Detection of enterovirulent *Escherichia coli* associated with diarrhoea in Seville, Southern Spain, with non-radioactive DNA probes

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Summary. To assess the role of diarrhoeagenic *Escherichia coli* in Southern Spain, faecal samples from 135 patients with diarrhoea and 40 healthy subjects from Seville, Andalusia, were investigated. In this prospective study, enterovirulent *E. coli* were identified by hybridisation with five non-radioactive DNA probes specific for enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and verocytotoxin-producing *E. coli* (VTEC). Probe-positive strains were isolated from four patients (3%) with diarrhoea and from none of the healthy controls. Two patients harboured ETEC and two patients had EPEC probe-positive strains in their faeces. No VTEC were isolated during this study. *Salmonella* spp. were the most frequently identified enteric pathogens, accounting for 10% of the cases, followed by *Campylobacter jejuni* (3%) and diarrhoeagenic *E. coli* (3%). This study indicates that enterovirulent *E. coli* play a modest role in the aetiology of diarrhoea among the indigenous population of Southern Spain.

Introduction

Escherichia coli is generally regarded as a benign commensal of the intestinal tract in man. However, within this species, there are pathogenic strains that are associated with intestinal infections.¹ These strains may be termed enterovirulent *E. coli* (EVEC).² Studies of the pathogenic mechanisms involved permit the separation of EVEC into four groups which differ in epidemiology and clinical picture.^{1,2} Enterotoxigenic *E. coli* (ETEC) causes diarrhoea in adults and children in developing countries and in travellers to these areas.^{3–6} The role of ETEC in diarrhoea amongst the indigenous population of European countries has been less clearly defined. Verocytotoxic *E. coli* (VTEC) has been identified as a significant cause of haemorrhagic colitis and haemolytic uraemic syndrome in the United Kingdom, North America, Czechoslovakia and Germany, in occasional outbreaks as well as in sporadic cases.^{7–12} Enteropathogenic *E. coli* (EPEC) is associated with outbreaks of acute diarrhoea in neonates.^{13,14} Hill *et al.* have recently reported individual cases of serious chronic diarrhoea associated with EPEC in children after the neonatal period.¹⁵ The last group is formed by the enteroinvasive *E. coli* (EIEC). The prevalence of EIEC is low.¹

In the past, identification of *E. coli* strains causing diarrhoea was possible only by testing culture supernates in tissue culture in combination with serotyping. The use of DNA probes has greatly facilitated detection. In a previous study we described a simplified and accurate hybridisation assay for detecting ETEC, VTEC and EPEC directly in stool smears with a set of five non-radioactive DNA fragment probes.¹⁶ Here we report the results of a prospective study of the frequency of EVEC in subjects with and without diarrhoea in Seville, Southern Spain.

Materials and methods

Study population

Stool specimens from 135 subjects (100 children and 35 adults) with diarrhoea and control faecal specimens from 40 subjects (14 children and 26 adults) without diarrhoea were collected in Seville, Andalusia, during 3 weeks in September 1991. Faecal samples were obtained from in-patient and out-patient settings.

Laboratory studies

For the identification of EVEC c. 1 g of faeces was placed in transport medium containing cysteine hydrochloride (Fluka, Switzerland), peptone (Oxoid)

and yeast extract (Merck, Germany); after homogenisation, these were stored at 4°C for a maximum of 15 days. Diarrhoeal specimens were cultured for common intestinal pathogens including *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Vibrio* spp., *Aeromonas* spp., *Plesiomonas* spp., *Campylobacter* spp. and were examined for *Giardia lamblia* by standard bacteriological techniques. Diarrhoeal samples and control samples were cultured for EVEC. Samples (100 µl) of a 10² or 10³ dilution (w/v) of faeces in transport medium were plated out on MacConkey agar plates without neutral red. After overnight incubation at 30°C, bacteria were replica-plated on to Z-probe filters (BioRad Laboratories). Bacteria were lysed and their DNA was fixed on the filters as described previously.¹⁶

DNA probe assay

Recombinant plasmids pWD299, pSLM004 and pJPN16 encoding for ETEC LT,¹⁷ ETEC STaII¹⁸ and EPEC¹⁹ respectively, were used as donors of the probes and were kindly provided by Dr P. J. Sansonetti, Institut Pasteur, Paris. Strains 6OR746 and 6OR363, carrying recombinant plasmids encoding VT1- and VT2-specific sequences to identify VTEC^{20,21} were kindly donated by Dr H. R. Smith, Central Public Health Laboratory, London. Probes were labelled with digoxigenin (Boehringer, Mannheim, Germany) by the polymerase chain reaction (PCR). The primers used for the generation of the probes, their length, the appropriate hybridisation temperatures, the composition of the PCR reaction mixture and hybridisation and detection methods have been described previously.¹⁶

Serotyping

Probe-positive *E. coli* strains were serotyped for the presence of O and K antigens by means of a mechanised microtechnique with all available O (O1–171) antigens and O:K antisera against the K antigens usually associated with each O antigen.²²

Antibiotic resistance patterns

Antibiotic susceptibility of probe-positive isolates was determined by an agar diffusion method with ciprofloxacin, amoxycillin, co-amoxycylav doxycycline and co-trimoxazole tablets (Rosco, Taastrup, Denmark).

