Journal of Medical Microbiology (2010), **59**, 345–352 DOI 10.1099/jmm.0.015560-0

Role and clinical course of verotoxigenic *Escherichia coli* infections in childhood acute diarrhoea in Argentina

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Received 28 August 2009
Accepted 16 October 2009

The aim of this study was to investigate the role and clinical course of verotoxigenic *Escherichia coli* (VTEC) infections in children with acute diarrhoea from Argentina, the country with the highest worldwide incidence of haemolytic uraemic syndrome (HUS). To accomplish this objective, 437 samples from children up to 6 years old with acute diarrhoea were collected and processed. More than 60 % of the children studied presented watery or mucous diarrhoea without blood, and in 25.2 % of the cases the samples contained blood. In a first screening, a multiplex PCR was performed to detect the presence of the vt_1, vt_2, *eae*, *ehxA* and *saa* virulence genes. The strains were then isolated and analysed to characterize their serotypes, virulence genes, antibiotic susceptibility profiles and verotoxin (VT) production. Forty-four of the 437 samples (10.1 %) were positive for VTEC virulence genes. VTEC-infected patients presented different types of diarrhoea (27.3 % belonged to the non-bloody type). Several serotypes and virulence genotypes were found. Isolates belonged to the serotypes O157 : H7, O145 : H^-, O26 : H11, O121 : H19, O111 : H2 and O118 : H2. HUS developed in 16 (36.4 %) patients positive for VTEC virulence genes. All of the VTEC isolates produced a cytopathic effect on Vero cell monolayers, confirming the ability to express VT. Despite most strains being sensitive to all of the antimicrobials studied, a positive association between clinical progression to HUS and antibiotic therapy was observed for the total number of patients studied, as well as for the $VTEC^+$ group. In conclusion, the data obtained in this study increase our knowledge of the role and clinical course of VTEC infection in childhood acute diarrhoea beyond bloody diarrhoea, and might be considered for the prevention, diagnosis and management of this disease. It is possible that the optimal approach for VTEC diagnosis could be using multiplex PCR to search for the presence of the vt_1, vt_2, *eae* and *ehxA* genes.

INTRODUCTION

Infection with verotoxigenic *Escherichia coli* (VTEC), also known as Shigatoxigenic *E. coli*, can cause a wide spectrum of clinical manifestations in humans, from asymptomatic infection, mild to moderate diarrhoea and haemorrhagic colitis to haemolytic uraemic syndrome (HUS) (Karmali, 1989; Proulx *et al.*, 2001; Tarr *et al.*, 2005). Watery diarrhoea develops in about 90 % of infected children and in most cases becomes bloody. HUS progression is observed in approximately 5–15 % of patients. There is no specific treatment for this infection (Karmali, 1989; Karch *et al.*, 2005; Tarr *et al.*, 2005; Goldwater, 2007). Therapy for VTEC infections with antibiotics or drugs that inhibit intestinal motility has been associated with an increased risk of developing HUS. Currently, treatment is only supportive, focusing on fluid and electrolyte management. Hospitalization during the diarrhoeal phase seems to benefit VTEC-positive patients. Precocious treatment is an absolute requirement for success and depends on early diagnosis, making rapid diagnostic techniques necessary (Wong *et al.*, 2000; Karch *et al.*, 2005; Tarr *et al.*, 2005; Goldwater, 2007).

E. coli O157 : H7 has been reported to be the most common VTEC serotype involved in human disease. However, other VTEC serotypes can cause a similar disease spectrum (Griffin & Tauxe, 1991; Karch *et al.*, 2005; Tarr *et al.*,

Abbreviations: HUS, haemolytic uraemic syndrome; IMS, immunomagnetic separation; VT, verotoxin; VTEC, verotoxigenic *Escherichia coli*.

2005). The virulence of VTEC is mainly determined by its ability to produce verotoxins (VTs), also called Shiga toxins. Most VTEC strains carry the gene encoding VT2 (vt_2) and about two-thirds carry that encoding VT1 (vt_1) (Karmali, 1989; Proulx *et al.*, 2001; Tarr *et al.*, 2005). Other virulence factors, pathogenic for human hosts, have been described: intimin (*eae*), by which *E. coli* mediates intimate attachment to epithelial cells, enterohaemolysin (*ehxA*) and an autoagglutinating adhesion factor called Saa (*saa*), associated with *eae*-negative Shigatoxigenic *E. coli* (Paton & Paton, 1998; Paton *et al.*, 2001; Tarr *et al.*, 2005).

