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High prevalence of atypical enteropathogenic *Escherichia coli* (EPEC) in Norwegian children with diarrhoea

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The aim of the present study was to investigate the relative contribution of enteropathogenic *Escherichia coli* (EPEC) as a cause of infectious diarrhoea in Norwegian children. Data from faecal specimens from children <2 years old with diarrhoea during the year 2001 were analysed. *E. coli* isolates with the attaching and effacing genotype (*eae*$^+$) were examined for the presence of the bundle-forming pilus (*bfpA*) and Shiga toxin genes by PCR, and for genetic relatedness by PFGE. During the 1-year period, 598 specimens from 440 patients <2 years old were analysed. Potential enteric pathogens were identified in 124 patients (28·2 %). EPEC was the most frequently identified agent (44 patients), followed by rotavirus (41 patients), *Campylobacter jejuni* (17 patients) and adenovirus (17 patients). All other agents were detected in five patients or less. Only one of the *eae*$^+$ *E. coli* isolates was classified as typical EPEC (*bfpA*$^+$). Among the 43 isolates that were classified as atypical EPEC (*bfpA*$^-$), eight strains belonged to EPEC serogroups, whereas the majority of strains ($n = 35$) were not agglutinated by EPEC antisera. None of the EPEC isolates were genetically related. This study demonstrates that atypical EPEC of non-EPEC serogroups is highly prevalent among Norwegian children with diarrhoea.

INTRODUCTION

Infectious diarrhoea is one of the world's leading causes of morbidity and mortality, resulting in about two million deaths per year (World Health Organization, 2002). The majority of cases of serious diarrhoea occur among children in developing countries (World Health Organization, 1995). In contrast to third-world countries, paediatric infectious diarrhoea is rarely fatal in industrialized countries. It does, however, frequently cause visits to physicians, as well as lost work-time for parents, and therefore gives rise to considerable medical expense (Avendaño *et al.*, 1993).

After the discovery of enteropathogenic *Escherichia coli* (EPEC) as a cause of childhood diarrhoea in 1945, EPEC was diagnosed frequently as a cause of paediatric diarrhoea in developed countries over the next three decades (Levine & Edelman, 1984). Then, for unknown reasons, the incidence of EPEC declined in this part of the world (Nataro & Kaper, 1998).

Abbreviations: A/E, attaching and effacing; EAF, EPEC adherence factor; EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *E. coli*.

For many years, diagnosis of EPEC was based on O : H serotype identification (Levine & Edelman, 1984). During the last two decades, the pathogenic mechanism of EPEC infection has been clarified (Nataro & Kaper, 1998); this has resulted in a change in diagnostic methods from serogrouping to phenotypic and genotypic methods.

The central mechanism of EPEC pathogenesis is a lesion called 'attaching and effacing' (A/E), which is characterized by intimate adherence of bacteria to the intestinal epithelium (Vallance & Finlay, 2000). The *eae* gene, which is located in the 'locus of enterocyte effacement' (LEE) pathogenicity island, and the *bfpA* gene, located on a plasmid called the EPEC adherence factor (EAF), have both been used for identification of EPEC and for subdivision of this group of bacteria into typical and atypical strains (Nataro & Kaper, 1998). *E. coli* strains with the A/E genotype (*eae*$^+$) that harbour the EAF plasmid (*bfpA*$^+$) are classified as 'typical EPEC'; most of these strains belong to certain O : H serotypes. Strains with the A/E genotype that do not posses the EAF plasmid (*bfpA*$^-$) are classified as 'atypical EPEC'. *E. coli* strains with the *eae*$^+$ genotype that harbour Shiga toxin genes (*stx1* and/or *stx2*) are classified as enterohaemorrhagic *E. coli* (EHEC).

