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Human Enteric Coronaviruses: Antigenic Relatedness to Human Coronavirus OC43 and Possible Etiologic Role in Viral Gastroenteritis

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In a group of infants with acute nonbacterial gastroenteritis, examination of paired sera for antibody to human coronavirus (HCV) OC43 and neonatal calf diarrhea coronavirus showed a peculiar pattern of serological response, restricted only to HCV OC43 surface antigens, in a significantly higher proportion than among age-matched controls. In another group of infants and young children with acute nonbacterial gastroenteritis, fecal excretion of coronavirus-like particles was detected by electron microscopy in 34 (16.3%) of 208 patients as compared with three (1.6%) of 182 controls ($P < .01$). Two strains of human enteric coronavirus (HECV) were purified from stools of two patients, and immune sera were raised in mice and guinea pigs. Immune electron microscopy showed a two-way cross-reactivity between HECV and HCV OC43 when tested with immune sera and convalescent-phase sera from patients with infection due to HECV or HCV OC43.

Human coronavirus (HCV) OC43 causes infections of the upper respiratory tract [1–3]. Neonatal calf diarrhea coronavirus (NCDCV) is a major cause of diarrhea in newborn calves [4, 5]. Antigenic relationships between these two coronavirus (CV) strains have recently been shown to be close only for antigens expressed in infected cells when mouse hyperimmune sera are used and also for surface antigens when convalescent-phase sera from natural hosts are used [6, 7]. In addition, CVs have possibly been involved in the etiology of human gastroenteritis, a suggestion based mainly on electron microscopic findings of CV-like particles in human stools and intestinal epithelium [8]. Because CVs are difficult to isolate and cultivate and because CV-like particles have been repeatedly reported to be excreted in stools for long periods, we first used some retrospectively selected clinical specimens for a

serological approach to the study of the suspected role of CVs in human gastroenteritis. We then examined more patients and controls for fecal excretion of CV-like particles as well as for serum antibody response to CVs. In addition, the antigenic relations between HCV OC43 and human enteric CV (HECV) were studied by immune electron microscopy (IEM) by using immune sera and convalescent-phase sera from patients with either infection.

Materials and Methods

Patient groups. Sixty-two inpatients, two to 12 months old, with the clinical diagnosis of acute gastroenteritis were selected during a period of a year (1967–1968) on the basis of negative tests for enteropathogenic bacteria (*Salmonella*, *Shigella*, enterotoxogenic *Escherichia coli*, *Yersinia*, and *Campylobacter*). As a control group 50 age-matched inpatients with the clinical diagnosis of acute nonbacterial disease other than gastroenteritis were selected during the same period. From each patient an acute-phase serum sample was obtained at admission; a convalescent-phase sample was drawn 10–20 days later. Paired sera were examined for antibody to HCV OC43, NCDCV, and HECV. These patients with gastroenteritis and the controls were selected retrospectively, and stools were not available.

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Stools and paired sera were then collected in the period 1981–1983 from 208 infants and young children, two months to four years old, with acute gastroenteritis and from 182 age-matched controls. Sera were tested for antibody to HCV OC43, NCDCV, and HECV. Fecal suspensions were examined by electron microscopy [9] after negative staining with 2% sodium phosphotungstate (pH 6.8) or 2% uranyl acetate (pH 4.5).

Purification of HECV. Stools from two patients with gastroenteritis were rich enough in CV-like particles to allow purification of virus. HECV from clarified fecal suspensions was purified by ultracentrifugation in sucrose density gradients, as reported for NCDCV by Sharpee et al. [10]. The gradient fractions were examined by electron microscopy for detection of typical CV particles.

Antisera to HECV. Using purified virus, we raised antisera to the two purified CV strains (HECV-24 and HECV-35) in CD-1 mice and in Hartley strain guinea pigs. Guinea pigs and four-week-old weanling mice received four 1.0-ml im inoculations, one week apart, of viral suspension in Freund's complete adjuvant. The total dose of immunogen was 10^{10} – 10^{11} viral particles (as determined by electron microscopy) per animal. Animals were bled seven days after the final inoculation.