Argentina has the highest worldwide incidence of reported HUS cases in children aged 5 years or under, with HUS being the major cause of acute renal failure and the second leading cause of chronic renal failure and renal transplantation in children. Currently, the mortality rate among children with this syndrome is about 5 %. However, routine microbiological detection of VTEC in stools from diarrhoeic children is not currently carried out. Only in some cases of bloody faeces is the search oriented towards detection of the O157 serogroup (Voyer, 1996; López *et al.*, 1998; Rivero *et al.*, 2004; Rivas *et al.*, 2006). Few studies have been performed to evaluate the role of VTEC infection in children with acute diarrhoea in Argentina. One such study, performed by López *et al.* (1998), included HUS patients, patients with grossly bloody diarrhoea, patients with watery diarrhoea and healthy children.

There are few studies on the clinical spectrum caused by VTEC infection in children in Argentina. Information on VTEC epidemiology in children with acute diarrhoea, particularly in the watery phase without blood, is also lacking. An early diagnosis, essential for establishing timely treatment and preventative measures, is not available for the general population. Hence, the present work was performed to study the role of VTEC infections in childhood acute diarrhoea, to generate information useful in the prevention, diagnosis and clinical management of the disease.

METHODS

Samples. Between December 2002 and April 2009, 437 children up to 6 years old with acute diarrhoea were included in the study. Acute diarrhoea was defined clinically as any sudden and significant increase in the frequency or decrease in the consistency of stools lasting less than 2 weeks. Stool samples from each patient were identified by a numeric code and placed in a transport medium. The enrolled patients had attended public or private healthcare institutions, including hospitals and clinics, in the cities of Tandil and its surroundings, Bahía Blanca, Morón (Province of Buenos Aires) and Río Cuarto (Province of Córdoba), Argentina. Parents and physicians were asked to fill out a questionnaire, providing information on age, sex, signs and symptoms, treatment, hospitalization and duration of illness of each patient.

Bacteriological procedures and analysis of virulence genes. Swabs from stools were plated onto MacConkey agar plates and incubated overnight at 37 °C. An aliquot of confluent growth of each sample was inoculated into 30 ml Luria–Bertani broth, incubated with shaking at 37 °C for 4 h and processed for DNA extraction as described previously (Sanz *et al.*, 1998). In a first screening, virulence genes (vt_1, vt_2, *eae*, *ehxA* and *saa*) were detected by means of a multiplex PCR, using primers and conditions described by Paton & Paton (2002). The *E. coli* strains used as positive controls were: EDL 933 (vt_1, vt_2, *eae*, *ehxA*; kindly supplied by Dr J. Blanco, Laboratorio de Referencia de *E. coli*, Spain) and HT 1-6 (vt_1, vt_2, *ehxA*, *saa*; Krüger *et al.*, 2006). Amplification products were analysed by 'submarine' gel electrophoresis (1.5 % agarose) and UV transillumination.

One millilitre from each vt^+ culture was kept frozen at −70 °C after the addition of 20 % glycerol for further isolation of individual vt^+ colonies on a MacConkey agar plate.

Isolation of O157 *E. coli* and non-O157 strains. Individual colonies were assessed to determine O157 and non-O157 serogroups. Samples positive for the vt_1 or vt_2 gene and *eae* gene were analysed by PCR for the presence of the *eae-γ* gene, which is characteristic of, although not exclusive to, the O157 serogroup. *E. coli* EDL 933 was used as a positive control. The reaction mixture and temperature profile for PCR amplification were as indicated by Gannon *et al.* (1993).

Following individual O157 strain isolation, those samples positive for the *eae-γ* gene were subjected to immunomagnetic separation (IMS), a rapid method used for detecting O157 strains. The methods described by Padola *et al.* (2004) were followed. Samples were inoculated in peptone water supplemented with antibiotics (0.05 mg cefixime l^{-1}, 8 mg vancomycin l^{-1}, 10 mg cefsulodin l^{-1}) and incubated with shaking at 37 °C for 6 h. One millilitre of each sample was then subjected to IMS with polystyrene magnetic beads coated with antibody specific for O157 (Dynal) as described by Lindqvist *et al.* (1998). The concentrated samples were inoculated onto MacConkey sorbitol agar plates, supplemented with 2.5 mg potassium tellurite l^{-1} and 0.05 mg cefixime l^{-1} as described by Blanco *et al.* (1996). Separate colonies were processed for amplification of vt_1, vt_2, *eae*, *ehxA* and *saa* (Paton & Paton, 2002).

