Human Calicivirus-Associated Diarrhea In Children Attending Day Care Centers

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We investigated human calicivirus (HCV)-associated diarrhea in children attending day care centers by using stool specimens collected in 1981–1983. We used a screening enzyme-linked immunosorbent assay (ELISA) derived from reagents prepared against the Sapporo strain of HCV and confirmed positive results with a blocking ELISA and immunosorbent electron microscopy. HCV was detected in 11 (2.9%) of 375 diarrheal stools and in none of 86 stools from asymptomatic contacts. This incidence rate was half that noted for rotaviruses and higher than that noted for *Campylobacter*, *Salmonella*, and *Shigella* in the original study. HCV was found in stool specimens from children in nine day care centers; HCV-associated diarrhea was sporadic, occurred with greater frequency in young children, and had a summer-fall predominance. Our results indicate that HCV is an important cause of diarrhea in day care centers and that frozen stool samples can yield epidemiological data on HCV infection.

In 1985, investigators from the Centers for Disease Control (CDC) reported on the incidence of diarrhea in day care centers in Maricopa County, Ariz [1]. An enteric pathogen (*Giardia*, rotavirus, *Campylobacter*, *Salmonella*, or *Shigella*) was identified in association with 20% of the diarrheal episodes. The majority of diarrheal episodes, whose etiology remained undetermined, were likely caused by viruses other than rotavirus. Viruses recently associated with childhood diarrhea and not tested for in that study include human caliciviruses (HCVs); adenovirus types 40 and 41; astroviruses; non-group-A rotaviruses; coronaviruses; and unclassified, small, round viruses [2].

Madeley and Cosgrove [3] detected HCVs in human stool specimens in 1976, and McSwiggan et al. [4] associated HCV with an outbreak of vomiting in children in 1978. Outbreaks of diarrhea associated with HCV have been reported in all age-groups in England, Japan, and Canada [4–10]. Diarrheal stools from children on four continents were examined by direct electron microscopy (EM) for detection of virus; caliciviruses have been found in 0.2%–6.6% of specimens [11–15]. Serological studies indicate that infection with HCV is common where studied in Asia, Australia, Africa, Europe, and North America [16–18]. The low prevalence of detectable virus in hospitalized patients with gastroenteritis and the contrasting high prevalence of antibody suggest, however, that although infection with HCV is common, most infections remain undiagnosed.

Serological cross-reactions of paired sera from outbreak-associated cases with the outbreak strains indicate that currently recognized HCVs may fall into five antigenic types: UK1, UK2, UK3, UK4, and Sapporo (Japan) [19]. HCVs are not abundant in clinical samples, and they have not yet been grown in the laboratory. A hyperimmune guinea pig antiserum prepared in 1983 against the Sapporo antigenic type of HCV (HCV [Sapporo]) and incorporated into an RIA [20] detects three of the other four known HCV types [19]. This RIA and ELISAs that incorporate recently prepared rabbit and mouse hyperimmune antisera to HCV (Sapporo) allow rapid screening and

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confirmation of HCV antigen and antibody to HCV in clinical specimens. Both the RIA and the ELISAs are more sensitive for HCV detection than is direct EM or immunoelectron microscopy (IEM) [18, 20].

Using the ELISAs, we initiated epidemiological studies of HCV infection in the United States. A study of age-specific antibody prevalence in Houston indicated that the highest incidence of antibody acquisition was in children between four months and four years of age [18]. We investigated HCV infection in day care centers because attendees are in this age range and because intestinal infections are common in this setting [21]. We were also interested in ascertaining whether tests of specimens previously collected and frozen for six years would yield epidemiological data and antigen for preparing additional laboratory reagents.

Materials and Methods

Description of the day care center study. The longitudinal study of diarrhea in day care centers in Maricopa County, Ariz, was conducted from September 1981 through September 1983 [1]. Twenty-two day care centers geographically distributed throughout greater Phoenix were selected. Through telephone contact with the day care centers, a nurse conducted active surveillance for diarrheal illness four days a week throughout the study period. A sporadic case of diarrhea was defined as unusually loose or frequent stools, as determined by the caregiver, in a child who had not had diarrhea within the preceding seven days. A single stool specimen was collected from each child with sporadic diarrhea on the day illness was identified. A diarrheal outbreak was defined as three or more cases in one day or five or more cases in any seven-day period in one day care center. Eighteen outbreaks occurred during the study period; stool specimens were obtained from all ill children.