Serological tests for CVs. HCV OC43 and NCDCV were adapted to both human embryonic lung fibroblasts and Vero cells [6, 7, 11]. OC43- and

NCDCV-hyperimmune sera were obtained from mice immunized with a CV strain adapted to suckling mouse brain [6]. The HAI and neutralization (NT) tests and detection of immunoperoxidase IgG antibody specific for CV (IgG IPA) were performed as previously described [6, 11]; test sera were treated with phospholipase C before the HAI and NT tests to remove nonspecific inhibitors of HA and infectivity. For detection of indirect fluorescence with IgG antibody to membrane-associated antigen (IgG IFAMA), OC43- and NCDCV-infected Vero cells grown in microtiter plates were used at 48 and 72 hr after infection, respectively [7].

Immune electron microscopy. IEM procedures [12] were performed on (1) HECV-24 and HECV-35 purified from stools and (2) crude harvests of cell culture-adapted HCV OC43 and NCDCV. Viral suspensions were diluted appropriately to give one to two particles per 400-mesh grid square by direct electron microscopy. About 0.2 ml of an appropriate serum dilution (determined in the homologous system) was mixed with 0.8 ml of each viral suspension, incubated at room temperature ($\sim 23^\circ\text{C}$) for 1 hr, and then centrifuged at $\sim 34,000\text{ g}$ for 90 min at 4°C . The pellet was resuspended in a few drops of distilled water, stained with 2% uranyl acetate (pH 4.5), and dropped onto 400-mesh grids that we coated with Formvar® carbon (Merck, Darmstadt, Federal Republic of Germany). The grids were examined with an electron microscope (model EM 201 or EM 301; Philips, Mahwah, NJ) at a magnification of $\sim 40,000\times$.

Table 1. Patterns of antibody response to HCV OC43 and NCDCV in human, bovine, and simian sera.

Species, pattern of antibody response to CV	Presence of antibody to indicated CV strain by							
	HAI		NT		IgG IPA		IgG IFAMA	
	OC43	NCDCV	OC43	NCDCV	OC43	NCDCV	OC43	NCDCV
Human								
H1	+	+	+	+	+	+	+	+
H2	+	–	+	–	–	–	–	–
H3	+	±	+	±	+	+	+	+
H4	+	±	+	±	±	±	±	±
Bovine								
B	+	+	+	+	+	+	+	+
Simian								
S	+	+	+	+	–	–	–	–

NOTE. Antibody titer of $\geq 1:20$ by HAI and IgG IFAMA and $\geq 1:80$ by the other tests = (+); antibody titer of $< 1:10$ by HAI and IgG IFAMA and $< 1:20$ by the other tests = (–); and antibody titer of $1:10$ by HAI and IgG IFAMA and $1:40$ by the other tests = (±).

Table 2. Examples of the four different human patterns of antibody response to HCV OC43 and NCDCV.

Pattern of antibody response	Patient/age (months)	Serum	Reciprocal antibody titer to indicated CV by							
			HAI		NT		IgG IPA		IgG IFAMA	
			OC43	NCDCV	OC43	NCDCV	OC43	NCDCV	OC43	NCDCV
H1	66/10	Acute	<10	<10	<20	<20	<20	<20	<10	<10
		Convalescent	40	10	160	40	160	80	40	10
H2	R-2/11	Acute	<10	<10	<20	<20	<20	<20	<10	<10
		Convalescent	40	<10	1,280	<20	<20	<20	<10	<10
H3	19/2	Acute	10	<10	40	20	20	20	10	10
		Convalescent	80	10	1,280	80	160	80	40	20
H4	1755/2	Acute	10	<10	40	<20	<20	<20	<10	<10
		Convalescent	20	<10	160	40	<20	<20	<10	<10

Sera examined. In addition to paired sera from patients with gastroenteritis and from controls, the following groups of sera were examined for antibody to both HCV OC43 and NCDCV by all four serological tests: (1) OC43- and NCDCV-hyperimmune mouse sera, (2) mouse and guinea pig antisera to HECV-24 and HECV-35, (3) single serum specimens from 40 calves, and (4) single serum specimens from 50 African green, 20 rhesus, and 30 cynomolgous monkeys. Some sera from patients with gastroenteritis and from controls, as well as antisera to CV, were also tested for IEM reactivity with the two HECV strains, HCV OC43 and NCDCV.