Following individual non-O157 strain isolation, 500–1000 colonies of samples negative for the *eae-γ* gene and samples positive for the *eae-γ* gene but unable to be isolated by means of IMS were subjected to PCR amplification of vt_1, vt_2, *eae*, *ehxA* and *saa* (Paton & Paton, 2002).

Confirmation of *E. coli*. Micro-organisms were confirmed as *E. coli* by identification of the *uspA* gene by PCR (Chen & Griffiths, 1998).

Serotyping. Identification of somatic (O) and flagellum (H) antigens was performed by agglutination with specific antisera, as described by Blanco *et al.* (1992). O antisera were obtained from the *E. coli* Reference Laboratory (Lugo, Spain), whilst H antisera were obtained from the Statens Serum Institut (Copenhagen, Denmark) (Orskov & Orskov, 1984; Pradel *et al.*, 2000).

Antimicrobial susceptibility test. Antimicrobial drug susceptibility testing was carried out using standard methods (disc diffusion test) using Mueller–Hinton agar and interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). VTEC strains were examined for susceptibility to cephalothin, amoxicillin/clavulanic acid, ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, streptomycin, sulfonamide, tetracycline and trimethoprim/sulfamethoxazole. Inhibition zones were classified as sensitive, intermediate (moderately sensitive) or resistant according to the procedures of the CLSI (2008). Isolates exhibiting resistance to at least two of the antimicrobial agents tested were considered to be multiresistant strains. *E. coli* ATCC 25922 and ATCC 35218 were used as quality-control strains to monitor the accuracy of the disc diffusion test (Bauer *et al.*, 1966; CLSI, 2008).

Cytotoxicity assay. VT production was confirmed by a cytotoxicity assay on Vero cell monolayers according to the protocol described by Blanco *et al.* (1997).

Data analysis. Data were analysed using Epi Info version 6.04d (Centers for Disease Control and Prevention, Atlanta, USA, and World Health Organization, Geneva, Switzerland, 2001). Descriptive statistics were used for data analysis. Associations between qualitative variables were evaluated by a χ^2 test or Fisher's exact test (Conover, 1971).

Ethical considerations. Recommendations established in the Declaration of Helsinki for Biomedical Research Involving Human Subjects, adopted by the 18th World Medical Assembly (Helsinki, Finland, 1964) and revised by the World Medical Assembly in Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996), Edinburgh (2000), Washington (2002), Tokyo (2004) and Seoul (2008), were followed.

RESULTS AND DISCUSSION

All samples

Unlike other studies conducted in our country, children (n=437) with different types of diarrhoea were sampled, and a large percentage of these samples were non-bloody. A description of the physical appearance of all collected samples is shown in Fig. 1. The signs and symptoms of all of the patients studied are shown in Table 1. The median age of the patients was 18 months (range 1–75 months). Two hundred and fifty-eight of the 437 patients (59.0 %) were children younger than 2 years old and 237 (54.2 %) of them were male. Eighty-four (19.2 %) of the 437 patients received antimicrobial therapy. We found a positive association between antimicrobial therapy and HUS development (P=0.01).

HUS$^+$ patients

Thirty-five (8 .0%) of all of the patients studied developed HUS. A description of the physical appearance of the stool samples belonging to the HUS$^+$ patients is given in Fig. 1. Watery diarrhoea without blood developed in nearly one-quarter of HUS$^+$ patients, similar to the results found by Giugno *et al.* (2007) in Argentina (25.8 %). Diarrhoea with blood developed in 65.7 % of cases, similar to the findings of Gerber *et al.* (2002) in Germany and Austria (57 %) and Espié *et al.* (2008) in France (59.4 %). The signs and symptoms of the HUS$^+$ patients are shown in Table 1. The median age was 22 months (range 6–70 months). Eighteen of the 35 HUS$^+$ patients (51.4 %) were children younger than 2 years old and 19 (54.3 %) were male. We found no significant differences between the occurrence of HUS in boys and girls (P=0.98), in agreement with other studies performed in our country (Rivas *et al.*, 2006; Giugno *et al.*, 2007). Additionally, 13 (37.1 %) of the 35 HUS$^+$ patients received antimicrobial therapy. Similar results have been reported in other studies. Giugno *et al.* (2007) reported that between 1999 and 2001, in Argentina, on average 44 % of patients with HUS were treated with antimicrobial drugs. In a study conducted by Banatvala *et al.* (2001) in the USA, 31 % of HUS patients had been treated. All patients with HUS were hospitalized and two of the 35 (5.7 %) died. The mortality rate was higher than that reported previously by Espié *et al.* (2008) in France (1 %), Gerber *et al.* (2002) in Germany and Austria (2 %), and Rivas *et al.* (2006) in Argentina (3.4 %).

