

Pleomorphic, Enveloped, Virus-Like Particles Associated with Gastrointestinal Illness in Neonates

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Pleomorphic, enveloped, virus-like particles were detected by electron microscopy in the stools of symptomatic infants during an outbreak of gastrointestinal illness in a neonatal intensive-care unit. To determine the incidence of virus-like particles in the stool and their relation to gastrointestinal symptoms, eight surveys of stools for the particles were conducted over 40 weeks. The incidence of virus-like particles in the stool decreased from 69% to <10% over the study period. Most infants surveyed were premature; overall, 32 (36%) of 88 neonates were positive for virus-like particles. Statistically significant associations were found between virus-like particles in the stool and gastrointestinal symptoms within one week of each survey. These symptoms included water-loss stools, blood in the stool, gastric retention, bilious gastric aspirates, and abdominal distention. Several infants with virus-like particles whose mothers had gastrointestinal or "flu-like" symptoms before delivery were identified in the community (not part of the survey study).

Coronaviruses have been reported to be associated with gastrointestinal illness in several mammalian species, including cats, dogs, mice, and calves [1-5]; newborn animals appear to be more susceptible to severe disease [2-5]. The symptoms of enteritis [2-5] are the most common; peritonitis [1] and systemic involvement [2-5] have also been described. "Coronavirus-like" particles have also been found in the stools of adults, children, and neonates, both with [6-8] and without [9-12] symptoms of acute gastroenteritis.

An outbreak of gastrointestinal illness among the infants in the neonatal intensive-care unit (NICU) at the Arizona Health Sciences Center, Tucson, in September 1979 was associated with the presence of pleomorphic, enveloped, virus-like particles detected in the stools of several of the sick infants. These particles were similar to previously reported coronavirus-like particles [6-12]. This finding prompted a longitudinal study to determine (1) the significance of virus-like

particles in the stools of patients in this setting, (2) the incidence of virus-like particles in the NICU over a 40-week period, and (3) the characteristics of gastrointestinal symptoms in infants positive for virus-like particles in comparison to infants negative for virus-like particles.

Patients and Methods

Patients. To determine the frequency of virus-like particles in the patients in the NICU, eight surveys of stools for the particles involving 88 infants from the NICU were conducted over a 40-week period (September 1979-June 1980). Twelve infants were screened more than once. Thirteen additional infants positive for virus-like particles whose mothers had gastrointestinal or "flu-like" symptoms before or shortly after delivery were identified in the community (not part of the survey study) between September 1979 and May 1981.

Electron microscopy of stool specimens. Stool specimens were diluted with 1-2 ml of distilled water; the amount of water used was dependent upon the consistency of the stool specimens. Rectal swab specimens were immersed and then shaken in 2 ml of distilled water. All of the specimens were vortexed and then centrifuged at 2,000 g for 30 min in a swinging-bucket clinical centrifuge (model no. J-6B; Beckman Instruments, Palo Alto, Calif.) to remove bacteria and debris. One drop of the supernatant was placed on the surface

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of each of two Formvar[®]-coated, 300-mesh copper grids (Ernest Fullam, Schenectady, N.Y.) that were placed in microtiter wells containing 1% agar. Grid no. 1 was immediately removed from the well and allowed to air-dry without blotting. Grid no. 2 was left in the well, and the drop was allowed to dry completely (~1 hr) onto the grid surface. Grid no. 1 was returned to the well, and both grids were negatively stained by adding several drops of 2% phosphotungstic acid (adjusted to pH 6.5 with 1 N KOH) directly to the well. The grids were removed after 3 min, blotted on filter paper, and examined under an electron microscope (model no. HU-12; Hitachi Scientific Instruments, Mountain View, Calif.). All of the stool specimens were photographed at a magnification of 60,000. Periodic calibration of the electron microscope with a carbon grating revealed a stable lens system during the entire period of observation.

