

## CONCISE COMMUNICATION

## Human Caliciviruses Are a Significant Pathogen of Acute Sporadic Diarrhea in Children of Santiago, Chile

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Human caliciviruses (HuCVs) are increasingly recognized as common pathogens that cause acute sporadic diarrhea in children; however, regional antigenic and genetic diversity complicate detection techniques. Stool samples from children seeking medical attention in 2 outpatient clinics, a large emergency department, and 2 hospital wards were evaluated for HuCVs by reverse transcription–polymerase chain reaction, using primers based on a conserved sequence of the polymerase region of a previously sequenced Chilean strain. HuCVs were detected in 53 (8%) of 684 children 1 month to 5 years of age (mean, 13 months). Detection occurred year-round without a clear seasonal peak, and detection frequency declined from 16% in 1997 to 2% in 1999. The decline may have been due to a change in virus genotype. HuCVs are a significant pathogen of acute sporadic diarrhea in Chilean children, and continuous characterization of genetic diversity will be crucial for appropriate detection.

Human caliciviruses (HuCVs) are the most common cause of viral diarrhea outbreaks in adults and are mainly associated with consumption of water and contaminated seafood [1]. The role of these viruses in nonoutbreak (sporadic) diarrhea in children is less clear. Seroprevalence studies suggest that HuCV infections occur in early childhood, especially in developing countries [2, 3]. In Chile, we demonstrated that by 5 years of age, anti-HuCV antibodies are present in >70% of children in a large metropolitan city (Santiago) and in the smaller southernmost city (Punta Arenas) [4]. Antibody presence was significantly associated with lower socioeconomic level and with consumption of seafood and uncooked vegetables.

Despite seroprevalence studies that indicate high exposure to HuCVs among children, detection of this pathogen in stools of children with diarrhea has been mostly unsuccessful [5, 6]. Because HuCVs cannot be cultured, detection methods rely on antigen-antibody reactions (ELISAs), using antisera to baculovirus-expressed capsids as antigens, or on amplification of

conserved regions by reverse transcription–polymerase chain reaction (RT-PCR). Problems with HuCV detection seem to be related to antigenic and genetic diversity within the HuCV family [7]. CVs that commonly cause human infections are currently classified into 2 antigenically distinct genera that include multiple distinct genetic clades of uncertain biologic significance [5–8].

In a previous study, we were relatively unsuccessful in detecting HuCVs in diarrhea samples from a large number of Chilean children [9]. By use of Norwalk virus (NV) and Mexico virus (MX) HuCV ELISAs, 0 of 677 stools from children 0–132 months of age with acute diarrhea were HuCV positive. The children attended child care centers (CCCs) or presented at outpatient clinics or emergency departments (EDs). A subset of samples was tested by RT-PCR with 3 different sets of primers (NV 35/36, Sapp 35/36, and JV 12/13), and only 1 sample was HuCV positive. That sample was from a CCC child with acute diarrhea. This HuCV strain was genetically distinct from known HuCVs and was closest in nucleotide identity (77% in the 3D genomic region) to HuCV/London/29845/92/UK, a Sapporo-like HuCV [9].

To design primers that might be more sensitive for HuCV detection, we aligned published and our unpublished NV-like and Sapporo-like sequences. We used the resulting primer pair, p289/p290 [10], to test diarrhea stools obtained prospectively over 2 years from children treated in different clinical settings.

### Patients and Methods

*Patient selection and stool sample collection.* The study was conducted from February 1997 through September 1999. Five

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**Table 1.** Human calicivirus (HuCV) positivity by year and by source of stool samples.

Source	Age in months, mean (range)	No. of HuCV-positive samples/ no. of samples tested (%), by year			
		1997	1998	1999	Total
<b>Diarrhea patients</b>					
Emergency department	13.7 (0.5–45)	8/39 (21) <sup>a</sup>	18/120 (15)	0/89	26/248 (10)
Outpatient clinic 1	17 (1–58)	3/25 (12)	0/42	0/13	3/80 (4)
Outpatient clinic 2	20.5 (0.5–60)	Not done	11/98 (11)	4/96 (4)	15/194 (8)
Hospital 1	7.6 (0.5–30)	1/12 (8)	5/30 (17)	0/18	6/60 (10)
Hospital 2	9.5 (1–48)	Not done	2/61 (3)	1/41 (2)	3/102 (3)
Total	15.2 (0.5–60)	12/76 (16) <sup>a</sup>	36/351 (10)	5/257 (2)	53/684 (8)
<b>Control patients</b>					
Emergency department	11.3 (0.5–40)	Not done	0/43	1/37 (3)	1/80 (1)
Hospital 1	6.7 (1–12)	Not done	0/11	0/7	0/18
Hospital 2	8.9 (0.7–52)	Not done	0/11	0/21	0/32
Total	9 (0.5–52)	Not done	0/65	1/65 (2)	1/130 (<1)