Results

The isolation rates of enteric pathogens among patients with diarrhoea are shown in the table. EVEC

Table. Frequency of isolation of enteropathogenic bacteria from faeces of 135 patients with diarrhoea in Seville, Southern Spain

Enteropathogens	Number (%) of patients
<i>Salmonella</i> spp.	14 (10%)
Enterovirulent <i>E. coli</i>	4 (3%)
<i>C. jejuni</i>	4 (3%)
<i>G. lamblia</i>	1 (0.7%)
Total	23 (17%)

were isolated from four (3%) patients with diarrhoea and from none of the control group.

One or more enteropathogenic micro-organisms were isolated from 23 (17%) of 135 faecal samples from subjects with diarrhoea. *Salmonella* spp. were detected from 14 (10%) patients, (10 children and four adults) and *C. jejuni* from four (3%) children. *G. lamblia* was detected in one (0.7%) patient. Three ETEC strains were isolated from two children with diarrhoea. One ETEC strain produced heat-labile toxin (ETEC LT) and was of serotype O25. Two different ETEC strains producing heat-stable toxin (ETEC ST) were isolated from another child; they belonged to the serotypes O115 and O15. One child and one adult with diarrhoea harboured EPEC probe-positive strains in their faeces. The serotypes of these strains were O127 and O23K18, respectively. No other *E. coli* serotypes were detected in the faecal samples from which the ETEC LT (serotype O25) and the EPEC (serotype O127) strains were isolated. On the other hand, the ETEC ST (serotypes O15 and O115) and the EPEC (serotype O23K18) strains comprised only 15–30% of the *E. coli* flora tested. During the study period, no VTEC strains were identified.

With respect to antibiotic susceptibility patterns, the EPEC strains and the ETEC LT strain were susceptible to all antibiotics tested. Both ETEC ST strains were resistant to amoxycillin and co-trimoxazole.

Discussion

Many studies have identified ETEC as the most common cause of travellers' diarrhoea in endemic areas in Africa, Asia and Latin America.^{5, 23, 24} In a recent study among Swedish travellers with diarrhoea, the isolation rate of ETEC was 20% in travellers visiting developing countries *versus* 13% in those travelling to Southern Europe.²⁵ As far as we know, the importance of ETEC as a cause of diarrhoea in the indigenous population of Southern Europe has been less well defined. In this prospective study, the isolation rate of ETEC was 2% in 100 children with diarrhoea in Seville in September 1991 (mean daily temperature 38°C). The affected children were 6 and 7 years old. Blanco *et al.* reported an isolation rate of 3.9% for ETEC in children with diarrhoea in North-Western

Spain.²⁶ In their study, ETEC were isolated more frequently from children with diarrhoea who were < 1 month of age than from older children. The investigators suggested that ETEC constituted an important cause of neonatal diarrhoea and, moreover, that ETEC infections may manifest as nosocomial outbreaks. We did not test faecal samples from neonates. However, when we compare our age group to the similar age group of Blanco, the isolation rates of ETEC are in the same order of magnitude. Two EPEC probe-positive strains were detected. The EPEC strain of serotype O127 was isolated from a child. This serotype belongs to classical EPEC serotypes and is associated with diarrhoea in children.¹ The EPEC of serotype O23K18 was detected in the stool of an adult with diarrhoea and does not belong to the classical serotypes of the EPEC species. Interestingly, we have isolated EPEC probe-positive strains with the same serotype from three adults with diarrhoea and from one adult without symptoms during an investigation

into the role of diarrhoeagenic *E. coli* in the Netherlands (unpublished data).

In the present study, filters with 50–500 *E. coli* colonies were hybridised with probes.¹⁶ This method provides rough information about the quantity of pathogens present in the stool. The two ETEC ST strains and one of the EPEC isolates were not present as pure cultures in the stool and would not have been diagnosed had only one-to-five *E. coli* colonies, isolated at random from each sample, been examined. In this study, the isolation rate of *Salmonella* spp. was 10%. Similar rates were demonstrated in previous studies.^{26,27} In agreement with these results, *Salmonella* spp. were identified in stools of Swedish travellers more often (26%) than ETEC (13%) after travel to Southern Europe.²⁵

This study indicates that enterovirulent *E. coli* play a modest role in the aetiology of diarrhoea in the indigenous population of Seville. The frequency was 3% and was similar to the isolation rate of *C. jejuni*.

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