Immune electron microscopy. A stool specimen that showed minimal spontaneous aggregation of the virus-like particles was chosen. Several grids were prepared by mixing 0.05 ml of this stool suspension with 0.05 ml of an infant's serum that was undiluted or diluted 1:2, 1:4, or 1:8 in 0.22% bovine serum albumin. A drop of this mixture was then placed on a grid in a microtiter well and processed as described above. Five random low-power fields (magnification, 10,000) were photographed from each grid. The specimens were examined without knowledge of the serum source.

Cultures. Combined nasopharyngeal-throat swabs, rectal swabs, and urine specimens were processed according to standard methods for isolating viruses [13]. Nasopharyngeal-throat and rectal specimens were inoculated onto cultures of primary cynomolgus monkey kidney cells, human lung cells (a continuous heteroploid cell line), and human embryonic tonsil cells (a diploid fibroblast cell strain). Urine specimens were inoculated onto human embryonic tonsil cells.

Stools from ill patients were also cultured for *Salmonella*, *Shigella*, and *Campylobacter* species when it was considered appropriate by the attending staff. In addition, selected samples were tested for the presence of *Clostridium difficile* toxin by a tissue-culture assay [14] and for human rotavirus antigen by an enzyme-linked immunosorbent assay [15].

Statistical analysis. Statistical significance was

determined by Student's *t*-test, χ^2 analysis, or by exact binomial confidence limits.

Results

Initial episode and survey. During an eight-day period in September 1979, eight (50%) of all 16 infants in the NICU developed acute gastrointestinal symptoms, including persistent abdominal distention and bilious gastric aspirates (three patients) or green, water-loss stools that contained blood and mucus (five patients). The illness was severe enough in five of the eight infants to warrant the discontinuation of oral feeding for at least five days. An infectious agent was suspected as the cause of gastrointestinal illness. Viral cultures and examination of stools by electron microscopy were performed. Five of the eight symptomatic infants had virus-like particles in the stool that were identified by electron microscopy. Echovirus type 30 was cultured from the rectal swab of another symptomatic infant whose stool was negative for virus-like particles (table 1).

To determine the incidence of virus-like particles in the NICU, the eight infants without gastrointestinal symptoms were also surveyed. Five of these infants had not yet received oral feedings, and six were positive for virus-like particles. Two of the six infants with virus-like particles had gastrointestinal symptoms either before or after the initial survey, and two others had signs of sepsis, including hepatosplenomegaly and abnormal white blood cell counts (neutropenia or neutro-

Table 1. Characteristics of eight infants with gastrointestinal illness in a neonatal intensive-care unit, September 1979.

Patient no.	No. of days off oral feeding	Viral culture of stool	Virus-like particles in the stool
1	8	Negative	Positive
2	7	Negative	Positive
3	6	Echovirus 30	Negative
4	13	Negative	Positive
5	9	Negative	Positive
6	0	ND	Negative
7	0	ND	Negative
8	0	Negative	Positive

NOTE. Patients no. 2, 4, and 5 were also tested for bacterial pathogens and were found to be negative. Stool specimens were examined for pleomorphic, enveloped, virus-like particles by electron microscopy. ND = not done.

philia), despite negative bacterial and viral cultures. One of these latter infants had large quantities of virus-like particles in the stool.

Subsequent surveys. All of the infants in the NICU were surveyed for virus-like particles in the stool at one, three, six, 11, 19, 25, and 40 weeks after the initial survey (figure 1). Although infants positive for virus-like particles were found at all times, the incidence decreased from 69% to <10% over the observation period. Overall, 32 (36%) of all 88 infants screened in the NICU over the study period were positive for virus-like particles.

Electron microscopy. In all of the positive stool specimens, virus-like particles were present in large numbers and were easy to find by electron microscopy. Rotaviruses, astroviruses, parvo- or picornaviruses, adenoviruses, and caliciviruses—all of which have been previously identified in our laboratory—were absent during the entire 40-week observation period. The virus-like particles were surrounded by a fringe, 13–18 nm in length (figure 2, top). In most instances the individual filaments composing the fringe appeared somewhat flexible and had a bulbous shape at their distal end. Some of the virus-like particles occurred in aggregates (figure 2, bottom).