Results

Patterns of antibody response to HCV OC43 and NCDCV in human and animal sera. In all humans tested two main patterns of antibody response to HCV OC43 and NCDCV were encountered: H1 and H2 (table 1). The H1 pattern shows the presence of NT and often HAI antibody to both CV strains in association with the presence of IgG antibody to both viruses by IPA and IFAMA. This pattern is the one usually found in sera from children and adults. In contrast, the H2 pattern shows the presence of NT and often HAI antibody only to HCV OC43 in the absence of IgG antibody by IPA and IFAMA to either CV strain. This pattern was found only in infants and less frequently in young children, but never in adults. In addition, two other patterns were far less frequently encountered in infants: H3 and H4 (table 1). The H3 pattern is similar to the H1 pattern, but the titer of NT antibody to HCV OC43 is at least 16-fold higher than the titer of NT antibody to NCDCV. The H4

pattern is similar to the H2 pattern, but the NT antibody response to HCV OC43 is associated with the presence of NT antibody to NCDCV. Examples of seroconversion for each of the four human patterns of antibody response to HCV OC43 and NCDCV described above are reported in table 2.

All 40 calves tested showed the presence of serum antibody to both HCV OC43 and NCDCV by all tests. However, as a rule homologous titers were at least fourfold higher than heterologous titers (pattern B; table 1). Twenty-three of 50 African green monkeys showed the presence of NT antibody to both HCV OC43 and NCDCV in the absence of IgG antibody by IPA and IFAMA (pattern S; table 1),

Table 3. Prevalence of different patterns of antibody response to HCV OC43 and NCDCV in 62 infants with acute nonbacterial gastroenteritis and in 50 age-matched controls with other nonbacterial infections.

Patterns of antibody response in paired sera	No. of infants (%) with antibody response	
	Gastroenteritis	Control
H1		
Seroconversion	2 (3.2)	2 (4.0)
Stable titers	13 (21.0)	11 (22.0)
H2		
Seroconversion	17 (27.4)*	1 (2.0)*
Stable titers	3 (4.8)	1 (2.0)
H3		
Seroconversion	2 (3.2)	2 (4.0)
Stable titers	3 (4.8)	4 (8.0)
H4		
Seroconversion	0	1 (2.0)
Stable titers	2 (3.2)	2 (4.0)

* Highly significant statistical difference ($P < .01$).

whereas none of 20 rhesus and 30 cynomolgous monkeys possessed specific antibody to HCV OC43 or NCDCV by any of the tests used.

Prevalence of different patterns of antibody response to HCV OC43 and NCDCV in infants. In the paired sera tested seroconversion and stable antibody titers of each pattern were detected in both the gastroenteritis group and the control group (table 3). The H2 pattern of CV seroconversion was observed in a much higher proportion of patients with gastroenteritis than in controls (27.4% vs. 2.0%, respectively). This difference was the only one that was statistically significant between the two groups. Stable titers of antibody to HCV OC43 and NCDCV in all four patterns were present in a comparable proportion of cases in both groups. However, stable antibody titers in the H1 pattern were largely predominant in both groups, a result suggesting that in the first year of life OC43-like infections (elic-

ing an antibody response to both HCV OC43 and NCDCV detectable by all the tests used) are the most common.

Fecal excretion of HECV by patients with gastroenteritis and by controls. Among the 208 infants and young children with acute gastroenteritis examined for fecal excretion of viral particles, CV-like particles were detected in 34 patients (16.3%), whereas they were detected only in three (1.6%) of the 182 age-matched controls ($P < .01$). The particles appeared pleomorphic, enveloped, and round, oval, or kidney shaped, with a diameter of 70–200 nm, surrounded by a fringe of surface projections 15–25 nm long (figure 1A). The buoyant density of the two HECV strains purified by sucrose density gradient ultracentrifugation, as determined by electron microscopic examination of each gradient fraction, corresponded to ~ 1.20 g/ml, a value in agreement with those reported for human and ani-