For sporadic cases and for ill and well children during outbreaks, epidemiological data about the child, the illness, and the household were obtained by using a telephone questionnaire. During the last 14 mo of the study, a follow-up questionnaire was used to determine the duration of symptoms, the level of medical care required by the child, and the occurrence of illness in household contacts. Diarrheal illness in children ≤ 36 mo of age was the basis of the published report [1].

Selection of stool specimens for HCV testing.

We tested for the presence of HCV in stool samples from sporadic and outbreak cases of diarrhea and in stools from contacts of outbreak cases. These specimens had previously been tested for the presence of *Salmonella*, *Shigella*, *Campylobacter*, rotavirus, and *Giardia lamblia*. The specimens had been stored in glass vials at -20°C since their collection.

A subset of available specimens was selected for testing, as follows: (1) stool samples from at least four cases and four contacts for each of the 18 outbreaks, (2) at least 10 diarrheal stool samples from each month of the study, and (3) at least 20 diarrheal stool samples from children in each of the age-groups from six months to three years. The first specimens that met these criteria were selected from a computer listing arranged by specimen number; specimen numbers had been assigned chronologically by date of collection. The presence of a previously detected pathogen was not considered when selecting specimens.

We used the definitions of the study population from the original report, with two exceptions. First, we did not exclude children >36 mo of age. Second, the original controls for the outbreaks could have diarrhea at the time of the stool collection but were excluded as outbreak-associated cases if their diarrhea began before the outbreak, as defined. We redefined outbreak controls to be outbreak cases if the child had diarrhea on the day of specimen collection. Three outbreak controls tested for HCV, including one with HCV detected in the stool specimen, were redefined as outbreak cases by this criterion.

Viruses and antisera. Stool specimens containing HCV (Sapporo); guinea pig, rabbit, and mouse hyperimmune antisera to HCV (Sapporo); and paired acute and convalescent sera from children with HCV diarrhea were used [6, 18, 20]. Ten percent (wt/vol) suspensions of stool specimens containing HCV (Sapporo), extracted with trifluoro-trichloroethane (RIP, Fort Worth, Tex), served as positive controls in the screening ELISA for HCV detection and in the confirmatory assays for HCV. Test stool specimens were treated similarly.

Assay for HCV detection. The screening ELISA for detecting HCVs that are antigenically related to HCV (Sapporo) has been described [18]. This ELISA uses hyperimmune guinea pig antiserum to HCV (Sapporo) (or the preimmune guinea pig serum as a control) to bind HCV antigen to a microtiter plate well. The bound antigen is detected by a hyperim-

mune mouse antiserum to HCV (Sapporo). Subsequently, bound goat antibody to mouse IgG conjugated to horseradish peroxidase (Hyclone, Logan, Utah) and the substrate 2,2'-azinobis(3-ethylbenz-thiazoline sulfonic acid) (ABTS; Sigma, St. Louis) generate a color reaction detected by absorbance at 414 nm by using an automated ELISA plate reader.

Results of the screening ELISA were considered positive if (1) the ratio of the A_{414} in the well coated with hyperimmune serum divided by the A_{414} in the well coated with preimmune serum (P/N ratio) was ≥ 2.7 (mean + 3 SD derived from a panel of negative stools) and (2) the A_{414} in the well coated with hyperimmune serum was at least 0.08. The A_{414} cut-off point was lowered from 0.1 [18] to 0.08 on the basis of further experience with the assay. Positive stool specimens have had A_{414} reactivities as high as 0.6 and P/N ratios as high as 35.

