

## Molecular Detection of Human Calicivirus among Spanish Children with Acute Gastroenteritis

E. Roman,<sup>1</sup> A. Negredo,<sup>2</sup> R. M. Dalton,<sup>2</sup> I. Wilhelmi,<sup>3</sup> and A. Sánchez-Fauquier<sup>2\*</sup>

*Servicio de Pediatría<sup>1</sup> and Servicio de Microbiología,<sup>3</sup> Hospital Severo Ochoa, Leganés, and Centro Nacional de Microbiología, Instituto de Salud Carlos III,<sup>2</sup> Madrid, Spain*

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**A survey was conducted among Spanish children with gastroenteritis treated in an emergency room. Reverse transcription-PCR with specimens negative for other enteric pathogens was used. The minimum incidence of human calicivirus infection was 7.7%, with Lordsdale as the predominant genotype. The clinical features and severity of calicivirus and rotavirus were similar.**

Norwalk-like virus (NLV) and Sapporo-like virus (SLV) are members of the family *Caliciviridae* (7), which has been considered one of the most common causes of nonbacterial gastroenteritis outbreaks and sporadic cases in adults and children (3, 6, 11, 25).

Routine detection is not yet possible due to the lack of a simple diagnostic test. Thus, the most-used human calicivirus (HuCV) detection assay is generic reverse transcription-PCR (RT-PCR) with the RNA polymerase gene (*pol*) as the target. Thus far, the RT-PCR assay has not been routinely used for the detection of childhood gastroenteritis etiologic agents. Although HuCV is emerging as a cause of sporadic gastroenteritis as a sole pathogen in young children, there are not many data about the incidence of this viral infection. During previous studies in other countries, this infection was detected in 3.5 to 20% of sporadic cases among young children (1, 2, 5, 9, 10, 12, 14, 15, 16, 22, 24). To date, there is to our knowledge a lack of data from Spain. In order to clarify the epidemiologic role and clinical significance of calicivirus-associated gastroenteritis in our country, we have conducted a surveillance study among children with gastroenteritis treated in an emergency room in Madrid, Spain. Our objectives were to determine the incidence of HuCV infection and the clinical characteristics of the disease and to establish the genetic diversity of HuCV strains. A total of 822 fecal specimens were collected from children of less than 4 years of age with gastroenteritis who visited the emergency room of Severo Ochoa Hospital in Madrid between October 1996 and September 1997. A gastroenteritis episode was defined as at least three looser-than-normal stools within a 24-h period or an episode of forceful vomiting with any loose stools. Clinical information was collected for all patients, including age, sex, presenting symptoms, and duration of illness prior to admission. Each episode was graded by using a 20-point severity score scale as previously described (17). Fecal specimens were screened for etiologic agents of diarrhea. Bacteria (*Salmonella*, *Shigella*, *Yersinia*, and *Campylobacter* spp. and *Vibrionaceae*) were detected by routine cultivation meth-

ods, and viruses (group A rotavirus, adenovirus, and astrovirus) were detected by commercial enzyme immunoassays.

No pathogens were detected in fecal specimens from 292 (35.5%) children. A subset of 201 of these samples with an adequate volume of fecal specimen was tested for the presence of HuCVs (NLV and SLV) by using RT-PCR with the RNA polymerase gene (*pol*) as the target. For this, RNA was extracted from 20% of the stool suspensions in phosphate-buffered saline by using the guanidine thiocyanate method (23) and the RNaid Spin kit (Q-BIOgene; Bio 101). Samples were first tested with the JV12-JV13 primer pair, which detects only NLV agents (21), and the samples that tested negative with this test were then analyzed with the p289-290 primer pair, which detects NLV and SLV agents (8). PCR products were analyzed by electrophoresis in 2% (wt/vol) agarose-Tris-borate-EDTA gels and detected by UV illumination after staining with ethidium bromide.