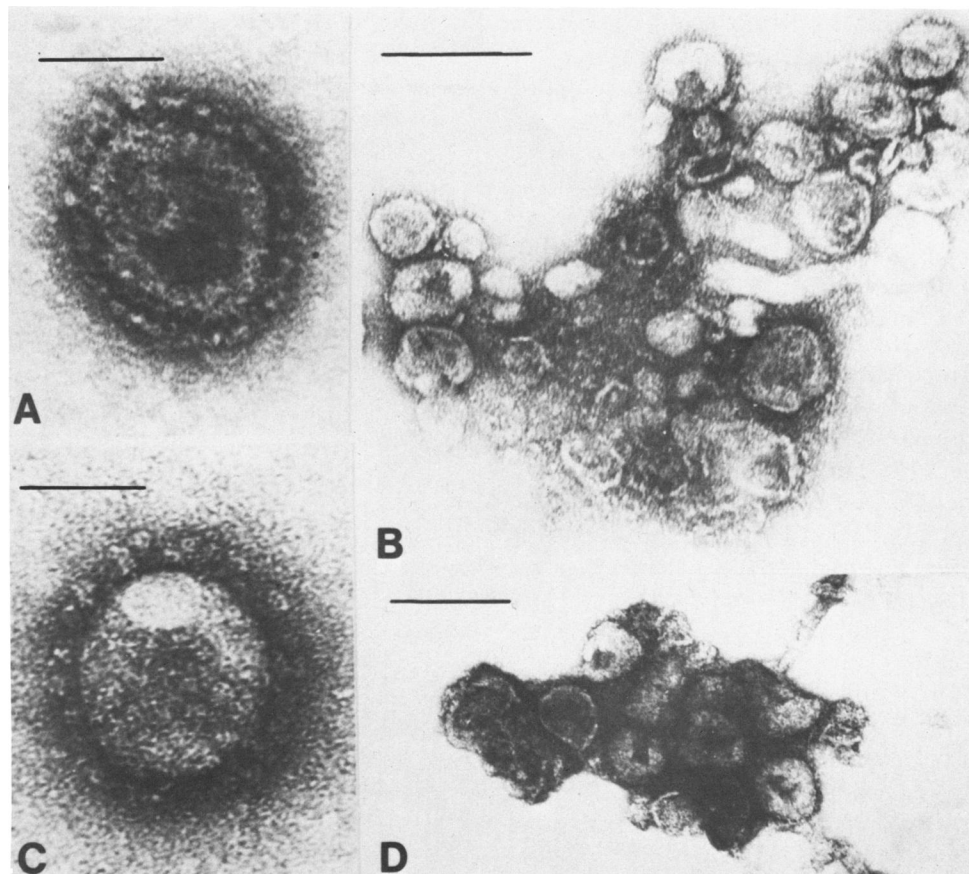


Figure 1. Morphology of CV particles: (A) strain HECV-35 and (C) HCV OC43. Bar = 100 nm. Immune aggregates formed with homologous guinea pig antiserum: (B) strain HECV-35 and (D) HCV OC43. Bar = 200 nm.

Table 4. Positivity for fecal excretion of HECV, alone or associated with other viruses, among 208 infants and young children with acute gastroenteritis.

Virus detected	No. positive (%)
HECV	25 (12.0)
HECV + AV	1 (0.5)
HECV + HRV	4 (1.9)
HECV + SRV	3 (1.4)
HECV + Astro + HRV	1 (0.5)

NOTE. AV = adenovirus; HRV = human rotavirus; SRV = "small round virus" particles; Astro = astrovirus.

mal CVs [13]. HECVs were detected by electron microscopy usually as single particles and sometimes associated with adenovirus, rotavirus, astrovirus, and "small round virus" particles (table 4). Among the few subjects who could be followed up for the length of HECV excretion, fecal shedding for periods of two to six months after recovery from gastrointestinal illness was observed in two patients. Most patients with gastroenteritis and most controls showed the presence of antibody to both HCV OC43 and NCDCV by all the serological tests used (H1 pattern with stable titers), and no statistically significant difference between these two groups was observed.

IEM reactivity of animal antisera and convalescent-phase sera from infants showing H2 seroconversion. Representative results obtained by IEM using animal antisera as well as data on the IEM reactivity of human convalescent-phase sera are reported in table 5. HCV OC43-immune mouse serum reacted very strongly with HCV OC43 (figure 1D), moderately with HECV-24 and HECV-35, and weakly with NCDCV, whereas NCDCV-immune

mouse serum reacted very strongly with NCDCV, weakly with HCV OC43, and very weakly with the two HECV strains. Conversely, antisera to HECV-24 and HECV-35 reacted strongly with the HECV strains (figure 1B) and weakly to moderately with HCV OC43, but they did not react with NCDCV (table 5).

Convalescent-phase sera from infants showing H2 seroconversion were also tested for IEM reactivity with HCV OC43, NCDCV, and the two purified HECV strains (HECV-24 and HECV-35). All the H2 convalescent-phase sera tested showed moderate reactivity with HCV OC43 and a strong reactivity with HECV-24 and HECV-35, but they did not react with NCDCV. In contrast, acute-phase sera were not reactive or were only very weakly reactive. H2 stable titers showed the same pattern of IEM reactivity as H2 convalescent-phase sera. On the other hand, H1 convalescent-phase sera reacted strongly with HCV OC43 and moderately with NCDCV and the two HECV strains. In most patients positive for fecal excretion of CV-like particles, antibody to HCV OC43 and NCDCV was detected by all the serological tests used. Convalescent-phase sera from the few patients who were seronegative for previous infections with HCV OC43 reacted by IEM with the two HECV strains and to a lesser extent with HCV OC43. Acute-phase sera from the same patients were poorly or not reactive by IEM.