Sera obtained three to four weeks after initial screening from four neonates who had been positive for virus-like particles were compared with the sera obtained from three healthy neonates from the same NICU who were negative for virus-like particles for their ability to cause immune aggregation of the virus-like particles. A striking aggregation of both envelopes and fringed particles was observed with the sera from the positive infants but not with the sera from the negative infants (figure 3). More aggregation occurred with the un-

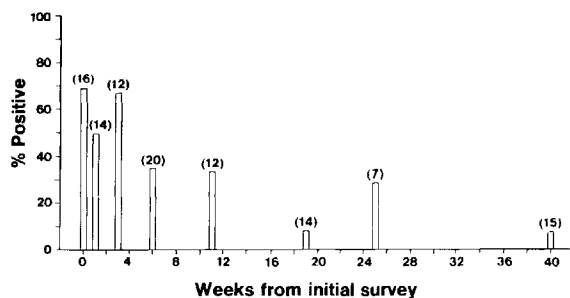


Figure 1. Incidence of pleomorphic, enveloped, virus-like particles in the stools of infants during an outbreak of gastrointestinal illness in a neonatal intensive-care unit. No. of patients surveyed is given in parentheses.

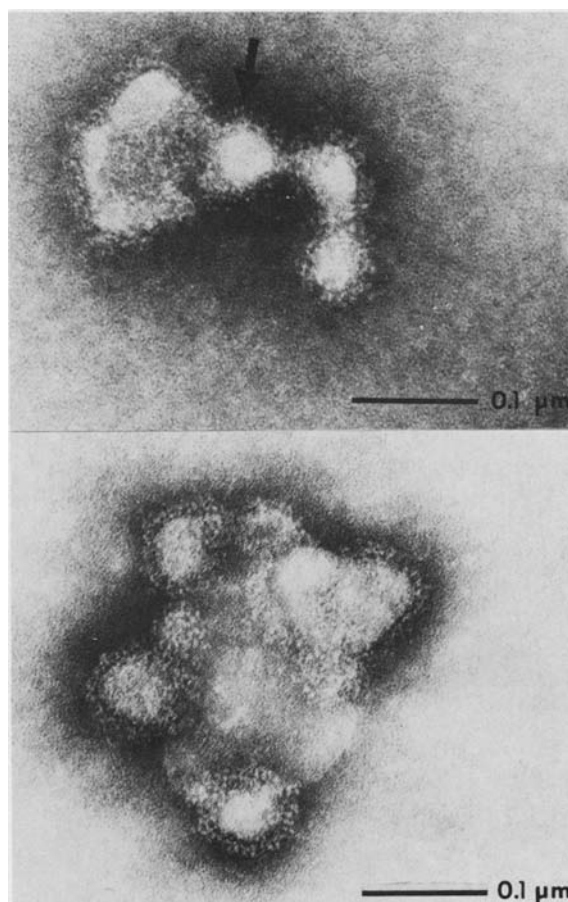


Figure 2. Pleomorphic, enveloped, virus-like particles detected by negative-contrast electron microscopy in the stools of infants during an outbreak of gastrointestinal illness in a neonatal intensive-care unit (negative contrast, phosphotungstic acid). *Top*, an irregularly shaped virus-like particle with a fringe that measures 15 nm in length. Most of the filaments that make up the fringe appear haphazardly arranged; in a few places the straighter filaments can be seen to have bulbous distensions at their distal end (arrow). *Bottom*, a cluster of virus-like particles. The length of the fringe measures 18 nm in some areas.

diluted than with the diluted sera. Although most of the immune aggregates contained a predominance of envelopes without characteristic fringes, definite virus-like particles could be identified at a high magnification (figure 4). Apparent antibody bridges could also be observed between adjacent particles (figure 4).