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Recently, after the introduction of new molecular diagnostic methods, EPEC has again been reported from several developed countries (Scotland *et al.*, 1991; Rademaker *et al.*, 1993; Morelli *et al.*, 1994; Forestier *et al.*, 1996; Giammanco *et al.*, 1996; Bokete *et al.*, 1997; Pelayo *et al.*, 1999; Tompkins *et al.*, 1999; Svenungsson *et al.*, 2000; Keskimäki *et al.*, 2001; Knutton *et al.*, 2001). The aim of the present study was to investigate the relative contribution of EPEC among other identifiable causes of infectious diarrhoea in Norwegian children.

METHODS

Patients. Data from analysis of clinical stool specimens received by the laboratory during the year 2001 from children <2 years of age were reviewed. Information on prolonged diarrhoea (⩾2 weeks duration at the time of examination by physician) was collected either from hospital records (hospitalized patients) or from the referral form (outpatients).

Culture and identification methods. Culture and biochemical identification of bacterial enteropathogens were done according to standard microbiological methods.

Bacterial growth on a primary-culture plate was agglutinated with polyspecific O-antisera Anti coli I (O26, O44, O114, O125, O142 and O158), Anti coli II (O55, O86, O111, O119, O126, O127 and O128) and Anti coli III (O157) according to the manufacturer's instructions (Sifin). Strains that were agglutinated by polyspecific antisera were retested by using monospecific O : K antisera at the Norwegian Institute of Public Health (Sifin and in-house antisera).

PCR. Screening for the presence of *eae*$^+$ and *stx*$^+$ bacterial strains was based on PCR of 2–3 cm streaks of bacterial growth on primary-culture plates. If the streak was PCR-positive, four distinct colonies were subcultured from the same primary-culture plate (stored at 4 °C) and retested. Isolates with the *eae*$^+$ genotype were stored at −70 °C until they were analysed for the presence of the plasmid-encoded bundle-forming pilus (*bfpA*) gene.

Bacterial growth from streaks was suspended in 4 ml physiological saline and further diluted 1 : 100. From agar plates with pure subculture, one bacterial colony was suspended in 1 ml physiological saline. Lysis of bacterial cells in suspension was done by heating at 94 °C for 15 min. Amplification of the *eae* gene was performed in a total volume of 50 µl, which contained 50 µM each dNTP, 0·5 µM each primer, 5 µl 10× PCR buffer (Roche), 1·5 mM $MgCl_2$, 1 U AmpliTaq Gold polymerase (Roche), 0·025 % BSA and 2 µl bacterial lysate as template. Other amplification reactions were carried out under similar conditions, except for the following: for detection of the *stx1* and *stx2* genes, multiplex amplification was carried out with 100 µM (each) dNTP and no BSA, and amplification of the *ial* gene of enteroinvasive *E. coli* was performed without BSA. Primers and cycling conditions are listed in Table 1. For all amplification reactions, the mixture was heated at 94 °C for 15 min prior to thermocycling. The mixture was held at 72 °C for 7 min after the final cycle before cooling at 4 °C. Amplified products were analysed by 2 % agarose gel electrophoresis and visualized by staining with ethidium bromide. As positive controls for PCR, clinical isolates of *E. coli* O157 : H7 (*eae*, *stx1* and *stx2*), *E. coli* strain B171 (*bfpA*) and *E. coli* O124 : H30 (*ial*) were used.

Genotyping of EPEC strains. *E. coli* isolates with the *eae*$^+$ genotype were analysed for genetic relatedness by PFGE according to standard methods. *Xba*I as the restriction enzyme and the following electrophoretic conditions were used: 14 °C, linear ramp of 5–60 s over 24 h, 120° switch angle and a gradient of 6·0 V cm^{-1}. Analysis of fragments was done by using Fingerprinting II software (Bio-Rad) and by visual inspection with the criteria of Tenover *et al.* (1995). Similarities between strains were analysed by the UPGMA clustering method, using the Dice coefficient at 2·5 % tolerance.

