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VEROCYTOTOXIN-PRODUCING *ESCHERICHIA COLI* INFECTION IN FAMILY MEMBERS OF CHILDREN WITH HEMOLYTIC UREMIC SYNDROME

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Summary Thirty-four hemolytic uremic syndrome (HUS) patients and ninety-five family members were studied to determine the frequency of infection with verocytotoxin-producing *Escherichia coli* (VTEC) in household contacts using three diagnostic criteria: VTEC strains isolation and characterization, detection of free fecal VT (FVT) and VT-neutralizing antibodies (VT-NAbs). Gastrointestinal tract symptoms occurred in one to six familiy members in 8 (23.5%) of the index cases, the week before admission to hospital or simultaneously. The control group consisted of 34 children with acute gastroenteritis who did not develop HUS. Cumulative evidence of VTEC infection was found in 13 (38.2%) of 34 HUS patients, in 30 (31.6%) of 95 family members and in 10 (29.4%) of 34 control children. The serotypes of VTEC isolated were O157: H7 and O25: H2. The prevalent VT type was VT2 in VTEC and FVT; and VT1 in VT-NAbs. Both parents had the same infection rate by fecal toxin or serological data (11.1% FVT, 32% VT-NAbs). These were higher than those detected in siblings (6.2% FVT, 23.5% VT-NAbs) and grandparents (0% FVT, 18% VT-NAbs). Of 16 patients without evidence of infection, 3 had household contacts with FVT and 13 with VT-NAbs. Our results show the wide dissemination of VTEC in the population of Argentina and that family members of HUS patients are usually infected. Therefore, person-to-person transmission may play an important role in the high incidence of HUS in our country.

Key words: hemolytic uremic syndrome, family members, verocytotoxin

Infection by verocytotoxin-producing *Escherichia coli* (VTEC), particularly strains of serotype O157: H7, can cause sporadic cases and outbreaks of diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS). Other *E. coli* serotypes (O5: NM; O26: H11; O111: H8; O113: H21; O128: NM; O145: NM; among others) share a similar pathogenic potential, and the group is called enterohemorrhagic *E. coli* (EHEC).Although diarrhea usually resolves within a week, 5-10% of patients develop HUS, characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. The case-fatality rate is 3-5%.

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Risk factors for developing HUS among patients infected by *E. coli* O157: H7 include: age (children under 5 years and elderly people); weak or absent expression of P1 and Pk antigens by red blood cells[1], elevated leukocyte count, and use of antimotility agents and antimicrobial therapy for diarrhea[2].

Years before HUS emerged as an important pediatric disease in North America, it was the major cause of acute renal failure in infants in the cone of South America, being hyperendemic in Argentina[3] and endemic in Chile[4].

The association between HUS and infection by VTEC, particularly strains of serogroup O157, was first demonstrated in Canada in 1983-1985[5] and has been subsequently confirmed by numerous studies conducted in different countries[6], including Argentina[7, 8].

Outbreaks of infection have been linked to the consumption of contaminated water[9] or foods of bovine origin, such as ground beef and unpasteurized milk[10, 11]. Person-to-person transmission has occurred among family members[12, 14] in day care centers[15, 16] and in nursing homes[17].

The aim of this study was to determine the frequency of infection with VTEC in family members of children with HUS using several diagnostic criteria.

Material and Methods

Study population:

Between January 1988 and December 1989 thirty-four patients with HUS (19 males, 15 females; mean age 14.3 months ± 9.9 months) were admitted to Hospital General de Niños «Pedro de Elizalde» of Buenos Aires. Patients with HUS were defined as previously healthy children who developed acute renal insufficiency, thrombocytopenia and microangiopathic hemolytic anemia, following an acute diarrheal prodromal illness. Bloody diarrhea was observed in 91% of the HUS patients.

Ninety-five family members of children with HUS were enrolled in this study. Family members were defined as persons who lived with an HUS index case in the same house at least 5 hours daily. Gastrointestinal tract symptons occurred in one to six household contacts in 8 (23.5%) of the index cases, within the prior week or simultaneously to admission to hospital of the HUS patient.

The control group consisted of thirty-four children with acute gastroenteritis (19 males, 15 females, mean age 10.0 months ± 6.4 months), who did not develop HUS and who were admitted to the hospital in the same period as the patient with HUS. Bloody diarrhea was observed in 5.7% of the control cases.