A subset of HuCV-positive samples was genetically characterized by either reverse line blot hybridization (RLB)

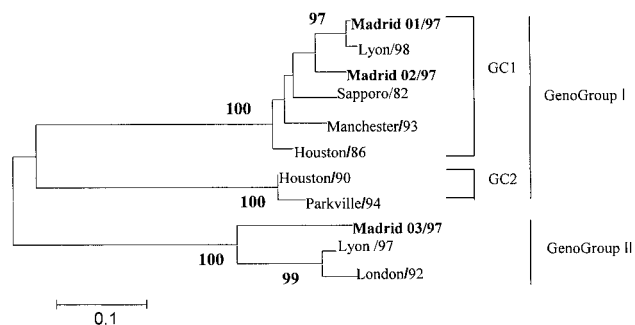


FIG. 1. Phylogenetic tree based on analyses of a 236-bp region of the RNA polymerase gene of three Spanish strains of SLV obtained in this study. Distances were calculated by the Kimura 2p method, available in the MEGA, version 2.1, analytical package. The tree was drawn by using the neighbor-joining method. Bootstrap values for each node are given if they are >80%. Genogroups and genetic clusters (GC) were named according to the Schuffenecker study of genetic classifications of SLVs (18). GenBank accession numbers for the SLV sequences used in this study are as follows: Lyon/98, AJ251991; Sapporo/82, U65427; Manchester/93, X86560; Houston/86, U95643; Houston/90, U95644; Parkville/94, U73124; Lyon/97, AJ271056; London/92, U95645. Spanish strains are marked in bold.

\* Corresponding author. Mailing address: Instituto de Salud Carlos III, Centro Nacional de Microbiología, Ctra. Majadahonda a Pozuelo Km. 2, 28220 Madrid, Spain. Phone: 34 91 509 79 01, ext. 3655. Fax: 34 91 509 79 66. E-mail: asanchez@isci.es.

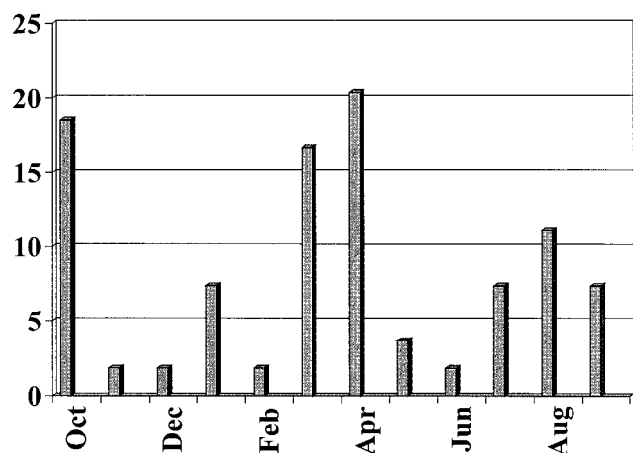


FIG. 2. Monthly distribution of HuCV-associated cases as percentages of HuCV-positive samples.

(20) or sequence analysis. The sequences were analyzed with CLUSTALX, version 1.8, methods.

Statistical analysis was performed with the Mann-Whitney U test method, which was used to compare the medians of clinical symptom scores between rotavirus- and HuCV-associated cases. For comparison of proportions, chi-square or Fisher's exact test was used. All tests were two-tailed and considered significant when the  $P$  value was  $\leq 0.05$ .

HuCV was detected by RT-PCR in 63 (31%) of 201 diarrhea samples that were negative for other common enteric pathogens. Of these 63 samples, 54 were positive with the JV12-JV13 (21) primer pair, which detects only the NLV genus, and 9 samples were positive with the p289-290 primer pair, which is able to detect the NLV and SLV genera (8).

Twenty-nine samples were genotyped either by RLB or by sequence analysis (the RLB method was used for 24 samples testing positive with the JV12-JV13 primer pair).