Symptoms related to the survey findings. To determine whether virus-like particles in the stool were associated with an increased incidence of gas-

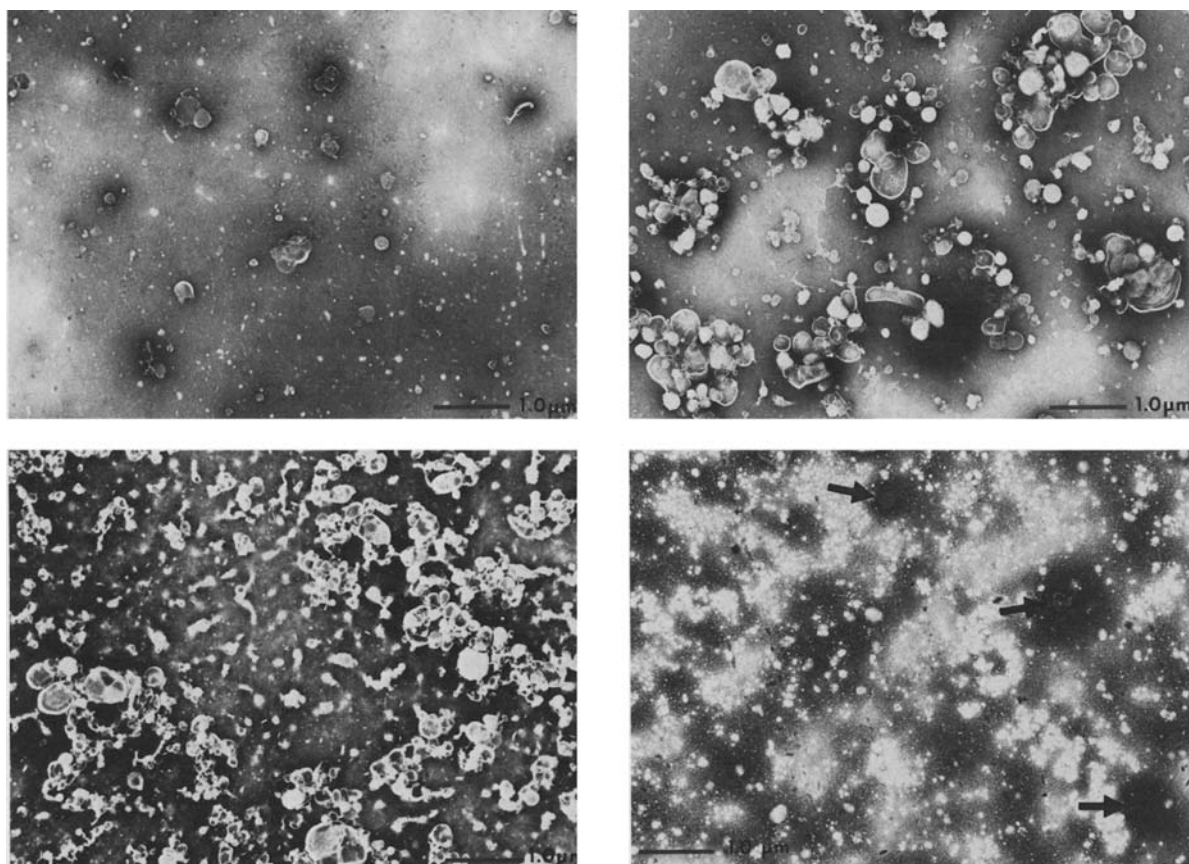


Figure 3. Immune electron micrographs of pleomorphic, enveloped, virus-like particles in a stool specimen obtained from an infant during an outbreak of gastrointestinal illness in a neonatal intensive-care unit. The stool specimen was diluted 1:1 with 0.22% bovine serum albumin and (*top left*) examined alone, (*top right*) mixed with a 1:8 dilution of convalescent-phase serum (day 25 after onset) from a symptomatic infant, (*bottom left*) mixed 1:1 with undiluted convalescent-phase serum from the same symptomatic infant as in *top right*, or (*bottom right*) mixed 1:1 with undiluted serum from an asymptomatic infant whose stool was negative for virus-like particles. Very little aggregation of viral envelopes is evident in *top left*, numerous small and large aggregates are seen in *top right*, and many more can be seen in *bottom left*. In *bottom right*, only three single envelopes can be seen (arrows), and no distinct clumping was observed anywhere on the grid. The small, round, amorphous particles in the background of *bottom right* are presumably lipoproteins or chylomicrons that are commonly found in serum.

