Enterotoxigenic *Escherichia coli* associated with infant diarrhoea in Galicia, north-western Spain

J. BLANCO*, E. A. GONZÁLEZ*, M. BLANCO*, J. I. GARABAL*, M. P. ALONSO*†, S. FERNÁNDEZ*, R. VILLANUEVA‡, A. AGUILERA‡, M. A. GARCIA‡, J. TORRES§, A. REY§, W. H. JANSEN¶ and P. A. M. GUINÉE¶

*Departamento de Microbiología y Parasitología, Facultad de Veterinaria, Universidad de Santiago, Lugo, †Sección de Bacteriología, Residencia Sanitaria Juan Canalejo, La Coruña, ‡Servicio de Microbiología, Hospital Xeral de Galicia, Santiago de Compostela, §Unidad de Microbiología, Hospital Xeral, Lugo, Spain, and ¶Department of Bacteriology, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands

Summary. To assess the role of enterotoxigenic *Escherichia coli* (ETEC) in infantile diarrhoea, 482 children with diarrhoea and 103 healthy controls, from three localities of Galicia, north-western Spain, were investigated between 1985 and 1988. Rotavirus (37·3%) and *Salmonella* spp. (12·8%) were the most common causal agents, followed by ETEC (3·9%), *Campylobacter jejuni* (2·3%), *Shigella* spp. (0·9%) and *Yersinia enterocolitica* (0·5%). ETEC were significantly more frequently isolated from children with diarrhoea who were under 1 month of age (26·5%) than from older diarrhoeic children (2·2%) ($p < 0·001$) or from healthy children who were under 1 month of age (0%) ($p < 0·05$). Among children who harboured ETEC, five of the nine children under 1 month of age developed diarrhoea in hospital, whereas none of the 10 children over 1 month of age did so. Seventeen ETEC isolates produced heat-stable enterotoxin (STa) only, four produced only heat-labile enterotoxin (LT), and two produced both toxins. Colonisation factor antigens CFA/I and CFA/II were detected in 11 (55·0%) of the 20 ETEC isolates that remained enterotoxigenic after maintenance in the laboratory. Most ETEC isolates belonged to serotypes O153:K—:H45 (nine STa⁺ CFA/I⁺ isolates), O27:K—:H7 (three STa⁺ isolates) or O6:K15:H16 (two LT⁺ STa⁺ CFA/II⁺ isolates). Our results suggest that ETEC constitute an important cause of neonatal diarrhoea in this part of Spain.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are responsible for 20–50% of cases of endemic diarrhoea in adults and children in countries with poor environmental hygiene and in travellers to these regions.^{1–5} In contrast, ETEC have been rarely associated with endemic diarrhoea in developed countries,^{6–10} although occasional outbreaks have been reported in North America¹¹ and Europe.^{12–14}

ETEC cause diarrhoea in man through the elaboration of a heat-labile (LT) and a heat-stable (STa) enterotoxin or both.¹⁵ For diarrhoea to result, ETEC must colonise the small intestine, and adhesive fimbriae such as colonisation factor antigens CFA/I and CFA/II promote attachment of bacteria to epithelial cells of the small intestine.^{15–18} CFA/I and CFA/II confer a relatively high degree of surface hydrophobicity to bacteria and are also responsible for mannose-resistant haemagglutination (MRHA) of certain types of erythrocyte.^{15–20} CFA/I and CFA/II,

as well as LT and STa, are encoded by plasmids.^{16, 21} Although many different serotypes have been described amongst ETEC, most strains belong to serotypes O6:K15:H16, O8:K40:H9, O15:H11, O25:K7:H42, O25:K98:H—, O78:H11, O78:H12 and O149:H10.^{22–24}

We have studied the role of ETEC in infants and young children with diarrhoea from three hospitals in Galicia, north-western Spain.