<sup>a</sup>  $P < .001$  by  $\chi^2$  test for trend of decrease in detection rates observed, 1997–1999.

health care facilities, including 2 outpatient clinics, a large ED, and 2 hospital wards of the Chilean National Health Care System, enrolled patients. Combined, these sites serve a population of ~2 million persons, the vast majority of whom are of low or mid-low socioeconomic status. At each site, all children  $\leq 60$  months of age with acute, nonbloody diarrhea of  $< 3$  days' duration were eligible for stool sample collection. Three sites participated from 1997 through 1999 and 2 from 1998 through 1999. Both participating outpatient clinics and 1 hospital ward (Roberto del Río Hospital; hospital 2) belong to the north health care area of Santiago. During 1998–1999, they had means of 1200 pediatric outpatient visits and 190 pediatric hospitalizations per month. The participating ED and the other hospital ward are part of the Sótero del Río Hospital (hospital 1) in southeast Santiago. These had means of 9200 ED visits and 311 hospitalizations for children per month during a similar period. Designated personnel obtained samples in the outpatient clinics and ED during weekday regular work hours. A stool sample was obtained by hospital staff from all children hospitalized with acute nonbloody diarrhea, as part of the routine workup for severe acute diarrhea.

**Sample processing.** All stool samples were fresh ( $< 30$  min old) and were stored at  $-70^\circ\text{C}$  until testing. Selected samples were processed for HuCVs at the University of Chile microbiology laboratory. Samples were processed for RT-PCR by the TRIzol extraction method (Gibco BRL, Gaithersburg, MD). A 500- $\mu\text{L}$  suspension of stool sample (1:5 in PBS and vortexed with 150  $\mu\text{L}$  of Genetron; Sigma, St. Louis) was centrifuged for 5 min at 18,000  $g$ . A mix that included 200  $\mu\text{L}$  of the supernatant, 600  $\mu\text{L}$  of TRIzol-R, and 160  $\mu\text{L}$  of chloroform was incubated for 3 min at room temperature and centrifuged at 13,000 rpm for 15 min at  $4^\circ\text{C}$ . Aqueous-phase RNA was extracted with isopropanol over 12 h at  $-20^\circ\text{C}$  and then precipitated with ethanol. The precipitant was resuspended in 20  $\mu\text{L}$  of RNase- and DNase-free distilled water [10].

Primers for RT-PCR 289/290 produce 319 and 331 nt products for NV-like and Sapporo-like HuCVs, respectively, in the 3D genomic region of open-reading frame 1, a relatively conserved region of the genome that encodes for the polymerase. For RT-PCR, 3  $\mu\text{L}$  of extracted RNA was mixed with 48  $\mu\text{L}$  of reaction mixture (8 U of avian myeloblastosis virus RT, 20 mM of each dNTP, 0.2  $\mu\text{g}$  of primer 289, 10 U of RNasin, 5  $\mu\text{L}$  of 1% bovine serum albumin, 5  $\mu\text{L}$  of  $10\times$  buffer, and 31  $\mu\text{L}$  of RNase- and DNase-

free distilled water). The reaction mixture was incubated for 1 h at  $42^\circ\text{C}$ . After this process, the tubes were spun, and 50  $\mu\text{L}$  of PCR mix containing 5  $\mu\text{L}$  of  $10\times$  buffer, 2  $\mu\text{g}$  of primer 290, 5 U of Taq polymerase, and 42  $\mu\text{L}$  of RNase- and DNase-free distilled water was added. Samples were denatured for 3 min at  $96^\circ\text{C}$  and were subjected to 40 cycles of amplification: 30 s at  $96^\circ\text{C}$ , 1 min at  $50^\circ\text{C}$ , and 2 min at  $72^\circ\text{C}$ . The RT-PCR product was separated on a 1% agarose gel, stained with ethidium bromide, and visualized by UV light.

## Results

**Population enrolled and selection of samples for HuCV testing.** In total, 1787 diarrhea samples were obtained from ED patients during the 31-month study period. For HuCV testing, we selected 10 samples per month or all available ED samples if 10 were not available, for a total of 248 samples (table 1). In addition, we tested 80 samples from age-matched control children without diarrhea who presented to the ED from April 1998 through August 1999. Of the diarrhea samples from hospitalized children, 280 were from hospital 1 and 300 from hospital 2 during the 25- and 17-month enrollment periods, respectively. For HuCV testing, we randomly selected 3 and 6 samples per month, respectively (or all available): 60 samples from hospital 1 and 102 from hospital 2. In addition, we tested 65 samples obtained from time- and age-matched children who were hospitalized during 1998 and 1999 for reasons other than diarrhea. We tested all 274 diarrhea samples from the 2 participating outpatient clinics for HuCVs. Control samples were not available from the outpatient clinics. As expected, children hospitalized for management of acute diarrhea were, in general, younger than children managed in the ED; the latter were younger than children managed in outpatient clinics (table 1).