Confirmatory tests for HCV. We used four methods to confirm positive ELISA results: a blocking ELISA and three types of EM. For the blocking ELISA, we used acute and convalescent sera from children with diarrhea caused by HCV (Sapporo) to block the reactivities of samples that were positive in the screening ELISA [18]. Specimens with reactivities that were not blocked by the acute serum and were blocked by the convalescent serum by $>50\%$ were considered positive for HCV. The reactivities of positive control specimens were blocked by the convalescent serum by $>90\%$.

Hyperimmune sera prepared against viral particles purified from stool may react with normal stool elements. Therefore, we sought to confirm the presence of HCV in the ELISA-positive specimens by a visual method. Three methods of EM—direct EM, IEM, and immunosorbent EM (ISEM)—were used to visually confirm the presence of HCV in specimens that were positive for HCV by ELISA. Direct EM and IEM were performed as previously described [18, 22]. For ISEM, antibody-coated grids were used to capture HCV particles, according to a technique modeled after that of Derrick [23] and Kjeldsberg [24]. The grids were placed for 15 min on a drop of an optimal dilution of rabbit hyperimmune antiserum to HCV (Sapporo). The grids were washed on six drops of diluent and were laid on drops from the same stool extracts used for the ELISAs. After a 2-h incubation at room temperature, the grids were washed, stained with phosphotungstic acid, and examined for 15 min. Photographs at a high magnification ($\times 73\,000$) documented the specificity of the

method by confirming the presence of particles with typical calicivirus structure.

Statistical methods. Fisher's exact test was used where appropriate.

Results

Detection and confirmation of HCV in day care stool specimens. Screening of the stool specimens by ELISA revealed 10 positive specimens; one additional specimen was positive by only one of the two ELISA criteria. The reactivities of nine of the 10 positive specimens and the one suspicious specimen were positive for HCV in the blocking ELISA. Direct EM failed to confirm HCV in any of the 11 specimens. IEM exhibited antibody-coated particles of the appropriate size for HCV in three specimens tested when mixed with hyperimmune rabbit antiserum to HCV and no particles when mixed with preimmune rabbit serum. Antibody-coated particles were visualized by IEM in one of the three specimens tested after a relatively large quantity of stool was concentrated and tested a third time.

Because of the apparently poor sensitivity of these visual methods, we evaluated ISEM as an alternative confirmatory method. The ISEM method visualized particles that were not coated by antibody and that had typical calicivirus structure (see figure 1, A–C). In preliminary studies, using positive control specimens containing HCV (Sapporo), no particles were captured by the preimmune serum, and 200–500 particles per grid square were captured at the optimal dilution of hyperimmune serum. All 11 specimens that tested positive by one or both screening ELISA criteria were positive by ISEM. Ten ELISA-negative diarrheal stool specimens that were tested as 10% suspensions or after a 4.2-fold concentration were also negative for HCV by ISEM.

Table 1 shows the ELISA and ISEM results for the HCV-positive specimens. Specimen 4, which originally met only one of the two criteria for positivity in the ELISA, had abundant HCV particles by ISEM. The specimen was concentrated and, on retesting by ELISA, had A_{414} reactivities of 0.205 in the wells containing hyperimmune antiserum to HCV and of 0.018 in the wells containing preimmune serum, for a P/N ratio of 11. The reactivities in the ELISA for the 11 specimens, by A_{414} density or P/N ratio, ranged greater than fivefold. Changes in the value of the ELISA reactivity did not correlate with (1) the density of particles captured on an antibody-

Figure 1. Immunoblot electron microscopy of specimen 6 (A; bar = 100 nm, 100 particles per square), specimen 3 (B; bar = 100 nm), and specimen 2 (C; bar = 50 nm) demonstrating high densities of HCV particles per grid square and details of typical calicivirus structure at higher magnifications. Direct electron microscopy of specimen 5 after purification of virus (D; bar = 100 nm) demonstrates the integrity of particles stored for six years at -20°C . Note the "Star of David" pattern in C and in D (arrowheads).

coated grid, (2) changes in the reactivity of the positive control specimen included on each plate, or (3) the number of days after onset of illness that the stool was collected.