gastrointestinal symptoms, we compared the frequency of various findings in infants positive for virus-like particles with those in infants negative for virus-like particles. Because the duration of viral shedding is unknown, we compared only the presence or absence of symptoms within seven days before or after each survey. These symptoms, routinely recorded in each infant's chart, were increases in stool frequency (more than eight per day), water loss or blood (occult or gross) in the stools, recurrent gastric aspirates (>2 ml) obtained >2 hr after feeding, bilious gastric aspirates, regurgitation, and abdominal distention. The symptoms were collected by a retrospective chart review.

The symptoms that occurred more often in infants with stools positive for virus-like particles are shown in table 2. Infants positive for virus-like particles were more likely to have multiple symptoms, including stools that contained both excessive water and blood ($P < 0.002$) and two or more additional symptoms in one of the following combinations: (1) gastric aspirates, regurgitation, and abdominal distention or (2) increased stool frequency, water-loss stools, and blood in the stool ($P < 0.002$).

Infants positive for virus-like particles were more likely to be symptomatic at any time during their hospitalization ($P < 0.01$) and within seven

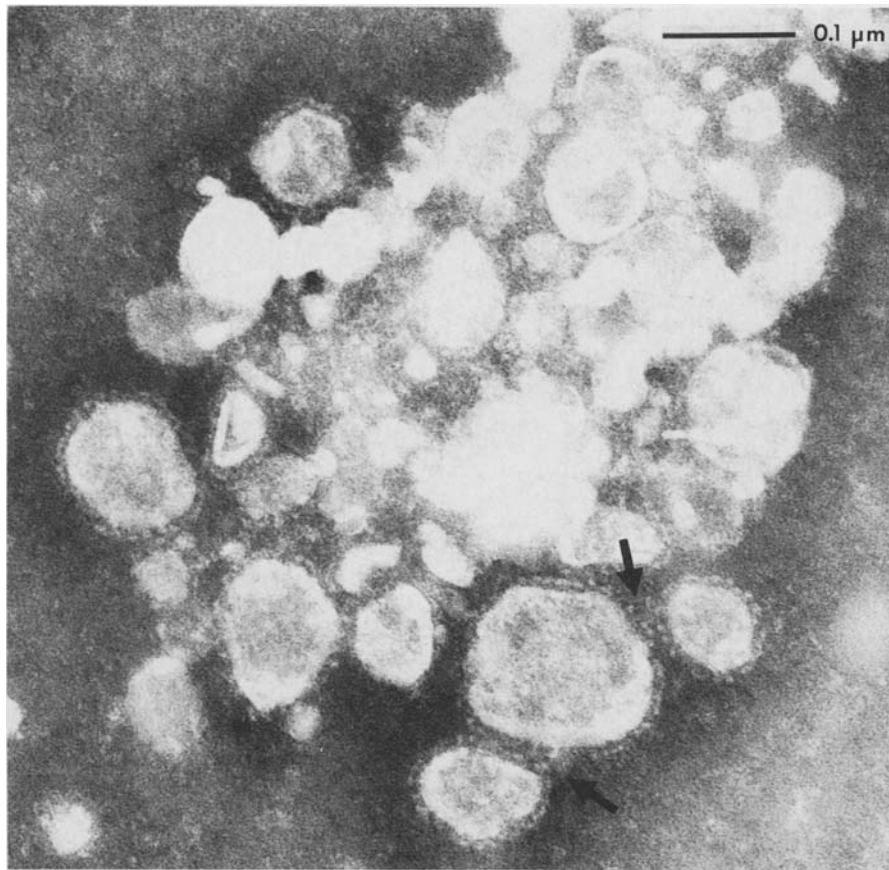


Figure 4. Immune electron micrograph of an infant's stool that was positive for virus-like particles combined with convalescent-phase serum from an infant with severe gastrointestinal illness whose stool was also positive for virus-like particles (negative contrast, phosphotungstic acid). The samples were obtained during an outbreak of gastrointestinal illness in a neonatal intensive-care unit. Several pleomorphic, enveloped, virus-like particles can be seen at the periphery of this immune aggregate. An apparent antibody bridge is evident between several of these particles (arrows). In the interior of the aggregate, some of the fringes have been obscured, presumably by excessive antibody coating.