Discussion

These results suggest a possible enteropathogenic role of an as yet unknown HCV OC43-related

Table 5. IEM reactivity of HCV OC43, NCDCV, HECV-24, and HECV-35.

Serum	IEM reactivity of			
	HCV OC43	NCDCV	HECV-24	HECV-35
HCV OC43, mouse	++++	+	++	++
NCDCV, mouse	+	++++	±	±
HECV-24, mouse	+ / ++	—	+++	+++
HECV-24, guinea pig	+	—	+++	+++
HECV-35, guinea pig	+ / ++	—	+++	+++
HCV OC43 human convalescent-phase*	+++	++	++	++
HECV, human convalescent-phase†	++	—	+++	+++

NOTE. The IEM reactivity (per 400-mesh grid square) was scored as follows: (—), no immune aggregates; (±), <1 immune aggregate; (+), 1 aggregate; (++) , 2–5 aggregates; (+++) , 6–10 aggregates; and (++++) , >10 aggregates.

* H1 pattern of antibody response to CV (see table 2).

† H2 pattern of antibody response to CV (see table 2).

HECV in infants and young children. This conclusion is supported by the following data. (1) In a fair proportion of subjects with nonbacterial gastroenteritis, a peculiar pattern (H2) of antibody response to HCV OC43 was observed, entirely distinct from that found in convalescent-phase sera from infants with respiratory infections due to HCV OC43 (pattern H1). (2) In infants and young children with acute gastroenteritis, fecal excretion of HECV was detected more frequently than in age-matched controls. (3) All convalescent-phase sera from infants showing H2 seroconversion showed IEM reactivity with HCV OC43 and also with HECV-24 and HECV-35. In addition, HCV OC43-hyperimmune serum, H1 convalescent-phase sera, and animal antisera to the two HECV strains purified from stools reacted with HCV OC43 as well as with HECV-24 and HECV-35. Thus a two-way cross-reactivity between surface antigens of HCV OC43 and HECV appears to exist. Furthermore, on the basis of preliminary findings the antisera to the two HECV strains and to a lesser extent HCV OC43-hyperimmune serum appear to react with the CV-like particles in stools from all our positive patients by conventional IEM and also by solid-phase IEM [14].

The H1 pattern has previously been reported as typical of primary respiratory infections with HCV OC43 [6, 11], whereas the H2 pattern is described here for the first time. The H1 and H2 patterns were identified on the basis of two main differences. (1) The H1 pattern presented reactivity to HCV OC43 by all the serological tests used, whereas the H2 pattern showed reactivity only to HCV OC43 surface antigens. (2) The H1 pattern also showed reactivity to NCDCV antigens, whereas in the H2 pattern reactivity to NCDCV was not detected. The complete dissociation between antibody reactivity to HCV OC43 surface antigens (as determined by HAI and NT) and antigens expressed in infected cells (as determined by IgG IPA and IgG IFAMA) was not previously reported but was surprisingly found also in sera from African green monkeys (pattern S). These findings could be important in view of the reported fecal excretion of CV-like particles in nonhuman primates [15, 16]. In contrast, in bovine sera a pattern of antibody response similar to the H1 pattern was encountered (pattern B). The H3 and H4 patterns infrequently found in humans appear to be very close to the H1 and H2 patterns, respectively. They are possibly due to infection by antigenically close strains

or to a different reactivity by different individuals to infections by the same CV strain.

The possibility that HECV gives rise to antibodies of only the IgM or IgA class to corona antigen (NT and HAI) as well as to CV antigens other than corona cannot be excluded. However, this suggestion seems unlikely because in all patients with acute infection due to HCV OC43 so far examined, we consistently detected the H1 pattern [6, 11]. Schmidt and Kenny [17], using two-dimensional immune electrophoresis, detected antibody only to the slow moving antigen (eliciting NT antibody) of HCV OC43 in human sera from acute infections with HCV OC43. This result was presumably determined by the low sensitivity of the technique used and is in agreement with our previous results showing a higher NT than IgG (as detected by IPA) antibody titer in convalescent-phase sera from patients with acute infection with HCV OC43 [6, 11]. Alternatively, because Schmidt and Kenny [17, 18] showed that only high immunogenic doses were able to elicit antibodies reacting with all three major CV antigens, it is possible that in infections with HECV the amount of antigen is too low to elicit an IgG antibody response other than NT or that the IgG antibody response to noncorona antigens is too weak to be detected.