Specimen collection:

Stool samples from HUS patients, their family members and control children were collected for culture and for cytotoxin determination after admission to hospital. Serum samples were obtained on admission to the study and, when possible, 20 days later.

Sample assays:

Three different diagnostic criteria were used to determine the frequency of infection with VTEC:

a) VTEC strains isolation[18], biotyping[19], serotyping[20] and virulence factors characterization

Ten lactose-fermenting colonies identified as *E. coli* were selected from a primary MacConkey agar culture to detect non-O157 VTEC. Ten sorbitol-negative colonies from a Sorbitol-MacConkey agar culture were picked to investigate O157 VTEC. Such isolates were subcultured onto Trypticase Soy agar. Single colonies from each subculture were inoculated into Penassay broth (Antibiotic Medium N° 3, Difco Laboratories, Detroit) that was incubated overnight at 37°C.

Bacterial supernatans and periplasmic cell extracts obtained by polymyxin B sulfate treatment of bacterial pellets[18] were assayed for cytotoxic activity on Vero cells[5].

E. coli virulence factors including cytotoxins, fimbrial adhesion (EHEC factor), and attaching and effacing factor (eae), were determined for all VTEC strains by biotin-d-UTP labeled gene probe under stringent conditions. Gene probes used were VT1 probe, a BamH1 fragment of 1.1 - kbp cloned from the pJN37 -19 plasmid; VT2 probe, a SmaI-PstI fragment of 0.84 - kbp cloned from the pNN110 - 18 plasmid; EHEC probe, a HindIII fragment of 3.4 - kbp cloned from the pCVD419 plasmid; eae probe, a SalI - Kpn1 fragment of 1 - kpb cloned from pCVD434[21].

Antibiotic susceptibility patterns were assayed by Kirby Bauer method for ampicillin, carbenicillin, cephalotin, chloramphenicol, streptomycin, gentamycin, nalidixic acid, colistin, and tetracyclin[22]. *E. coli* strains were serotyped with specific antisera for presence of different known O and H antigens by standard methods[20].

b) Detection of specifically neutralizable free fecal VT (FVT)

Equal volumes of the fecal specimen and PBS (0.01 M; pH 7.2) were thoroughly mixed and then centrifuged. A bacteria - free filtrate of the supernatant was assayed to VT activity. Cytotoxic activity of fecal extracts was assayed on Vero cells (ATCC CCL81) as previously described[5]. Specific toxin activity was evidenced by neutralization test using VT1 and VT2 specific monoclonal antibodies (MAb 13C4 and BC5BB12, respectively) provided by NA Strockbine, Center for Infectious Diseases, Atlanta, Georgia, USA.

c) *Serological test* to detect VT-neutralizing antibodies (VT-NAbs) was performed using 2 CD50 units of toxin preparations from reference *E. coli* strains [C-984 (VT1); 1271-84 (VT2); E32511 (VT2c)][5].

Fourfold or greater rises in titer were considered to indicate seroconversion.

Results

The detection of VTEC, FVT and VT-NAbs in children with HUS, in their family members and in

TABLE 1.— *Detection of verocytotoxin-producing* Escherichia coli *in children with Hemolytic Uremic Sindrome (HUS) and their family members*

Study Population	N° with VTEC (%)	Type of VT	Serotype	Biotype	Antibiotic Resistance Pattern
HUS Patients					
(n = 16)	3 (18.7)+	VT_2	O157: H7 ++	D	–
			O25: H2++		Am-Cb-Cf-C-Gm-St
Family members					
(n = 9)	1 (11.1)+	VT_2	O157: H7	D	Te

VTEC: Verocytotoxin-producing *Escherichia coli*; Am: Ampicillin; Cb: Carbenicillin; Cf: Cephalotin; C: Chloramphenicol; Gm: Gentamycin; St: Streptomycin; Te: Tetracyclin.
+: P>0.05 (Fisher's Exact Test).++: Two VT_2 - Producing *Escherichia coli* isolated from one patient.