Materials and methods

Children

Stool specimens were obtained from 482 children with diarrhoea (patients) and from 103 healthy children (controls) between July 1985 and January 1988. Only children under 3 years of age were examined. Faecal samples from children with diarrhoea were collected from 109 outpatients and 373 inpatients in three hospitals in different towns of Galicia: Residencia Sanitaria Juan Canalejo (La Coruña), Hospital Xeral de Galicia (Santiago de

Compostela), and Hospital Xeral (Lugo). These three towns are approximately 100 km apart. Infants developing diarrhoea after admission to hospital and subjects who had received antimicrobial treatment for the current illness were not excluded.

Microbiological methods

Fresh stool samples were cultured for bacterial enteropathogens, including *E. coli*, *Salmonella*, *Shigella*, *Campylobacter* and *Yersinia* spp., by standard methods;²⁵ 134 stools were also investigated for rotavirus with a commercial slide Rota-kit (Bio-Mérieux, France) or a Rotazyme II kit (Abbot, USA). A single sample was taken from each child and three to five *E. coli* colonies isolated from each sample were examined for enterotoxin production.

Enterotoxin assays

LT production was determined by the Vero cell assay²⁶ and STa by the infant mouse test.²⁷ For detection of STa and LT, bacteria were grown for 20 h in Tryptone Soya Broth (Oxoid) at 37°C (with shaking at 200 rpm) and centrifuged (6000 g) for 30 min at 4°C; the supernates were filtered through 0.22-µm membrane filters.

Detection of CFA/I and CFA/II

ETEC isolates were inoculated into Mueller-Hinton Broth (Difco) and incubated at 37°C for 5 days until a definite pellicle was formed. From this pellicle, bacteria were inoculated on to casamino acid-yeast extract agar (CFA agar) plates¹⁶ and incubated at 37°C for 18 h. Bacteria grown on CFA agar were examined for CFA/I and CFA/II by an improved staphylococcal co-agglutination test with adsorbed specific anti-CFA/I and anti-CFA/II rabbit sera.¹²

Haemagglutination and hydrophobicity

Tests for MRHA and mannose-sensitive haemagglutination (MSHA) were done by the rocked-tile method with human (blood groups A and O), calf, guinea-pig, adult chicken, sheep and pig erythrocytes as previously described.²⁸ Isolates were grouped according to their MRHA patterns in six MRHA types (I–VI).²⁸ Bacterial surface hydrophobicity was determined by the improved salt aggregation test (ISAT).²⁰ For these tests, bacteria were grown in batch culture in Mueller-Hinton Broth at 37°C for 5 days and the pellicle formed was plated on CFA¹⁶ and Minca-Is²⁹ solid media and incubated at 37°C for 18 h.

Serotyping

The presence of O, K and H antigens was determined as previously described³⁰ with all available

O (O1–O167) and H (H1–H56) antisera, and O:K antisera against the K antigens usually associated with each O antigen.

Antibiotic resistance patterns

ETEC strains were tested for antibiotic resistance by the disk diffusion method.³¹ Antibiotics tested were: amikacin, co-amoxiclav, ampicillin, cefotaxime, cefoxitin, cephalothin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, neomycin, nitrofurantoin, polymyxin B, streptomycin, sulphadiazine, tetracycline, tobramycin and co-trimoxazole.

Control strains

Reference *E. coli* strains used as positive and negative controls were: m452-C₁ and m633-C₁ (O63:K–:H–, LT⁺, STa⁺, CFA/I⁺), PB176 and B2C (O6:H16, LT⁺, STa⁺, CFA/II⁺), and K12-185 (non-toxigenic strain).

Statistical analysis

Results were compared by the χ^2 test with Yates's correction for continuity.