Infants showing H2 seroconversion as well as infants and young children positive for detection of CV-like particles in feces had diarrhea for two to three days, had no fever, and subsequently recovered rapidly. So far the suspected etiologic role of CVs in human gastroenteritis was based on electron microscopic detection of CV-like particles in stools from subjects with diarrhea [8]. However, Moscovici et al. [19] recently described an outbreak of acute hemorrhagic enterocolitis (in immature infants) associated with both detection of CV particles in stools and appearance of an IEM antibody response to a strain of HECV. More recently Vaucher et al. [13] reported an outbreak of gastrointestinal illness in neonates with similar electron microscopic and IEM findings. The serological results here reported add further support to the suspected enteropathogenic role of CVs in infants and children. There may be several reasons for the delay in obtaining serological support for the association between CV infection and gastroenteritis in humans. First, specific NT and HAI tests have only recently been made reliable by eliminating CV infectivity inhibitors and non-specific inhibitors of CV HA [6, 11]. Second, com-

parative studies of the reactivity of human sera with known human and animal CVs have not usually been performed in patients during the first year of life. Antibody to HCV OC43 is acquired early in life [20], and previous antibody responses to infection by different respiratory strains of CV related to HCV OC43 could have hidden the antibody response to HECV in most young children positive for fecal excretion of CV-like particles. Third, HECV strains detected by electron microscopy in human stools have not been studied for reactivity with convalescent-phase sera from infants with acute nonbacterial gastroenteritis until recently [13, 19].

A study of the antigenic relationships among HECV, HCV OC43, and NCDCV is reported for the first time in this article. A two-way cross-reactivity between HCV OC43 and the two HECV strains was consistently observed. The antigenic relatedness to HCV OC43, the typical morphology, and the buoyant density in sucrose—in agreement with those reported for known human and animal CVs [21]—suggest that the CV-like particles detected by electron microscopy were actually HECVs. Furthermore, on the basis of preliminary findings some HECV strains from stools of our patients can be propagated on HRT 18 cell monolayers [22], but only with partial success. This cell line appeared to be susceptible to HECV only at low population-doubling levels. Although only a few patients could be followed up for the length of viral excretion, fecal excretion of HECV was detected for as long as six months after recovery from diarrhea. Our results are in accord with those of several authors [8] who reported fecal shedding of CV-like particles by patients for periods of several months after cessation of symptoms.

The following conclusions can be drawn. (1) HECV infection appears to be associated with some cases of acute gastroenteritis, and the possible enteropathogenic role of HECV must be further investigated. (2) HECVs are antigenically related to HCV OC43. (3) With use of specific hyperimmune antisera, fecal HECV excretion could be readily detected in the near future by methods such as ELISA, which is more widely used than is electron microscopy or IEM [9]. This technical advance will be of help in defining the relation between HECV infection and gastrointestinal illness.

Addendum

After the submission of this article, it was reported that coronavirus-like particles detected by electron

microscopy in stools from humans show a serological relationship with the Breda virus [23] recently isolated from calves with diarrhea [24]. A similar virus, referred to as the “Berne” virus, was isolated from a horse with enteritis [25]. Both these viruses resemble coronavirus-like particles by electron microscopy and have recently been assigned to the newly proposed family of Toroviridae (M. C. Horzinek, M. Weiss, and G. N. Woode, unpublished observation). These viruses have been shown by serological tests to be members of the same group and not to be cross-reactive with three animal members of the Coronaviridae family [25]. In addition, the Berne virus has been shown to direct the synthesis of polypeptides with a pattern of molecular weights quite different from those of coronaviruses [26]. The genome polarity seems also to be different from coronaviruses [26]. However, in an epidemiological survey antibodies to the Berne virus were found in several animal species but not in humans [25]. Thus recent findings showing an antigenic relationship between the Breda virus and coronavirus-like particles from human stools [23] require confirmation. We cannot now exclude the possibility that in humans as well as in animals two groups of coronavirus-like particles (one belonging to the Coronaviridae and the other to the new Toroviridae family) may be associated with gastroenteritis.

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