Other methods. Stool specimens were examined for adenovirus by enzyme immunoassay (IDEIA; Dako), viral cell culture and PCR, and for rotavirus antigens by enzyme immunoassay (IDEIA; Dako). In addition to the routine analysis above, some specimens were tested for

Table 1. PCR for detection of enteropathogenic, enterohaemorrhagic and enteroinvasive *E. coli*

Abbreviations: *bfpA*, bundle-forming pilus gene; *eae*, attaching and effacing-associated gene; EIEC, enteroinvasive *E. coli*; *ial*, invasion-associated locus of EIEC; *stx*, Shiga toxin gene. Forward and reverse primers are indicated by F and R, respectively.

Category	Target	Nucleotide sequence (5′→3′)	Amplification conditions	No. cycles	Size of amplified product (bp)	References
EPEC (all)	*eae*	F, GTGGCGAATACTGGCGAGACT; R, CCCCATTCTTTTTCACCGTCG	94 °C (1 min), 60 °C (1 min), 72 °C (2 min)	35	891	Gannon *et al.* (1993)
EHEC	*stx1*	F, AAATCGCCATTCGTTGACTACTTCT; R, CAGTCGTCACTCACTGGTTTCATCA	94 °C (1 min), 64 °C (1 min), 72 °C (15 s)	35	370	Brian *et al.* (1992)
	stx2	F, TGCCATTCTGGCAACTCGCGATGCA; R, GGATCTTCTCCCCACTCTGACACC	94 °C (1 min), 64 °C (1 min), 72 °C (15 s)	35	283	Brian *et al.* (1992)
EPEC (typical)	*bfpA*	F, AATGGTGCTTGCGCTTGCTGC; R, GCCGCTTTATCCAACCTGGTA	94 °C (1 min), 60 °C (1 min), 72 °C (2 min)	30	326	Gunzburg *et al.* (1995)
EIEC	*ial*	F, CTGGATGGTATGGTGAGG; R, GGAGGCCAACAATTATTTCC	94 °C (1 min), 43 °C (2 min), 72 °C (3 min)	26	320	Schoolnik (1993)

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other enteropathogenic agents when suspected clinically: by electron microscopy (calicivirus and astrovirus, $n = 2$), by enzyme immunoassay for *Giardia lamblia* (CELISA, Cellabs; $n = 105$), by microscopy for other parasites ($n = 65$) or by PCR for enteroinvasive *E. coli* ($n = 1$).

RESULTS AND DISCUSSION

Patients

During the 12-month study period, 598 stool specimens from 440 children <2 years old were examined. Among the study population, 135 children (30·7 %) were <6 months old, 99 (22·5 %) were 6–11 months old and 206 (46·8 %) were 12–23 months of age, with a sex distribution of 241 male (54·8 %) and 199 female (45·2 %) patients.

All potential enteropathogens

A potential or established microbial pathogen was detected in 124 patients (28·2 %), either as a single agent (114 patients) or in combination with other agents (10 patients). EPEC was the most frequently identified agent (44 patients), followed by rotavirus (41 patients), *Campylobacter jejuni* (17 patients) and adenovirus (17 patients) (Table 2). All other agents were each diagnosed in five or fewer patients.

Table 2. Potential and established enteropathogens isolated from Norwegian children <2 years old with diarrhoea ($n = 440$)

Isolates	No. patients (%)
EPEC (*eae*$^+$, *stx*$^-$)	44 (10·0)
Alone	39 (8·9)
Combined with other pathogens:	
+*Campylobacter jejuni*	2 (0·5)
+Rotavirus	1 (0·2)
+Rotavirus + calicivirus*	1 (0·2)
+Adenovirus	1 (0·2)
EHEC (*stx*$^+$)†	4 (0·9)
Alone	1 (0·2)
Combined with *Campylobacter jejuni*	3 (0·7)
Non-*E. coli* pathogens	76 (17·3)
Alone:	
Rotavirus	39 (8·9)
Adenovirus	16 (3·6)
Campylobacter jejuni	11 (2·5)
Salmonella spp.	3 (0·7)
Shigella spp.	1 (0·2)
*Giardia lamblia**	2 (0·5)
Yersinia enterocolitica	1 (0·2)
Calicivirus*	1 (0·2)
Combinations of pathogens:	
Salmonella sp. + *Campylobacter jejuni*	1 (0·2)
Salmonella sp. + *Shigella* sp.	1 (0·2)
Total	124 (28·2)

*Investigation done only when suspected from clinical history.