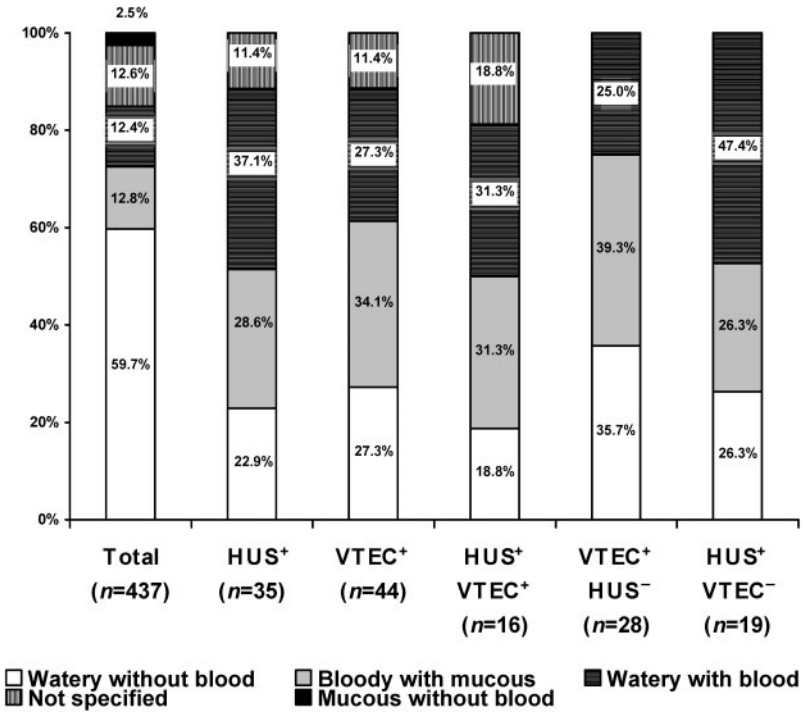

Fig. 1. Bar diagram giving details of the physical appearance of stool samples from total, HUS$^+$, VTEC$^+$, HUS$^+$ VTEC$^+$, VTEC$^+$ HUS$^-$ and HU$^+$ VTEC$^-$ patients.

Table 1. Signs and symptoms expressed in percentages (frequencies) from all, HUS$^+$, VTEC$^+$, HUS$^+$ VTEC$^+$, VTEC$^+$ HUS$^-$ and HUS$^+$ VTEC$^-$ patients

The results were calculated according to the information that was available from the patient questionnaires; not all information was available for each patient.

Symptom	All patients	HUS$^+$	VTEC$^+$	HUS$^+$ VTEC$^+$	VTEC$^+$ HUS$^-$	HUS$^+$ VTEC$^-$
Vomiting	54.5 (218/400)	75.0 (24/32)	59 (23/39)	64.3 (9/14)	56.0 (14/25)	83.3 (15/18)
Abdominal pain	61.9 (237/383)	81.8 (27/33)	68.4 (26/38)	80.0 (12/15)	60.9 (14/23)	83.3 (15/18)
Fever	50.4 (201/399)	54.5 (18/33)	41.0 (16/39)	53.3 (8/15)	33.3 (8/24)	55.6 (10/18)
Anuria	4.5 (11/244)	83.3 (10/12)	26.3 (5/19)	83.3 (5/6)	0.0 (0/13)	83.3 (5/6)
Oliguria	7.9 (27/342)	91.7 (22/24)	32.4 (11/34)	100.0 (11/11)	0.0 (0/13)	84.6 (11/13)
Sensory alteration	2.5 (6/239)	27.3 (3/11)	15.8 (3/19)	50.0 (3/6)	0.0 (0/13)	0.0 (0/5)
Weakness	6.3 (15/240)	18.2 (2/11)	15.8 (3/19)	33.0 (2/6)	7.7 (1/13)	0.0 (0/5)
Paleness	6.7 (16/239)	63.6 (7/11)	15.8 (3/19)	50.0 (3/6)	0.0 (0/13)	80.0 (4/5)
Neurological compromise	1.8 (6/337)	18.2 (2/11)	7.1 (2/28)	40.0 (2/5)	0.0 (0/23)	0.0 (0/6)
HUS	8.1 (35/434)	100.0 (35/35)	36.4 (16/44)	100.0 (16/16)	0.0 (0/28)	100.0 (19/19)
Total (*n*)	437	35	44	16	28	19