Results

Frequency of isolation of enteropathogens

ETEC were isolated from 19 (3.9%) of 482 children with diarrhoea and from 1 (1.0%) of 103 controls ($\chi^2 = 1.46$, $p < 0.3$; not significant). However, ETEC were isolated significantly more frequently from children with diarrhoea who were under 1 month of age than from diseased children older than 1 month ($\chi^2 = 42.84$, $p < 0.001$) or from healthy children under 1 month of age ($\chi^2 = 5.11$, $p < 0.05$) (table I). The isolation rate of ETEC was higher in inpatients (18 of 373; 4.8%) than in outpatients (1 of 109; 0.9%), but this difference was not significant ($\chi^2 = 2.45$, $p < 0.2$). Among children who harboured ETEC, five of the nine children under 1 month of age developed diarrhoea while in hospital, whereas none of 10 children older than 1 month of age did so.

Rotavirus was the most frequently identified enteric pathogen, accounting for 37.3% of the cases, followed by *Salmonella* spp. (12.8%), ETEC (3.9%), *C. jejuni* (2.3%), *Shigella* spp. (0.9%) and *Y. enterocolitica* (0.5%) (table II). The isolation rates for enteropathogens other than ETEC did not differ significantly among different age groups (fig. 1). The incidence of *Salmonella* spp. and ETEC was higher during warm months, whereas rotavirus predominated during the cold months (fig. 2).

Table I. Frequency of ETEC in children with diarrhoea and in healthy children of different age groups

Age group (months)	Number of children with ETEC strains/number investigated (%)				
	Children with diarrhoea from				Healthy controls
	La Coruña	Santiago	Lugo	Total	
<1	3/10 (30.0)	3/11 (27.3)	3/13 (23.1)	9/34 (26.5)	0/22
1-5	0/60	0/27	1/31 (3.2)	1/118 (0.8)	0/22
6-11	2/52 (3.8)	1/24 (4.2)	0/26	3/102 (2.9)	1/28 (3.6)
12-23	3/64 (4.7)	1/61 (1.6)	0/25	4/150 (2.7)	0/20
24-35	2/27 (7.4)	0/40	0/11	2/78 (2.6)	0/11
Total	10/213 (4.7)	5/163 (3.1)	4/106 (3.8)	19/482 (3.9)	1/103 (1.0)

Table II. Frequency of isolation of various enteropathogenic micro-organisms in children with diarrhoea

Enteropathogen	Number (%) of children from	
	La Coruña (n=213)	Lugo (n=106)
ETEC	10 (4.7)	4 (3.8)
<i>Salmonella</i> spp.*	28 (13.1)	13 (12.3)
<i>Shigella</i> spp.	3 (1.4)	0
<i>C. jejuni</i>	5 (2.3)	NT
<i>Y. enterocolitica</i>	1 (0.5)	NT
Rotavirus†	18/50 (36.0)	32/84 (38.1)

NT, not tested.

* *S. enteritidis* (38 isolates) and *S. typhimurium* (three isolates).

† Only 134 patients were examined for rotavirus; results show the number found to be positive/number examined.

Clinical findings in ETEC diarrhoea

Severity of disease ranged from mild to severe; 94.7% had a watery diarrhoea, but only 31.6% were febrile; vomiting occurred in 63.2% and 42.1% became dehydrated. Persistence of diarrhoea ranged from 3 to 12 days (mean 5 days). All infants with ETEC

Fig. 1. Isolation rate of ETEC (■), *Salmonella* spp. (▨) and rotavirus (□) in various age groups of patients. **Fig. 2.** Incidence of ETEC (■), *Salmonella* spp. (▨) and rotavirus (□) by season. Cold months: Nov.-March; temperate months: April, May, Sept. and Oct.; warm months: June-Aug.

diarrhoea recovered after treatment. Nevertheless, after 5 days at home, one child was admitted to hospital and died 4 days later with an umbilical cord infection and gastro-enteritis. Blood and urine culture performed 4 days before death were negative, but faecal culture was not performed during the second period of hospitalisation.