†Three EHEC isolates were *eae*$^+$.

EPEC

PCR from growth on the primary-culture plate was positive for the *eae* gene in 65 patients (14·8 %). In 18 of these patients, however, an *eae*$^+$ organism could not be recovered after subculture of four colonies. We chose not to record these 18 patients as EPEC-positive. As these patients probably had a low number of *eae*$^+$ organisms, they were less likely to have symptomatic disease due to this organism (Nataro & Kaper, 1998). In three patients, *E. coli* isolates were classified as EHEC due to the presence of the *stx* genes. Among the remaining 44 patients from whom EPEC was isolated, 39 had EPEC as their only diagnosed potential pathogen and five had this organism in combination with other enteropathogenic agents (Table 2). Only one patient (0·2 %) had typical EPEC (a *bfpA*$^+$ isolate), whereas 43 patients had atypical EPEC (*bfpA*$^-$ isolates).

Surprisingly, EPEC was the most frequently identified potential pathogen in this study, identified in 44 of 440 patients (10·0 %). This is a high prevalence compared to most other reports (Forestier *et al.*, 1996; Bokete *et al.*, 1997; Tompkins *et al.*, 1999; Svenungsson *et al.*, 2000; Keskimäki *et al.*, 2001; Knutton *et al.*, 2001; Vieira *et al.*, 2001). Typical EPEC (*bfpA*$^+$) was uncommon, a finding that is consistent with other reports of low prevalence of typical EPEC in Europe (Rademaker *et al.*, 1993; Morelli *et al.*, 1994; Forestier *et al.*, 1996; Giammanco *et al.*, 1996; Scotland *et al.*, 1996; Svenungsson *et al.*, 2000; Keskimäki *et al.*, 2001; Knutton *et al.*, 2001; Paciorek, 2002) and North America (Bokete *et al.*, 1997; Caeiro *et al.*, 1999). Atypical EPEC, which constituted all but one isolate in this study, seemed to be widely spread among children with diarrhoea in Norway.

Patients admitted to hospital

Altogether 135 patients in this study (30·7 %) were admitted to hospital. Potential enteropathogens were detected with similar frequency in admitted patients and outpatients ($n = 38$, 28·1 %). As expected, rotavirus was the most frequently identified agent in this group of patients, whereas atypical EPEC was identified in four children (3·0 %). None of the patients with EPEC who were admitted to hospital had severe acute gastroenteritis, in contrast to 16 of the 18 patients with rotavirus infection (88·9 %). These data support the hypothesis that atypical EPEC rarely causes acute serious gastroenteritis among children in Norway.

Disease duration

Prolonged duration (⩾2 weeks) of gastroenteritis symptoms at the time of examination was recorded in 12 of 38 patients (31·6 %) with atypical EPEC. The fact that almost one-third of patients with atypical EPEC had a protracted course of diarrhoea was surprising and raises the question of whether atypical EPEC can cause prolonged diarrhoea. This finding could, however, also be due to the selection of patients from whom specimens were sent to the laboratory. This should be clarified in a prospective study.

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Genetic relatedness among EPEC isolates

Macrorestriction patterns of 38 EPEC strains were analysed by PFGE. Typically, *Xba*I digestion yielded between eight and twelve restriction fragments, whilst two strains were non-typable. Among the 36 typable strains, each displayed a unique genotypic pattern (data not shown). The genetic diversity of strains in this study demonstrates that the high prevalence of EPEC in the paediatric population in Norway is not caused by clonal spread, but rather that there are several distinct *eae*$^+$ *E. coli* strains present in this patient population.