TABLE 2.— *Detection of free fecal verocytotoxin (FVT) in children with Hemolytic Uremic Syndrome (HUS), in their family members and in control children with acute gastroenteritis*

Study Population	N° with FVT (%)	Type of Verocytotoxin VT_1	VT_2	VT_1-VT_2
HUS Patients (n = 34)	12 (35.3)*+	2	10	0
Family Members (n = 69)	7 (10.1)*	0	5	2
Control Children (n = 34)	1 (2.9)+	0	0	1

*+ P < 0.01 (χ^2 test with 2-tailed Yates's correction).

control children with acute gastroenteritis is shown in Tables 1, 2 and 3.

VTEC O157: H7, biotype D, VT2, susceptible to all the antibiotics tested was found in 3 (18.7%) of 16 patients. Two VT2 - producing *E. coli* belonging to serotypes O157: H7 and O25: H2 were isolated from one patient[23].

VTEC O157: H7, biotype D, VT2, resistant to tetracyclin, was detected in 1 (11,1%) of 9 family members (Table 1). VTEC strains were not detected in the control group.

There was no significant difference (p>0.05) in the VTEC strains isolation between HUS patients and their family members.

The long interval between onset of symptoms and stool collection (9.8 days ± 6.9 days); and the antimicrobial therapy administered to 76.5% of our HUS patients may have affected the VTEC strains isolation.

All O157: H7 *E. coli* strains were positive to VT2, fimbrial adhesion and eae factors with DNA probes.

The O25: H2 *E. coli* strain produced VT2 as shown by cytotoxic and neutralization assays on Vero cells and had a multiresistance antibiotic pattern. This strain hybridized with the EHEC gene probe but not with VT1, VT2 and eae gene probes.

FVT was detected in 12 (35.3%) of 34 patients; in 7 (10.1%) of 69 family members and in 1 (2.9%) of 34 control cases (Table 2). Significant differences (p<0.01) were found in FVT detection comparing HUS patients with their family members and with the control group.

FVT persists longer than VTEC strains in stool. In one patient FVT was detected 33 days after onset of symptoms.

A fourfold or greater rise in VT-NAbs titer was found in 3 (8.8%) of 34 patients. VT-NAbs were detected in 25 (27.5%) of 91 family members; 7 with seroconversion to VT and 18 with ≥ 1: 4 titers. VT-NAbs were detected in 10 (52.6%) of 19 children of the control group; 2 with seroconversion to VT and 8 with ≥ 1: 4 titers (Table 3). The prevalent VT type was VT2 in VTEC and FVT; and VT1 in VT-NAbs. These results are in agreement with previous reports from several countries[24, 27].

Both parents had the same infection rate according to fecal toxin or serological data (11.1% FVT, 32% VT-NAbs), these were higher than those detected in siblings (6.2% FVT, 23.5% VT-

TABLE 3.— *Detection of Verocytotoxin neutralizing antibodies (VT-NAbs)* in children with Hemolytic Uremic Syndrome (HUS) in their family members and in control children with acute gastroenteritis

Serological Data	HUS Patients (n = 34)	Family Members (n = 91)	Control Children (n = 19)
Presence of VT-NAbs: Titer ≥ 1: 4 against:			
VT_1	0	5	7
VT_2	0	6	0
VT_1 - VT_2	0	7	1
	0 (0.0%)	18 (19.8%)	8 (42.1%)
Seroconversión against:			
VT_1	1	3	1
VT_2	1	1	0
VT_1 - VT_2	1	3	1
	3 (8.8%)	7 (7.7%)	2 (10.5%)
Total	3 (8.8%)*+	25 (27.5%)*	10 (52.6%)+

* $P < 0.05$ (χ^2 test with 2-tailed Yates's correction). + $P < 0.01$ (Fisher's Exact Test).

NAbs) and grandparents (0% FVT, 18% VT-NAbs). Of 16 patients without evidence of infection, 3 had family members with FVT and 13 with VT-NAbs positive data.

Different infection patterns in family members of HUS patients in Argentina are shown in Figure 1.

Figure 1a shows a child with FVT and seroconversion to VT and his father with seroconversion to VT.

Figure 1b shows a girl with HUS and her mother with positive results for FVT, VTEC and seroconversion to VT-NAbs.

Figure 1c shows a HUS patient without meeting any of the three diagnostic criteria while her mother and brother presented seroconversion to VT-NAbs and her father and grandmother, positive serology to VT. This case shows the usefulness of evaluating family members when it cannot be demonstrated VT - associated illness in the index case.