Abundance and characteristics of ETEC strains

A total of 64 ETEC colonies were isolated from 19 children with diarrhoea. ETEC colonies were predominant in 13 (68.4%) of the 19 positive samples. In 16 samples, all ETEC colonies isolated showed identical characteristics; in two samples two types of ETEC were detected and in one sample three different ETEC were observed. Characteristics of the 23 ETEC isolates are summarised in table III. Of these isolates, 17 were STa⁺, two LT⁺STa⁺ and four LT⁺. However, three isolates (LD73b, LD76a and LD76b) lost their enterotoxigenic activity during maintenance in the laboratory.

CFA/I or CFA/II was detected in 11 (55.0%) of the 20 ETEC isolates that still showed enterotoxigenic activity after maintenance in the laboratory (table IV). CFA/I was found in nine (64.3%) of 14 STa⁺ isolates; CFA/II was found in the two (100%) LT⁺STa⁺ isolates; and no isolate that produced LT only expressed either colonisation factor. Most ETEC belonged to serotypes

Table III. Characteristics of ETEC isolates from children with diarrhoea

Isolate no.	Serotype	Toxins	Colonisation factors	Haemagglutination*		Hydrophobicity†		Antibiotic resistance
				CFA agar	Minca-Is medium	CFA agar	Minca-Is medium	
CD20b	O8:K(A)?	LT	...	MRHA-VI MSHA	MRHA-VI MSHA	>2.0	>2.0	AxC C Cx
CD49a	O9:K(A)?	LT	...	...	...	AA	AA	None
CD68a	O25:K?	LT	...	MSHA	MSHA	2.0	2.0	None
SD25a	O71:K-	LT	...	MRHA-IVb MSHA	MRHA-IVb MSHA	AA	AA	Ap Sm Nm Km Tc Sd
LD102b	O6:K15:H16	LT STa	CFA/II	MRHA-II	MRHA-II	0.4	0.4	None
LD105a	O6:K15:H16	LT STa	CFA/II	MRHA-II	...	0.4	>2.0	None
CD5a	O27:K-:H7	STa	...	MSHA	MRHA-VI MSHA	0.4	0.4	None
SD52a	O27:K-:H7	STa	...	MSHA	MRHA-VI MSHA	0.4	0.4	None
CD15a	O27:K-:H7	STa	...	MSHA	MRHA-VI MSHA	0.4	0.4	Ap Sm Sd
CD68b	O153:K-:H45	STa	CFA/I	MRHA-Ia	MRHA-Ib	0.06	0.1	Ap Sm Nm Km Sd
SD53a	O153:K-:H45	STa	CFA/I	MRHA-Ia	MRHA-Ia	0.06	0.06	Ap Sm Nm Km Sd Tc
SD72a	O153:K-:H45	STa	CFA/I	MRHA-Ia	MRHA-Ia	0.06	0.06	Ap Sm Nm Km Sd Tc
CD22d	O153:K-:H45	STa	CFA/I	MRHA-Ia	MRHA-Ib	0.06	0.06	Ap Sm Sd Tc
CD69a	O153:K-:H45	STa	CFA/I	MRHA-Ia	MRHA-Ib	0.06	0.06	Ap Sm Sd
CD79a	O153:K-:H45	STa	CFA/I	MRHA-Ia	MRHA-Ib	0.06	0.06	Ap Sm Sd
CD80c	O153:K-:H45	STa	CFA/I	MRHA-Ia MSHA	MRHA-Ib MSHA	0.06	0.06	Ap Sm Sd
CD99a	O153:K-:H45	STa	CFA/I	MRHA-Ia MSHA	MRHA-Ia MSHA	0.06	0.06	Ap Sm Sd
SD165b	O153:K-:H45	STa	CFA/I	MRHA-Ia	MRHA-Ia	0.06	0.06	Ap Sm Sd
CD68c	NT	STa	...	...	...	AA	>2.0	Sd
LD73c	NT	STa	...	MSHA	MSHA	AA	AA	Sm Sd Tc
LD73b†	O24:K?:H15	STa	...	MSHA	MSHA	>2.0	AA	Sm Sd Tc
LD76a†	O89:K-:H15	STa	...	MSHA	MSHA	1.0	1.0	None
LD76b†	NT	STa	...	MSHA	MSHA	1.4	1.4	None