VTEC$^+$ patients

Forty-four of our patients were positive for VTEC. The frequency and characteristics of VTEC infections in children with different types of diarrhoea in our country are not well established. López *et al.* (1998) found evidence of VTEC infection (by using a free faecal cytotoxin assay, PCR and/or DNA probes) in 57 % of HUS patients, 38 % of patients with diarrhoea with blood, 21 % of patients with watery diarrhoea without blood and none of the healthy patients. In this work, we studied patients who, in nearly 60 % of the cases, presented watery diarrhoea without blood. VTEC was found in 10.1 % of all stool specimens analysed and in 45.7 % of stool cultures from patients with HUS, with bloody and non-bloody diarrhoea. The physical appearance of stool samples of VTEC$^+$ patients is shown in Fig. 1. The signs and symptoms of these patients are shown in Table 1. Their median age was 21.5 months (range 6–72 months). It is interesting that, in nearly 30 % of the VTEC$^+$ samples, faeces were watery. Twenty-five of the 44 VTEC$^+$ patients (56.8 %) were children under 2 years old and 20 (45.5 %) were male. Fourteen (31.8 %) of the 44 VTEC$^+$ patients received antimicrobial therapy. We could establish a positive association between HUS development and antibiotic therapy for the patients with evidence of VTEC infection (P=0.03).

HUS$^+$ VTEC$^+$ patients

Infection with VTEC was diagnosed in 16 of the 35 HUS$^+$ patients (45.7 %), results similar to other studies carried out in Argentina (32.8, 57 and 59.1 %) but lower than those described in Germany and Austria (83 %), France (86 %, 66 %) and the USA (72 %) (López *et al.*, 1998; Banatvala *et al.*, 2001; Gerber *et al.*, 2002; Rivas *et al.*, 2006, 2008; Espié *et al.*, 2008). These differences may reflect regional variations, or may be because the identification of specific antibodies or free faecal toxin was not performed, but instead PCR was used to determine VTEC infection. The physical appearance of the stool samples of the HUS$^+$ VTEC$^+$ patients is shown in Fig. 1. It should be noted that, in nearly 20 % of HUS cases positive for VTEC, the stools were non-bloody. The signs and symptoms of the HUS$^+$ VTEC$^+$ patients are shown in Table 1. The median age of the HUS$^+$ VTEC$^+$ patients was 23 months (range 6–60 months). Nine of the 16 HUS$^+$ VTEC$^+$ patients (56.3 %) were children under 2 years old, and eight (50 %) were male. Seven (43.8 %) of the 16 HUS$^+$ VTEC$^+$ patients received antimicrobial therapy. All patients with HUS and with evidence of VTEC infection were hospitalized and one of the 16 (6.3 %) died.

VTEC$^+$ HUS$^-$ patients

The physical appearance of the stool samples of the VTEC$^+$ patients without HUS (n=28) is shown in Fig. 1. Although the differences were not significant (P=0.68), this group presented more watery diarrhoea without blood than the HUS$^+$ VTEC$^+$ patients. The signs and symptoms of these patients are shown in Table 1. The occurrence of vomiting, fever and abdominal pain was more common in the HUS$^+$ VTEC$^+$ group than in the VTEC$^+$ HUS$^-$ group, similar to results reported by Oshima (1997), although the differences were not statistically significant (P=0.61, P=0.21 and P=0.29, respectively). The median age of the VTEC$^+$ HUS$^-$ patients was 17 months (range 7–72 months). Sixteen of the 28 VTEC$^+$ HUS$^-$ patients (57.14 %) were children under 2 years old, and 12 (42.85 %) were male.