Epidemiology of HCV infection in the day care population. A total of 461 stool specimens were tested (table 2). Of these, 266 came from sporadic

cases of diarrhea, 109 from cases of diarrhea in outbreaks, and 86 from asymptomatic contacts of outbreak cases. HCV was detected in seven (2.6%) of the specimens from sporadic cases and in four (3.7%) from the outbreak cases, for an overall rate of 2.9% (11 of 375) in diarrheal cases. HCV was not detected in any of the 86 stool specimens obtained from

Table 1. Results of ELISA and immunoblot electron microscopic (ISEM) testing of stool specimens containing HCV from day care center attendees.

Subject no.*	ELISA		ISEM
	Absorbance: hyperimmune wells/preimmune wells	P/N ratio†	
1a	179/34	5.3	90
2a	121/22	5.5	40
3	192/15	13.0	>100
4	48/11	4.4	30
5b	96/8	12.0	10-20
6c	231/15§	15.0	100
7c	235/11§	21.0	30
8b	135/15	9.0	20-50
9	140/44	3.2	20
10	125/19	6.6	100
11	174/32	5.4	1/3

* Letters indicate specimens tested on the same ELISA plate.

† P/N ratio indicates the ratio of absorbance at 414 nm for a specimen tested in a well coated with hyperimmune guinea pig antiserum to HCV (P) or preimmune guinea pig serum (N).

‡ Numbers represent the mean number of particles per grid square determined for each of five squares at a magnification of 46 000.

§ A 1:2 dilution of stool extract was tested.

Table 2. Results of testing for HCV, according to case and control groups.

Group	Total no. of patients	No. positive for HCV/no. tested (%)
Sporadic cases	603	7/266 (2.6)
Outbreak cases	176	4/109 (3.7)
Outbreak controls	176	0/86 (0)
Total	955	11/461 (2.4)

NOTE. Fisher's exact test was used for statistical analyses. $P = .10$ for outbreak cases vs. outbreak controls and for all cases of diarrhea vs. outbreak controls. Of all diarrheal stool specimens tested, 2.9% were positive for HCV.

asymptomatic contacts of the outbreak cases ($P = .10$). The sporadic and outbreak cases were initially analyzed separately for each parameter presented below. Because no significant differences with respect to the incidence of HCV infection were noted between the two groups, the results were combined.

HCV was found in specimens from children in nine day care centers. Two sporadic cases occurred in the same week at one day care center. The four outbreak cases were distributed among three outbreaks. HCV was detected in two of five cases in the first outbreak, one of six cases in the second, and one of five cases in the third ($P = .06$; Fisher's exact test was used for differences in detection between cases and controls in the three outbreaks). There was no significant difference in the detection of HCV between facilities in which outbreaks occurred and facilities in which outbreaks did not occur. Two (20%) of the 10 child household contacts and none of the 21 adult household contacts of 9 HCV-positive cases were ill (vomiting or diarrhea) in the same week as the day care case ($P = .09$). The overall rate of illness among household contacts of HCV cases (6%) was similar to that observed for household contacts of children with diarrhea who did not have HCV in their stools.

The age-specific rates of HCV-associated diarrhea in all children with diarrhea who were tested is presented in table 3. Three (15%) of 20 children with diarrhea who were less than six months of age at the onset of their illness had HCV in their stool. Beyond this age, the rate was significantly lower (2.3%, $P = .02$).

Demographic and clinical features of children with HCV-associated diarrhea. Table 4 shows the demographic and clinical characteristics of the 11 children

Table 3. Age-specific HCV attack rates in children with diarrhea.

Age (mo)	No. screened	No. (%) positive
0-5	20	3 (15)*
6-11	82	2 (2.4)
12-23	167	4 (2.4)
24-35	96	2 (2.1)
36	8	0 (0)
Total	373†	11

* $P = .02$, by Fisher's exact test, for a difference in incidence when compared with older age-groups.