LT, heat-labile toxin; STa, heat-stable toxin; CFA, colonisation factor antigen; MRHA, mannose-resistant haemagglutination; MSHA, mannose-sensitive haemagglutination; AA, autoagglutinating; NT, not tested; Ap, ampicillin; AxC, co-amoxiclav; C, chloramphenicol; Cx, cefoxitin; Sm, streptomycin; Nm, neomycin; Km, kanamycin; Tc, tetracycline; Sd, sulphadiazine.

* MRHA haemagglutination types: Ia, MRHA with human A and O, bovine, and adult chicken erythrocytes; Ib, MRHA with human A and O, bovine, adult chicken, and pig erythrocytes; II, MRHA with calf and adult chicken erythrocytes; IVb, MRHA with human A and O, and pig erythrocytes; VI, MRHA negative with human erythrocytes and MRHA variable with the remaining five erythrocyte species.²⁸ *E. coli* CD20b MRHA VI⁺ showed haemagglutination with pig erythrocytes only, whereas MRHA VI⁺ isolates of serotype O27:K-:H7 showed MRHA with calf and sheep erythrocytes.

† Minimal molar ammonium sulphate concentration in which bacteria aggregated in the improved salt aggregation test (ISAT).

‡ Enterotoxin activity lost during laboratory culture.

O153:K-:H45 (nine STa⁺ isolates), O27:K-:H7 (three STa⁺ isolates) and O6:K15:H16 (two LT⁺ STa⁺ isolates) (table III). All isolates of serotype O153:K-:H45 were CFA/I⁺ and the two isolates of serotype O6:K15:H16 were CFA/II⁺. CFA/I⁺ isolates showed a high level of bacterial surface hydrophobicity, aggregating in 0.06 M ammonium sulphate, when grown either on CFA agar or on Minca-Is medium. CFA/I⁺ isolates grown on CFA agar showed MRHA characteristic of CFA/I: MRHA with human A and O, bovine and adult chicken erythrocytes.

Table IV. Relationship between enterotoxin production and the presence of CFA/I and CFA/II in ETEC isolates

Enterotoxin produced	Number (%) of isolates		
	tested	CFA/I ⁺	CFA/II ⁺
LT	4	0	0
LT STa	2	0	2 (100)
STa	14	9 (64.3)	0
Total	20	9 (45.0)	2 (10.0)

However, when they were grown in Minca-Is medium, some of the CFA/I⁺ isolates also agglutinated pig erythrocytes in the presence of mannose. CFA/II⁺ isolates expressed a significant level of bacterial surface hydrophobicity, aggregating in 0.4 M ammonium sulphate, and showed MRHA with bovine and adult chicken erythrocytes. Enterotoxigenic isolates of serotype O27:K-:H7 were CFA/I⁻, CFA/II⁻, but, like CFA/II⁺ isolates, they were moderately hydrophobic (0.4 M ammonium sulphate) and showed mannose-resistant haemagglutination. ETEC belonging to serotype O27:K-:H7 expressed MRHA with calf and sheep erythrocytes when grown on Minca-Is medium, but not on CFA (table III).

Of the 23 ETEC isolates 15 (65.2%) were resistant to at least one of the antibiotics tested (table III).