days before or after the survey ($P < 0.005$). They were also more likely to be taken off oral feeding ($P < 0.05$), especially for more than three days ($P < 0.005$). Gastrointestinal symptoms lasted significantly longer ($P < 0.05$) in infants positive for virus-like particles than in infants negative for virus-like particles (mean \pm SEM, 9.2 ± 1.7 vs. 5.2 ± 0.8 days). No significant differences were found between the infants positive for virus-like particles and those negative for virus-like particles with regard to birth weight, gestational age, day surveyed, or antibiotic usage.

Cultures and assays for toxin or antigen. The only virus isolated was an enterovirus found in the group of neonates negative for virus-like particles. The stool samples from 78 patients (30 positive

and 48 negative for virus-like particles) were negative for human rotavirus antigen as tested by enzyme-linked immunosorbent assay. In addition, only one of 33 stool specimens from the positive patients contained detectable *C. difficile* toxin.

Additional observations. Twelve infants whose stools were positive for virus-like particles on their first screening were studied at least once more one to six weeks later. Five of these infants were either continuously or intermittently positive for virus-like particles on the subsequent surveys, two of the infants excreted virus-like particles for at least six weeks, and another two infants continued shedding for at least three weeks. Because of discharges from the NICU, we could not determine precisely how long shedding continued to occur.

Table 2. Gastrointestinal symptoms associated with virus-like particles in the stool in an outbreak of gastrointestinal illness in a neonatal intensive-care unit, September 1979.

Gastrointestinal symptoms	Virus-like particles in the stool		P
	Positive (n = 32)	Negative (n = 56)	
Water-loss stools	15	10	<0.004
Blood (occult or gross) in the stool	15	14	<0.01
Persisting for more than two days	9	3	<0.003
Abnormal gastric aspirates	13	9	<0.02
Persisting for more than two days	9	2	<0.001
Bilious for more than two days	7	3	<0.02
Abdominal distention	8	5	<0.05
Persisting for more than two days	5	0	<0.01
Increased stool frequency	5	9	NS
Regurgitation	7	8	NS

NOTE. Stool specimens were examined for pleomorphic, enveloped, virus-like particles by electron microscopy. NS = not significant. P values were determined by Student's *t*-test.

Most of the infants (11 of 12) were asymptomatic at the time of subsequent screening.

Eleven (34%) of 32 infants positive for virus-like particles were found to shed the particles at <72 hr of age (range, 6–65 hr); four of these infants were <24 hr old. Three infants were delivered by Caesarean section, and two infants had unruptured membranes until operative delivery. None of the infants had a history of prolonged rupture of membranes (mean \pm SEM interval between rupture and delivery, 1.9 ± 0.8 hr; range, 0.2–7.5 hr).

Four infants positive for virus-like particles were thought to be septic on the basis of one or more of the following characteristics: respiratory distress, hepatosplenomegaly, lethargy, and abnormally low or elevated white blood cell counts. The bacterial cultures were negative for pathogens in all four of the infants. Three of the infants had viral cultures done, which were also negative; two of these infants died. A postmortem examination, performed on only one infant, demonstrated pneumonia and hepatosplenomegaly but no gastrointestinal lesions. Virus-like particles were not seen in postmortem sections of the lung or the liver, and attempts to culture virus from these organs were unsuccessful.