† Age was not recorded for two outbreak cases that were not HCV positive.

with HCV in their diarrheal stools (age range, two to 29 months; five were boys and six were girls). Infection occurred throughout the entire study period. Nine of the cases occurred in the months of June through November, two in other months. All children had diarrhea, seven had respiratory symptoms, five had decreased appetite, three had vomiting, two had fever, and two had a rash. One child was taken to a physician; none were admitted to the hospital. Among those with HCV-associated diarrhea, the portion with and the duration of other noted symptoms did not differ significantly from the group of 42 children with rotavirus-associated diarrhea originally identified in the same study population (data not shown; range of P values = .25-.61). HCV was found in nine (3.5%) of 257 diarrheal stools collected on the day clinical illness was noted, or the day after, and in two (1.6%) of 126 diarrheal stools collected two or more days after clinical illness was noted ($P = .24$). No repeat HCV infections were found by examining 12 previous or subsequent, temporally remote stools from these HCV-positive cases.

Specimens containing rotavirus; adenovirus types 1, 2, 5, 7, 40, and 41; echovirus types 2, 5, 6, 7, 11, 14, 15, 20, 21, 23, 25, and 29; Coxsackievirus types A9 and B1; *Campylobacter*; and *Giardia* ($n = 33$) were negative for HCV by ELISA (data not shown).

Discussion

We provide the first description of HCV activity in the day care center setting. We detected HCV in 11 stool specimens collected for a longitudinal study of diarrhea in children attending day care centers in Maricopa County, Ariz. Previous evidence of HCV infection in North America includes the following:

Table 4. Demographic and clinical features of the 11 children with HCV-associated diarrhea.

Patient no.	Age in mo/sex	Onset date*	Symptoms and duration (d) [†]			
			Diarrhea	Decreased appetite	Respiratory symptoms	Others
1	10/F	10/81 (0)	NA	NA	NA	NA
2	6/M	10/81 (6)	NA	NA	NA	NA
3	23/F	6/82 (0)	>7	1-2	none	none
4	21/F	8/82 (0)	3-4	3-4	3-4	[giardia] v (1), f (5-6)
5 [‡]	29/F	11/82 (0)	1	5-6	>7	[rash] v (5-6)
6 [‡]	25/M	11/82 (0)	1	7	7	f (1) [giardia]
7	20/M	11/82 (0)	1-2	none	>7	none
8	13/F	1/83 (1)	>7	none	7	none
9 [‡]	5/M	5/83 (0)	>7	none	>7	none
10	2/F	7/83 (0)	1-2	1-2	none	[rash] v (5-6)
11 [‡]	4/M	8/83 (9)	NA	none	>7	none

* The number in parenthesis indicates the day of illness on which the stool specimen was collected; 0 = stool specimen collected on the day illness was noted.

[†] NA = duration of symptoms for these patients was not available, v = vomiting, and f = fever; the number in parenthesis is the no. of days of duration of the symptom. Additional signs or symptoms of infection are shown in brackets.

[‡] Indicates outbreak-associated cases.

(1) surveys of gastroenteritis conducted in Buffalo, NY [25], and Tucson [15] of children with diarrhea, most of whom were hospitalized, in which HCV was found by EM in 5 (0.4%) of 1160 and 2 (0.2%) of 862 diarrheal stools, respectively; (2) a nosocomial outbreak of HCV among children at a hospital in Montreal [5]; and (3) the detection of antibody to all five antigenic types in pooled immunoglobulin preparations from Canada and of antibody to UK4 in the United States [17]. HCVs have also been noted by EM in Washington, D.C. [26] and in California [19]. HCVs antigenically related to HCV (Sapporo) have been found in the diarrheal stools of hospitalized children in Houston [18] (D. O. M. and M. K. E., unpublished data). This report extends the geographic distribution of HCV (Sapporo) infection to Phoenix and demonstrates that most HCV infections are not detected by direct EM or IEM. HCV infections caused by agents antigenically related to HCV (Sapporo) are probably widespread in the United States.

Of 375 sporadic and outbreak-associated diarrheal episodes, we found HCV in 11 (2.9%). In the previous analysis of these same specimens, *Giardia* was found in 13% of diarrheal stool specimens, rotavirus in 6%, *Campylobacter* in 2%, and *Salmonella*

and *Shigella* each in 0.2% [1]. The HCV rate that we detected in a day care center population was >15-fold higher than that detected by direct EM examination of diarrheal stools from hospitalized children in a concurrent study in Tucson [15]. HCV activity in the day care centers was widespread, sporadic, and occurred throughout the study period. The rate of reported illness in household contacts implies person-to-person transmission of HCV, a pattern consistent with that reported for some HCV outbreaks [27]. These data imply that caliciviruses are important agents of diarrhea in the day care setting.