HUS$^+$ VTEC$^-$ patients

Nineteen of the HUS$^+$ patients (54.3 %) showed no evidence of VTEC infection. The physical appearance of the stool samples of the HUS$^+$ VTEC$^-$ patients is shown in Fig. 1, and their signs and symptoms in Table 1. This group presented no neurological complications (sensory

alteration, weakness or neurological damage) and had more vomiting and slightly more fever and abdominal pain than the HUS$^+$ VTEC$^+$ patients. The median age of the HUS$^+$ VTEC$^-$ patients was 21 months (range 8–70 months). Nine of the 19 HUS$^+$ VTEC$^-$ patients (47.37 %) were children under 2 years old, and 11 (57.89 %) were male. No association was found between HUS development and antibiotic therapy for the patients who showed no evidence of VTEC infection (P=0.5). All patients with HUS and without evidence of VTEC infection were hospitalized and one of the 19 (5.26 %) died. There was no difference between the mortality rate of HUS$^+$ VTEC$^+$ patients and HUS$^+$ VTEC$^-$ patients (P=1).

Individual strain isolation

Similar to results from other studies, we isolated individual strains from 34 (77.3 %) of the 44 VTEC$^+$ samples. Paton *et al.* (1996), Piérard *et al.* (1997), Blanco *et al.* (2004) and Espié *et al.* (2008), using a protocol similar to the present one, were unable to isolate VTEC colonies in 24, 19, 13 and 16 % of PCR-positive stools, respectively. This could be explained by the high sensitivity of the PCR technique, which detects a positive result when a VTEC colony is mixed with commensal *E. coli* even at a ratio as low as $1:10^{-4}$ or $1:10^{-8}$.

Strain characterization

A number of VTEC serotypes and genotypes were characterized and the results are shown in Table 2. As in other studies conducted in Argentina, the USA, France, Belgium, Germany and Austria, O157 : H7 was the serotype found most frequently in stool cultures from paediatric patients with HUS, and VTEC could be isolated and characterized in 92.3 % of samples (Piérard *et al.*, 1997; Banatvala *et al.*, 2001; Gerber *et al.*, 2002; Rivas *et al.*, 2006, 2008; Giugno *et al.*, 2007; Espié *et al.*, 2008). In addition, in the current work, an O145 : H$^-$ strain was isolated from an HUS patient. Rivas *et al.* (2006) characterized 48 VTEC strains recovered from bloody and non-bloody stools from patients with diarrhoea and HUS contacts. Of these, 81.2 % were *E. coli* O157 : H7 and the remainder were VTEC non-O157 : H7. Unlike in the study of Rivas *et al.* (2006), the presence of the O157 : H7 serotype was found in only 38.1 % of the stool samples from patients without HUS in our study. These differences could be due to our sampling of children with different types of diarrhoea, rather than being oriented towards the study of bloody diarrhoea or to children in contact with HUS patients. Remarkably, López *et al.* (1998) found that the incidence of O157 : H7 was only 2–18 % for HUS patients and 3 % in the case of the bloody diarrhoea group. In Australia, most of the non-O157 : H7 VTEC serotypes (mainly O111 : H$^-$) were found in sporadic HUS cases, and these serotypes were also associated with outbreaks (Goldwater & Bettelheim, 1998; Elliott *et al.*, 2001).

Table 2. Serotypes and virulence genotypes of the 34 VTEC strains

Serotype	Virulence genotype	No. of isolates	Percentage
O157 : H7	vt_2, *eae*, *ehxA*	20	58.8
0145 : NM	vt_2, *eae*, *ehxA*	6	17.7
O26 : H11	vt_1, *eae*, *ehxA*	2	5.9
O121 : H19	vt_2, *eae*, *ehxA*	2	5.9
O111 : H2	vt_1, *eae*, *ehxA*	1	2.9
O118 : H2	vt_1, *eae*, *ehxA*	1	2.9
O? : H?	vt_2, *eae*, *ehxA*	1	2.9
O? : H?	vt_1, vt_2, *ehxA*	1	2.9
Total		34	100.0

Unlike the findings of researchers from other countries, our strains corresponding to serotype O157 : H7 possessed the vt_2 gene only and all of them were motile. In the USA, Slutsker *et al.* (1997) found that 86.4 % of O157 : H7 strains produced vt_1 and vt_2, 11.9 % produced vt_2 only and 1.7 % produced vt_1 only. They also found *E. coli* O157 isolates that were non-motile (Slutsker *et al.*, 1997). In contrast, Willshaw *et al.* (2001) found that 76 % of the O157 : H7 isolates in England and Wales carried the vt_2 gene only, 23.3 % encoded both vt_1 and vt_2, and vt_1-only strains were very rare. The strains either possessed the flagellar antigen H7 or were non-motile. In two different studies conducted by Piérard *et al.* (1997) and Klein *et al.* (2002), all O157 : H7 isolates possessed the vt_2 gene in combination with vt_1 in 31 and 71 % of cases, respectively. Non-motile strains were also found by Piérard *et al.* (1997).

