur R. Finsen: HIP FRACTURES

1988

30. Rigmor Austgulen: TUMOR NECROSIS FACTOR: A MONOCYTE-DERIVED REGULATOR OF CELLULAR GROWTH.
31. Tom-Harald Edna: HEAD INJURIES ADMITTED TO HOSPITAL.
32. Joseph D. Borsi: NEW ASPECTS OF THE CLINICAL PHARMACOKINETICS OF METHOTREXATE.

33. Olav F. M. Sellevold: GLUCOCORTICOIDS IN MYOCARDIAL PROTECTION.
34. Terje Skjærpe: NONINVASIVE QUANTITATION OF GLOBAL PARAMETERS ON LEFT VENTRICULAR FUNCTION: THE SYSTOLIC PULMONARY ARTERY PRESSURE AND CARDIAC OUTPUT.
35. Eyvind Rødahl: STUDIES OF IMMUNE COMPLEXES AND RETROVIRUS-LIKE ANTIGENS IN PATIENTS WITH ANKYLOSING SPONDYLITIS.
36. Ketil Thorstensen: STUDIES ON THE MECHANISMS OF CELLULAR UPTAKE OF IRON FROM TRANSFERRIN.
37. Anna Midelfart: STUDIES OF THE MECHANISMS OF ION AND FLUID TRANSPORT IN THE BOVINE CORNEA.
38. Eirik Helseth: GROWTH AND PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN GLIOMAS AND BRAIN METASTASES - WITH SPECIAL REFERENCE TO TRANSFORMING GROWTH FACTOR BETA AND THE EPIDERMAL GROWTH FACTOR RECEPTOR.
39. Petter C. Borchgrevink: MAGNESIUM AND THE ISCHEMIC HEART.
40. Kjell-Arne Rein: THE EFFECT OF EXTRACORPOREAL CIRCULATION ON SUBCUTANEOUS TRANSCAPILLARY FLUID BALANCE.
41. Arne Kristian Sandvik: RAT GASTRIC HISTAMINE.
42. Carl Bredo Dahl: ANIMAL MODELS IN PSYCHIATRY.

1989

43. Torbjørn A. Fredriksen: CERVICOGENIC HEADACHE.
44. Rolf A. Walstad: CEFTAZIDIME.
45. Rolf Salvesen: THE PUPIL IN CLUSTER HEADACHE.
46. Nils Petter Jørgensen: DRUG EXPOSURE IN EARLY PREGNANCY.
47. Johan C. Ræder: PREMEDICATION AND GENERAL ANAESTHESIA IN OUTPATIENT GYNECOLOGICAL SURGERY.
48. M. R. Shalaby: IMMUNOREGULATORY PROPERTIES OF TNF-α AND THE RELATED CYTOKINES.
49. Anders Waage: THE COMPLEX PATTERN OF CYTOKINES IN SEPTIC SHOCK.
50. Bjarne Christian Eriksen: ELECTROSTIMULATION OF THE PELVIC FLOOR IN FEMALE URINARY INCONTINENCE.
51. Tore B. Halvorsen: PROGNOSTIC FACTORS IN COLORECTAL CANCER.

1990

52. Asbjørn Nordby: CELLULAR TOXICITY OF ROENTGEN CONTRAST MEDIA.
53. Kåre E. Tvedt: X-RAY MICROANALYSIS OF BIOLOGICAL MATERIAL.
54. Tore C. Stiles: COGNITIVE VULNERABILITY FACTORS IN THE DEVELOPMENT AND MAINTENANCE OF DEPRESSION.
55. Eva Hofsli: TUMOR NECROSIS FACTOR AND MULTIDRUG RESISTANCE.
56. Helge S. Haarstad: TROPHIC EFFECTS OF CHOLECYSTOKININ AND SECRETIN ON THE RAT PANCREAS.
57. Lars Engebretsen: TREATMENT OF ACUTE ANTERIOR CRUCIATE LIGAMENT INJURIES.
58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

1991

65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.

68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
76. Harold C Sexton: STUDIES RELATING TO THE TREATMENT OF SYMPTOMATIC NON-PSYCHOTIC PATIENTS.
77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
78. Terje Johannessen: CONTROLLED TRIALS IN SINGLE SUBJECTS.
79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

1993

82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

1994

92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

1995

104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.

109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANSER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

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124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

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132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.

139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

1999

141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.

168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM

198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL

224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
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