Figure 1d shows a HUS patient with positive results for FVT and VTEC and his father with FVT.

Figure 1e shows a HUS patient without evidence of VT - associated infection but her parents, siblings and grandmother presented positive serology to VT indicating the prior exposition to VTEC of the whole family.

Figure 1f represents a HUS patient with FVT, her mother who had positive serology to VT and her father with seroconversion to VT.

Discussion

Since Gasser's early description[28], and due to a rise in the incidence of HUS, several etiologic hypotheses have been presented.

The association between VT - producing organisms and idiopathic HUS, as demonstrated by Karmali et al.[5] made etiologic diagnosis possible. However, for this instance, multiple assays are required: detection of FVT, isolation and characterization of VTEC and serologic assays for presence and/or seroconversion to VT-NAbs and to lipopolysaccharide (LPS) antibodies[29].

HUS bears a prodromic period, ranging from 6 to 10 days and is generally characterized by bloody diarrhea. *E. coli* O157: H7 is easily isolated from feces in the first week after symptoms appear; afterwards if becomes difficult to isolate[15]. DNA probes to the VT genes, used to screen hundreds of *E. coli* colonies per isolation plate, provide a sensitive way to detect the small amount of organisms present late in infection[30]. Methods of detecting FVT have the advantage of detecting cytotoxins produced by any VTEC in the abscence of living organisms[5, 31].

Cumulative evidence of VTEC infection was found in 13 (38.2%) of 34 HUS patients and in 30 (31.6%) of 95 family members using microbiological and serological detection methods. However, 16/21 (76.2%) patients who met no diagnostic cri-

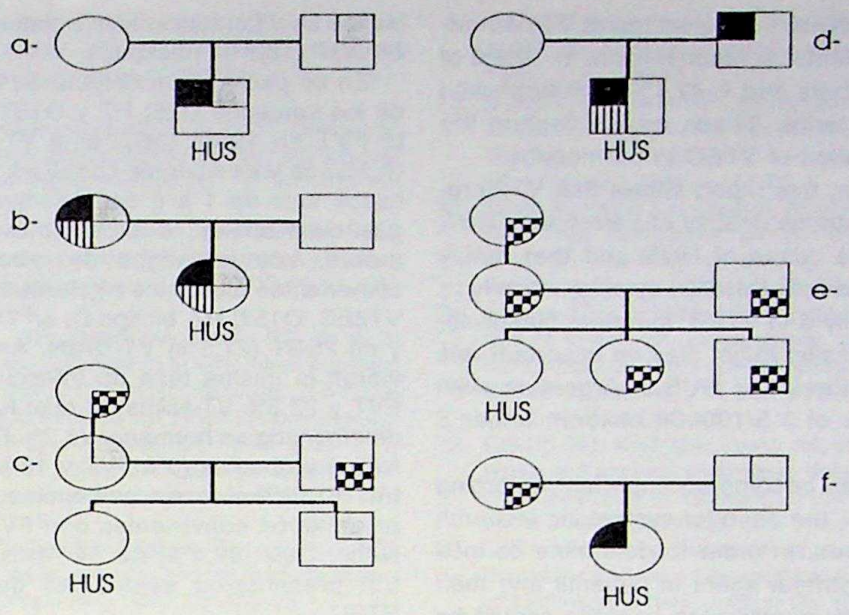

Fig. 1.— Evidence of infection with VT-producing *Escherichia coli* in family members of six children with Hemolytic Uremic Syndrome (HUS).

Female
FVT
Seroconversion
Male
VTEC
Positive Serology

teria had, at least, one family member with evidence of infection.

Our work has demonstrated that the use of several diagnostic criteria increases the possibility of establishing an association between HUS and VTEC infection. In addition to this, evaluation and analysis of family members of HUS patients are useful for an accurate and early diagnosis, in order to determine the appropriate therapy to prescribe.

Evidence of VTEC infection could be understimated because LPS serology was not performed in this work. Further studies including investigation of serological response to LPS are necessary to assess the real frequency of O157:H7 infection in our country.

The occurrence of most HUS cases after a diarrheal prodrome, and the tendency for cases to occur in clusters within communities and families led many researchers to postulate several routes of transmission.