Almost all of the strains isolated in the present work were positive for the *eae* and *ehxA* genes, which coincides with the group of strains that are more frequently associated with severe disease, HUS and young age, as described by Beutin *et al.* (1998). López *et al.* (1998) also found a positive association between the presence of the *eae* and *ehxA* genes and HUS development.

Antimicrobial resistance

In the present study, most of the isolates were sensitive to all of the antimicrobials tested. The percentages of VTEC isolates that exhibited a sensitive, intermediate or resistant phenotype with respect to each antimicrobial agent are shown in Table 3. Five (14.7 %) of the 34 isolates were resistant to at least one antimicrobial agent. Of these five isolates, four were resistant to only one antimicrobial agent. There was one O157 : H7 strain resistant to amoxicillin/clavulanic acid, and two O145 : H$^-$ and one O157 : H7 resistant to cephalotin. Three of these four strains belonged to HUS cases, and the other was an O145 : H$^-$ strain isolated from a bloody and mucous stool. The multiresistant strain corresponded to an *E. coli* O157 : H7, isolated from a 9-month-old male HUS patient from the city of Bahía Blanca. He did not receive

Table 3. Percentages of VTEC strain isolates showing a sensitive (S), intermediate (I) or resistant (R) phenotype with respect to antimicrobial agents, as determined by the agar disc diffusion method

Antimicrobial agent	S (%)	I (%)	R (%)
Amoxicillin/clavulanic acid	94.1	0	5.9
Ampicillin	97.05	2.95	0
Cefotaxime	100	0	0
Cephalotin	50	41.15	8.85
Chloramphenicol	97.05	2.95	0
Ciprofloxacin	100	0	0
Gentamicin	100	0	0
Imipenem	100	0	0
Streptomycin	100	0	0
Sulfonamide	97.05	0	2.95
Tetracycline	97.05	0	2.95
Trimethoprim/sulfamethoxazole	97.05	2.95	0

antimicrobial therapy. The strain showed resistance to amoxicillin/clavulanic acid, tetracycline and sulfonamide. Similarly, Slutsker *et al.* (1997) found antibiotic resistance in 8.5 % of the O157 : H7 strains studied. The resistance observed by these authors was to ampicillin, tetracycline, streptomycin, sulfisoxazole, chloramphenicol, gentamicin and trimethoprim/sulfamethoxazole. However, Willshaw *et al.* (2001) found a mean of 20 % of O157 : H7 strains resistant to one or more antimicrobial tested. The resistance was observed to streptomycin, sulfonamide and tetracycline.

Despite most of the strains being sensitive to all of the antimicrobials tested, a positive association between HUS and antibiotic therapy, both for the total number of patients studied and for the VTEC$^+$ group, was observed in the present work. These results agree with the hypothesis of Wong *et al.* (2000), who suggested that antibiotic treatment of children with *E. coli* O157 : H7 infection increases the risk of HUS. This could perhaps be due to other VTEC, as in the present work, where one patient with O145 : H$^-$ infection was treated with antibiotics and developed HUS.

VT production

All of the VTEC isolates produced a cytopathic effect on Vero cell monolayers.

Clinical findings related to serotype

Twelve out of 20 O157 : H7 strains (60.0 %) were isolated from stools corresponding to HUS patients. However, only one O145 : H$^-$ of the 14 non-O157 : H7 strains (7.1 %) was recovered from an HUS patient. In contrast to our results, Klein *et al.* (2002) observed HUS development in 18 % of patients with O157 : H7 and none in patients with non-O157 : H7 VTEC infection. In addition, Wong *et al.* (2000) and Tarr *et al.* (2005) observed HUS development in 14 and 15 % of patients with O157 : H7 VTEC infection, respectively. The results of the present study were quite different: we found HUS development in 60 and 7.1 % of patients with O157 : H7 and non-O157 : H7 infection, respectively. The relatively high frequency of HUS may be due to the fact that samples were from children under 75 months, who are supposed to have a higher risk of developing HUS once infected by VTEC than older children or adults (Goldwater, 2007).