HCV-associated diarrhea was more frequent in young infants (age, six months or less) than in older infants or toddlers. This pattern was also observed for rotavirus-associated diarrhea and contrasts with the older age of onset for *Giardia*-associated diarrhea in the same population [1]. The age distribution of the 11 children with HCV-associated diarrhea (table 3) closely parallels that in two previous studies [13, 17]; however, neither of those two reports provided age-specific incidence rates. We found that HCV-associated diarrhea occurred predominantly in the summer and fall, although the total number of cases was small. This trend contrasts with that observed by Cubitt and McSwiggan [13] in northwest

London, where, during a 2.5-y period, 22 (56%) of 39 identified cases occurred from November through January.

The ~3% incidence of HCV-associated diarrhea we detected is likely to be an underestimate. The hyperimmune guinea pig serum prepared against HCV (Sapporo) [20] reacts with four (UK1, UK2, UK4, and Sapporo) of the five known HCV antigenic types but not with the UK3 strain [19]. Studies of the same five antigenic types by IEM [19] find them to be antigenically distinct, a result indicating that HCVs have both unique and common epitopes. In Houston, we have found HCVs that were antigenically related to HCV (Sapporo) and others that were antigenically distinct (D. O. M. and M. K. E., unpublished data). We expect that a proportion of HCV infections in Phoenix are caused by HCVs not detected by our ELISA.

Others have reported that HCVs disintegrate with freezing [10, 19]. Our results, however, contrast with these in that all specimens positive by the ELISA contained intact HCV particles. We have purified intact particles from two stool samples (subjects 5 and 6) that were frozen for six years at -20°C (see figure 1D) and produced rabbit and guinea pig hyperimmune antiserum to HCV. Although we cannot exclude the possibility that some specimens that originally contained HCV remained undetected due to deterioration of virus, our results indicate that epidemiological data concerning HCV and HCV material to produce laboratory reagents can be obtained from frozen stool specimens.

Caliciviruses are reported to be shed in stools for up to 13 d [5, 13, 28], but 50% of the patients apparently have no detectable virus five to seven days after the onset of clinical illness [28]. In the 11 cases discovered here, nine of the stool specimens containing HCV were collected on the first two days of illness and one each on day 6 and day 9. A more accurate estimate of the duration of shedding of virus after infection will come from studies that include multiple stool specimens from each diarrheal case and in which HCV can be cultivated.

The clinical features of HCV-associated diarrhea were generally mild and were indistinguishable from those of rotavirus-associated diarrhea in the same population. This similarity in the spectrum of symptoms associated with HCV and rotavirus diarrhea was also found by Cubitt and McSwiggan [13] in northwest London. None of the nine children who

responded to questionnaires and who were identified in this report were admitted to the hospital, whereas 26 of 28 respondents in northwest London were hospitalized. Questionnaires for this study were applied uniformly to all children with diarrhea, whereas questionnaires were distributed only to HCV-positive patients in the northwest London study. The two children with a rash are of interest, because infections with many animal caliciviruses cause a vesicular exanthem [29]; this feature has not yet been noted for HCV infections. Studies with other designs will be required to define the complete spectrum of illness caused by HCV.

Finally, ISEM proved to be a sensitive and specific method for confirming ELISA-positive results. By using ELISA-positive specimens from three epidemiological studies, we have had the following experience with the confirmatory assays: ELISA blocking, 26 of 27 confirmed; direct EM, 0 of 27 confirmed; IEM, 12 of 17 confirmed; and ISEM, 27 of 27 confirmed. In addition, ISEM detected HCV in two specimens that tested negative by ELISA (including one reported here). These results confirm that laboratories with EM facilities and an HCV antiserum may use ISEM to detect HCV [24]. These results will facilitate future epidemiological studies.

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