Outbreaks might be originated by simultaneous exposure of several individuals to a common foodborne source. In sporadic cases there might exist a primary infection, strongly associated with the ingestion of contaminated food or water; secondary person-to-person transmission may occur in small communities or families. Familial outbreaks of HUS[13, 14], and bloody diarrhea in siblings of patients[12] have been reported, even though they have not been numerous.

A higher incidence of HUS has been reported in Canadá[32] in children who have a father who is either a physician or a lawyer, attributed to their parent's habit of eating in fast-food restaurants for reasons of professional duties. Besides, children might acquire the infection in day care centers[33] or kindergartens. We have found a similar, and higher rate of infection in parents than in siblings and grandparents.

Furthermore, Rowe et al[34] have shown that patients with HUS were more exposed to family or non-family contacts with gastroenteritis than healthy controls, this implies that person-to-person transmission could be an important factor in the development of HUS in children.

It has been demonstrated that 20% of Argentinean healthy children belonging to the risk

group have VT-NAbs[8]. Also we found VT-neutralizing activity, without seroconversion, in 19.8% of the family members and in 42.1% of children with acute gastroenteritis. These results confirm the wide dissemination of VTEC in our country.

In conclusion, this report shows that VT2-producing *E. coli* strains, mainly of serogroup O157, cause sporadic cases of HUS and that family members are usually infected symptomatically or asymptomatically with VTEC; therefore person-to-person transmission might play an important role in the high incidence of HUS in Argentina, with an annual rate of 7.8/100000 children under 5 years of age.

As VTEC has become an important emerging pathogen lately, the need for systematic research must be stressed, in order to determine its incidence as a diarrheal agent in patients and their family members; surveillance in cattle should be performed as a way of identifying reservoirs and routes of transmission to break the epidemiologic chain. Laboratories must use Sorbitol-MacConkey agar for the detection of VTEC O157 and determine if non-O157 strains are VT producers by means of Enzyme Linked Immunosorbent Assay, DNA probe hybridization; PCR techniques and/or specific cytotoxicity assay in tissue cultures. Since therapy is limited and only supportive, public heath efforts must be directed to the prevention of infection and disease.

Resumen

Infección con Escherichia coli *productor de verocitotoxina en convivientes de niños con síndrome urémico hemolítico*

Se estudiaron 34 pacientes con el sindrome urémico hemolítico (SUH) y 95 convivientes para determinar la frecuencia de infección con *E. coli* productor de verocitotoxina (VTEC), utilizando distintos criterios diagnósticos. El grupo control consistió en 34 niños con gastroenteritis aguda que no desarrollaron SUH. Se obtuvieron muestras de materia fecal y suero en el momento de internación del caso índice y 20 días después. Los criterios diagnósticos utilizados fueron: a) aislamiento de VTEC y su biotipificación, serotipificación y caracterización de factores de virulencia; b) detección de verocitotoxina libre en materia fecal (FVT); c) detección de anticuerpos neutralizantes (NAbs) a-VT1 y a-VT2. Se aisló VTEC O157:H7, biotipo D, VT2, susceptible a todos los antibióticos en 3/16 (18,7%) pacientes.

En un paciente se detectaron 2 cepas VT2EC de los serotipos O25: H2 y O157: H7. Se detectó FVT en 12/34 (35,3%) y VT-NAbs en 3/34 (8,8%) de los pacientes con SUH. El 23,5% de los casos tuvo de 1 a 6 convivientes con síntomas gastrointestinales, la semana previa o simultáneamente. Los hallazgos de laboratorio en los convivientes fueron los siguientes: en 1/9 (11,1%) VT2EC, O157: H7, biotipo D; en 7/69 (10,1%) FVT y en 25/91 (27,5%) VT-NAbs. Ambos padres tuvieron la misma tasa de infección (11.1% para FVT y 23.5% VT-NAbs) la cual fue mayor que la determinada en hermanos (6.2% FVT y 23.5% VT-NAbs) y abuelos (0% FVT y 18% VT-NAbs). Entre 16 pacientes sin evidencias de infección, 3 presentaron convivientes con FVT y 13 con VT-NAbs. Diez (29,4%) de 34 niños del grupo control presentaron evidencias de infección por VTEC.

Nuestros resultados muestran el carácter endémico de la infección por VTEC, que los convivientes de los pacientes con SUH están usualmente infectados y que la transmisión persona a persona puede jugar un rol importante en la alta incidencia de la enfermedad en nuestro país.

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