Discussion

These results show that ETEC is a frequent cause of diarrhoea in children younger than 1 month in Galicia. In infants below the age of 1 month, ETEC strains were isolated more frequently from children

with diarrhoea than from controls and significant differences were observed between the incidence of ETEC in infants under 1 month of age and in older infants. Among the children under 1 month of age who developed ETEC infections, about half developed diarrhoea in hospital. This interesting result is in keeping with several reports of nosocomial outbreaks of ETEC affecting neonates in hospitals in different parts of Spain.^{12, 13}

The isolation rate of ETEC (3.9%) in this study was lower than the 10.5% found by Čobeljčić *et al.*³² in Yugoslavia and the 5.9% found by Escribano *et al.*¹³ in Valencia on the Mediterranean coast of Spain. However, the rate was higher than that observed in other studies carried out in Spain,^{7, 33} in other European countries^{6, 10} and in North America.^{8, 9}

Rotavirus was the most frequently identified enteropathogen, followed by *Salmonella* spp. and ETEC; as in other studies^{2, 33, 34} infections by ETEC and *Salmonella* spp. had seasonal peaks in the warmer months, whereas infections by rotavirus were more frequent during colder months.

Reports from other geographical areas indicate that LT⁺STa⁺ and STa⁺ strains, which usually express CFA/I or CFA/II, are isolated mainly from ill children, whereas LT⁺ strains, normally without colonisation factors, have been found in similar proportions in sick and healthy children.^{3, 4} In our study, CFA/I and CFA/II were produced by 55% of the ETEC isolates. This incidence is higher than the 18–41% reported in most published studies.^{17–19, 35, 36} However, similar³⁴ or higher^{1, 37} incidences have been reported from other parts of the world. In this study, CFA/I was detected in most STa⁺ isolates and CFA/II in the two LT⁺STa⁺ isolates, but no isolate producing LT alone expressed colonisation factors.

Most ETEC detected in this study belonged to a few serotypes and expressed CFA/I or CFA/II. ETEC of serotype O27:K—:H7 did not express CFA/I or CFA/II, but were hydrophobic and showed MRHA with calf and sheep erythrocytes when grown on Minca-Is medium. We think that the mannose-resistant haemagglutinin expressed by O27:K—:H7 ETEC may represent a new putative colonisation factor, unrelated to others that have recently been described, which lack MRHA, or in which it is expressed with human and calf erythrocytes.^{15, 17, 18} Further studies are necessary to establish the role of the mannose-resistant haemagglutinin expressed by ETEC strains of O27:K—:H7.

E. coli O153:K—:H45 strains producing STa and CFA/I were the most common ETEC isolates in this study. Similar strains have been found to be responsible for three outbreaks of nosocomial diarrhoea in neonates in two hospitals in Madrid¹² and in a hospital in Valencia¹³ in recent years. ETEC strains of this serotype have also been found to be the most common ETEC isolates in Chile,¹ but have been detected only occasionally in Italy,¹³ Germany,³⁸ Liberia,¹³ the Central African Republic,³⁹ Somalia⁴⁰ and Thai-

land.²¹ Of the other ETEC isolates in this study, serotype O6:K15:H16 is commonly reported from different parts of world,^{1, 8, 22–24, 36} and serotype O27:K—:H7 has been detected previously in Somalia,⁴⁰ Kenya³⁶ and Thailand²² as well as in travellers with diarrhoea who visited Asian countries.²⁴ However, *E. coli* O27:K—:H7 was not found commonly among ETEC isolates in other countries.

Our results suggest that ETEC strains, mainly STa⁺ CFA/I⁺ belonging to serotype O153:K—:H45, are an important cause of neonatal diarrhoea among infants in Galicia. We recommend routine screening for ETEC in Spanish clinical microbiology laboratories, especially in stools from newborn children under 1 month of age and in outbreaks of diarrhoea. Most ETEC strains could be detected by means of simple and rapid slide agglutination or co-agglutination tests with specific anti-CFA/I and anti-CFA/II sera, and another serum against the putative new colonisation factor associated with ETEC strains of O27:K—:H7. ETEC strains may be the most important cause of diarrhoea in Spain after rotavirus and *Salmonella* spp.

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