**Characterization of HuCV positivity.** In total, 53 (8%) of 684 diarrhea stool samples were positive for HuCV (table 1). During 1998–1999, 41 (6.7%) of 608 diarrhea samples and 1 (0.8%) of 130 control samples were positive for HuCV ( $P = .01$ ,  $\chi^2$  test with Yates's correction). HuCV-positive samples were obtained from all sites throughout the study period. There

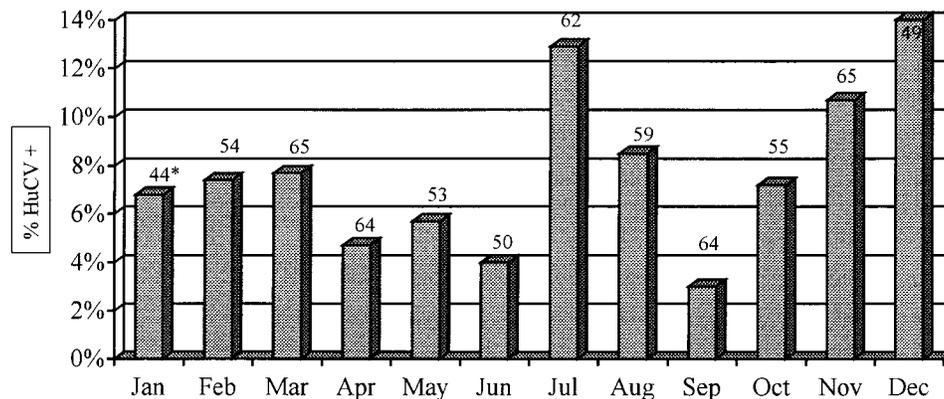


Figure 1. Monthly distribution of human calicivirus (HuCV) positivity. \*No. of samples tested.

was a trend toward a higher HuCV detection rate among children with diarrhea managed at the ED than among children managed in outpatient clinic 1 during the study period (10% vs. 4%;  $P = .06$ ,  $\chi^2$  test). HuCV detection rates declined progressively from 16% in 1997 to 2% in 1999 ( $P < .001$ ,  $\chi^2$  test for trend), a decline observed at all study sites. Preliminary analysis did not suggest that the decline was associated with a compensatory increase in other enteric viruses (authors' unpublished data). HuCVs were detected in children of all ages (1–60 months), and the mean ages of HuCV-positive and -negative children were similar (14.6 months vs. 15.3 months, respectively;  $P = .73$ , Student's  $t$  test). Figure 1 shows the monthly distribution of HuCV detection rates for all sites. HuCVs were detected year-round, without a clear seasonal predominance.

## Discussion

HuCVs are significant pathogens of infantile gastroenteritis. In recent studies in which ELISA and RT-PCR were used to detect HuCV in children, HuCVs caused 14% and 20% of the cases of acute gastroenteritis in French and Finnish children, respectively [11, 12]. ELISAs that use antibodies from baculovirus-expressed antigens may be highly specific but are of low sensitivity, due to the antigenic variability of capsids on circulating strains [6, 13]. In addition, genetic diversity is also high among HuCVs, even in the most conserved genomic regions. Efforts to improve detection yields must focus on development of either universal primers or primers targeted toward regional circulating strains [7, 14].

A previous seroprevalence study from Chile suggested that HuCV exposure occurs early in life [4], but detection rates were extremely low (<0.5%) with the use of NV and MX virus baculovirus-expressed antigen ELISAs or RT-PCR using well-recognized primers (NV 35/36, Sapp 35/36, and JV 12/13) [9]. The only HuCVs detected had a distinct polymerase region when compared with other known HuCVs. With this in mind, we developed new primers closely related to the previously isolated

strain. This strategy significantly increased our yield to 8%, a yield that may be improved with the genetic characterization of more strains. This 8% yield is a cross-sectional estimate for this population and represents "sporadic" episodes that occur year-round among children with diarrhea, not in recognized outbreaks. It is possible that some cases may have occurred in small, undetected outbreaks.

Of note, the detection rate decreased from 16% in 1997 to 2% in 1999, a decline that occurred at all participating sites. This decline probably is a consequence of genetic variation that decreased the sensitivity of our RT-PCR, rather than a true decline in HuCV circulation; a demonstration of this hypothesis, however, will require future study. Although the number of HuCV detections was not high enough to clearly define overall epidemiology, we detected HuCVs year-round without clearly identifiable seasonal peaks. Seasonal trends of HuCV infection have been reported in some studies [12] but not in others [6], and for Chile a definite answer on seasonality will require larger studies.

In summary, we have demonstrated that, in developing countries like Chile, HuCVs represent an important cause of acute sporadic gastroenteritis and that appropriate primers designed for local circulating strains significantly improve detection rates. Use of appropriate primers in well-designed epidemiologic studies will define more precisely the role of HuCVs in acute diarrhea of children in developing countries.

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