Other features

A diagnosis of EPEC was significantly more common among children who were 12–23 months old (33/206 patients, 16·0 %) than in those who were <12 months of age (11/234, 4·7 %; χ^2 test, $P < 0{\cdot}001$). Only four of 135 patients (3·0 %) who were <6 months old were diagnosed with EPEC.

A marked seasonal distribution was seen in the isolation rate of EPEC strains. Twenty-one (47·7 %) EPEC isolates were identified during the 3-month period from July to September, in contrast to rotavirus, which was diagnosed mainly during the late-winter season (data not shown).

Among the four patients who were infected with EHEC, none developed haemolytic uraemic syndrome. All EHEC isolates belonged to serogroups other than O157.

Atypical EPEC of non-EPEC serogroups

Strains with the *eae*$^+$ genotype that lack the EAF plasmid (*bfpA*$^-$) and Shiga toxin genes (*stx*$^-$) are classified as atypical EPEC by some authors only if they belong to recognized EPEC serogroups (Trabulsi *et al.*, 2002). Strains with the *eae*$^+$ genotype that do not belong to EPEC serogroups were reported recently to constitute a heterogeneous group that may resemble EHEC, enteroaggregative *E. coli* or diffusely adherent *E. coli* (Vieira *et al.*, 2001; Trabulsi *et al.*, 2002). However, most such strains have virulence profiles similar to those of atypical strains that belong to EPEC serogroups (Vieira *et al.*, 2001); at least some of these strains are able to induce A/E lesions on human intestinal biopsies (Knutton *et al.*, 2001; Vieira *et al.*, 2001). We therefore included all *eae*$^+$, *stx*$^-$ and *bfpA*$^-$ *E. coli* strains in the category of atypical EPEC. Among the 43 atypical EPEC strains in our study, eight (1·8 %) belonged to recognized EPEC serogroups, whereas the majority of strains ($n = 35$, 8·0 %) were not agglutinated by EPEC antisera. Classification of EPEC strains diagnosed during the 1-year period is presented in Table 3. Atypical EPEC of non-EPEC serogroups was diagnosed more frequently in this study than the value of 1–6 % that has been reported from other parts of Europe (Knutton *et al.*, 1991, 2001; Forestier *et al.*, 1996; Keskimäki *et al.*, 2001) and North America (Bokete *et al.*, 1997).

The role of atypical EPEC in endemic diarrhoea has not been established conclusively. Absence of the EAF plasmid (*bfpA*$^-$) probably results in reduced virulence (Levine *et al.*, 1985), but some atypical strains have been shown to have other virulence factors that may compensate for this (Scaletsky *et al.*, 2002; Trabulsi *et al.*, 2002). There have been reports of atypical EPEC associated with both endemic diarrhoea (Pedroso *et al.*, 1993; Bokete *et al.*, 1997; Vieira *et al.*, 2001) and outbreaks (Viljanen *et al.*, 1990; Hedberg *et al.*, 1997; Yatsuyanagi *et al.*, 2002). To elucidate the role of atypical EPEC in diarrhoeal disease among Norwegian children, both epidemiological studies and further investigation of the virulence properties of these strains are warranted.

Table 3. Classification of *eae*$^+$ and *stx*$^-$ *E. coli* isolates identified among 440 Norwegian children <2 years old with diarrhoea

Serogroup	*E. coli* (*eae*$^+$, *stx*$^-$)	
	bfpA$^+$	*bfpA*$^-$
EPEC serogroup*	1†	8‡
Non-EPEC serogroup	0	35
Total	1	43

*As defined by the World Health Organization (1987).

†Serogroup O111 : K58.

‡Serogroups O26 : K60 (four isolates), O55 : K59 (one isolate), O127 : K63 (two isolates) and O128 : K67 (one isolate).

In conclusion, atypical EPEC is prevalent among small children with diarrhoea in Norway and was diagnosed in 9·8 % of patients in this study. Typical EPEC was rare and most atypical EPEC strains did not belong to recognized EPEC serogroups. Few EPEC isolates were diagnosed in hospitalized patients, supporting the hypothesis that this group of organisms does not often cause serious acute disease.

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