This genotype analysis showed that 26 samples belonged to the NLV genus and 3 samples belonged to the SLV genus. Of the 26 NLV samples, 23 were related to the Lordsdale virus, 2 were related to the Mexico virus, and 1 was related to the Queen Arms virus. Of the three SLV samples, two were related to genogroup I genetic cluster 1 (Sapporo/82) and one was related to genogroup II genetic cluster 1 (London/92) (Fig. 1) (18).

The median age of children with HuCV was 15.12 months (range, 1 to 47 months). The seasonal distribution of gastroenteritis episodes caused by HuCV is shown in Fig. 2. HuCV was detected year-round, with two peak seasons in October and in March and April.

From the 63 cases positive for HuCV, 52 (82.5%) were associated with vomiting, 20 (31.7%) were associated with fever, 15 (24%) were associated with mild dehydration, and 1 (1.6%) was associated with severe dehydration. Hospitalization was required in 8 (12.6%) cases.

To further assess the medical importance of HuCV infection, the clinical characteristics of children infected with HuCV were compared with those found in patients infected with rotavirus (205 of 822) (Table 1). The clinical features and the severity of HuCV and rotavirus gastroenteritis episodes were similar, although children with HuCV infection were less likely to be dehydrated ( $P < 0.05$ ), despite the longer duration of diarrhea observed among these patients.

This is the first report, to our knowledge, of HuCV detection among Spanish children with sporadic gastroenteritis. Our results demonstrate that HuCV might be an important and under-appreciated cause of diarrhea in Spanish children. This study provides the lowest possible estimate of the magnitude of the problem (63 of 822 cases, 7.7%) because we screened only those specimens which had no other pathogens. In addition, some positive samples could escape detection with the primer pairs used.

Our results confirm those of studies done in The Netherlands (10), South Africa (24), France (Rouen) (12), and China (16). However, this incidence is lower than that found in studies from Australia (9), France (Dijon) (1), Finland (14, 15), Mexico (4), and Chile (13). The reasons for this discrepancy could be attributable to the different methods used to diagnose HuCV as well as the diversity of the studies.

As described in previous studies, most of the HuCVs identified worldwide belonged to the NLV genus (89.6%) and the majority of the strains were related to the Lordsdale virus (4, 5, 9, 19). SLV appears to be an uncommon cause of severe gastroenteritis in young Spanish children, although further studies are needed to confirm this data.

The clinical severity of HuCV infection has been reported only in Finnish (14) and Japanese (26) studies up until now. Our findings showed that the mean HuCV clinical severity was equal to that in rotavirus gastroenteritis, as in the Japanese

TABLE 1. Clinical characteristics of acute gastroenteritis associated with HuCV or rotavirus

Variable	Median (range) for patients with:		Statistical significance ( $P$ ) <sup>a</sup>
	HuCV ( $n = 63$ )	Rotavirus ( $n = 205$ )	
Diarrhea (days)	2 (1-13)	1 (1-8)	<0.05
Diarrhea (maximum no. of times/day)	4.5 (1-15)	5 (1-20)	NS
Vomiting (days)	1 (0-7)	1 (0-5)	NS
Vomiting (maximum no. of times/day)	3 (0-15)	3 (0-20)	NS
Maximum fever (°C)	37 (37-40)	37 (36-40)	NS
Severity score (points) <sup>b</sup>	10 (2-16)	10 (1-16)	NS
Dehydration (%)	26	41	<0.05
Hospitalization (%)	12.6	19.5	NS

<sup>a</sup> NS, not significant.

<sup>b</sup> The maximum possible severity score was 20 points.

study (26). However, our data are at variance with those of the Finnish study (15).

Undoubtedly, future studies should be applied to determine the true epidemiologic role of HuCV diarrhea among Spanish children.

**Nucleotide sequence accession numbers.** GenBank accession numbers for the SLV sequences analyzed in this study are as follows: AY102703 (Madrid01/97), AY102704 (Madrid02/97), and AY102705 (Madrid03/97).

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