We found a positive association between the O157 : H7 serotype and HUS development (P=0.001), as well as with higher hospitalization rates (P=0.004) and abdominal pain (P=0.01) (Table 4). Moreover, 81.3 % of the O157 : H7 isolates were from children with bloody diarrhoea, whereas 53.8 % of the non-O157 : H7 strains were isolated from bloody stools. These results are similar to those found by Gerber *et al.* (2002) and Klein *et al.* (2002). It could be that O157 : H7 is associated with more severe disease and HUS because all of the strains contained vt_2 only, which is more virulent than vt_1 alone or the combination of vt_1 and vt_2 (Proulx *et al.*, 2001; Tarr *et al.*, 2005). Non-O157 : H7 serotypes were found more frequently in children under 2 years old (57.1 %), and O157 : H7 serotypes were found equally in both groups (50.0 % each), which agree with the results obtained by Beutin *et al.* (1998) in Germany and Gerber *et al.* (2002) in Germany and Austria.

Although O157 : H7 VTEC$^+$ patients presented higher rates of hospitalization and HUS, we believe that all of the strains analysed carried a virulence genotype capable of causing severe disease. Furthermore, all of the VTEC isolates produced a cytopathic effect on Vero cell monolayers, confirming their ability to express VT. Most of the non-O157 : H7 serotypes that we found have been described previously in human disease and were associated with HUS (Griffin & Tauxe, 1991; Goldwater & Bettelheim, 1998; Gerber *et al.*, 2002; Johnson *et al.*, 2006).

Conclusions

In conclusion, the data obtained in this study further our current knowledge on the role and clinical course of VTEC

Table 4. HUS development, hospitalization and abdominal pain in patients infected with O157 : H7 and non-O157 : H7 serotypes

Variable	Category	O157 : H7	Non-O157 : H7	*P* value
HUS	Yes	12	1	0.001
	No	8	13	
Hospitalization	Yes	15	2	0.004
	No	4	11	
Abdominal pain	Yes	14	4	0.01
	No	3	8	

infections in childhood acute diarrhoea and may be useful for the prevention and diagnosis and early management of this disease. The present data increase the evidence that VTEC is present in bloody and non-bloody stools from children with acute diarrhoea with and without HUS. We found a large variety of VTEC strains belonging to different serotypes and presenting different virulence genotypes capable of causing severe disease. It is necessary to improve the detection and isolation of VTEC in cases of acute diarrhoea. Children with evidence of VTEC infection should be monitored carefully in a hospital and treated to prevent the onset or progression of HUS. Early diagnosis of infection should help in making clinical decisions regarding the administration of fluids and antibiotics. These interventions should be helpful in reducing morbidity and mortality associated with the disease. Finally, rapid diagnosis can serve to limit the spread to contacts and allow public health authorities the timely detection of new cases of VTEC and of contamination sources. Detection of VTEC infection should be carried out at least in paediatric patients with bloody diarrhoea and/or HUS, who are at greater risk of developing severe disease when infected with these pathogens. A rapid, sensitive, accurate and non-expensive diagnostic method should be incorporated into clinical laboratory protocols. As many VTEC serotypes can cause severe disease, the diagnosis should be independent of serotype. It is possible that the optimal approach to VTEC diagnosis could be by multiplex PCR analysis for the presence of vt_1, vt_2, *eae* and *ehxA* genes, which are present in most of the VTEC strains isolated from HUS patients.

ACKNOWLEDGEMENTS

The authors are greatly thankful to 'Sociedad Argentina de Pediatría', Hospital 'Ramón Santamarina', Laboratorio 'Pérez Cambet', 'Nueva Clínica Chacabuco', Municipalidad de Bahía Blanca, Hospital 'Dr José Penna', Hospital 'Alejandro Posadas' and Nuevo Hospital de Río Cuarto 'San Antonio de Padua' for their collaboration in sample collections. We also thank María R. Ortiz, Marcelo E. Sanz, Hilda Etchevarría and Guillermo H. Arroyo for their technical assistance. M. A. R. is a holder of a fellowship from the National Council of Scientific and Technical Research (CONICET). A. E. P. is a member of the Scientific Research Commission of the Province of Buenos Aires (CICPBA). This work was partly supported with grants from CICPBA, CONICET, Fundación Alberto J. Roemmers and the Fund for Scientific and Technological Research (FONCYT).

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