Mother-infant pairs. In an effort to determine whether vertical transmission occurred, 13 other infants positive for virus-like particles whose mothers had gastrointestinal or flu-like illnesses within two weeks of delivery were identified in the community (not part of the survey study) (table 3). All of the mothers except one had symptoms that were predominantly gastrointestinal, including nausea, vomiting, diarrhea, and occasional fever. Five of the mothers went into premature labor soon after becoming symptomatic. Two of the four mothers examined also had virus-like particles in the stool. The transmission of virus-like particles across the placenta or intact membranes is suggested by the delivery of four infants—patients no. 7 and no. 8 (twins; Caesarean section) and patients no. 2 and no. 9 (vaginal deliveries)—at which the amniotic membranes were not ruptured until the time of delivery. Virus-like particles were present in meconium passed on the first day of life in all four infants.

Four of the 13 infants had no gastrointestinal symptoms or other evidence of infection. Two of these infants died at <24 hr of age; their deaths were due to extreme prematurity and respiratory failure. Six of the remaining infants developed loose stools, distention, and/or bilious aspirates, and three infants had clinical and/or hematologic (leukopenia or leukocytosis) evidence of infection with negative bacterial and viral cultures. The only potential pathogen identified was an echovirus in the stool of patient no. 1. Three of the infants had hepatomegaly and/or persistent conjugated hyperbilirubinemia.

Discussion

Our results suggest that the presence of virus-like particles in the stool is associated with symptoms of gastrointestinal illness in neonates. Infants positive for virus-like particles were more likely to have gastrointestinal symptoms, including water-loss stools containing gross or occult blood, bilious gastric aspirates, and abdominal distention. Multiple symptoms and the need to discontinue oral feeding for several days were more frequent in infants positive for virus-like particles.

The frequency with which virus-like particles were associated with gastrointestinal symptoms would tend to be underestimated as a result of our study design, because we considered only the presence or absence of symptoms seven days before or

Table 3. Clinical characteristics of 13 infants positive for virus-like particles in the stool and of the infants' mothers.

Patient no.	Birth weight (g)	Gastro-intestinal symptoms	Age at onset (duration)*	Age at detection of virus-like particles*	Viral cultures	Characteristics of infant's mother	
						Symptoms	Virus-like particles in the stool
1	2,585	Yes	6 (2)	7	Echovirus 11	Fever, diarrhea two days before onset of infant's symptoms	ND
2	1,200	No†	...	<1	ND	Fever, vomiting, diarrhea 15 hr before delivery	ND
3	1,680	Yes	2 (5)	4	ND	Diarrhea for five days before delivery	ND
4	1,565	Yes	2 (5)	6	ND	Fever, vomiting five days before delivery	ND
5	2,977	Yes	1 (3)	<1	Negative	Fever, diarrhea, hepatic dysfunction two days before delivery	Positive
6	1,440	No	...	6	Negative	Fever, myalgia, chills, upper respiratory infection, loose stools 10 days before delivery	ND
7‡	1,700	No	...	<1	ND	Vomiting, abdominal cramps, diarrhea for nine days before delivery; father and older sibling had similar symptoms	Positive
8‡	1,600	No	...	<1	ND		
9	910	No†	...	<1	Negative	Vomiting, diarrhea for one week before delivery	Negative
10	3,800	No	...	<1	Negative	Fever, vomiting, diarrhea, chills, one day before delivery	Negative§
11	1,928	No	...	<1	ND	Fever, vomiting four days before delivery; sibling had similar symptoms	ND
12	2,050	Yes	5 (8)	<1	ND	Vomiting, diarrhea four days before delivery	ND
13	2,525	Yes	9 (11)	17	Negative	Diarrhea two weeks before delivery	ND

NOTE. These infants were identified by a survey of the community (September 1979–May 1981) and were not involved in an outbreak of gastrointestinal illness in a neonatal intensive-care unit that is described elsewhere in the present report. Gastrointestinal symptoms included water-loss stools, blood in the stool, gastric retention, bilious gastric aspirates, and abdominal distention. Stool specimens were examined for pleomorphic, enveloped, virus-like particles by electron microscopy. ND = not done.

* Expressed in days.

† Died of unrelated causes at less than one day of age.

‡ Patients no. 7 and no. 8 were twins.

§ Tested seven days after delivery.

after the time of screening. The infants studied who had such findings more than seven days before or after the screening were considered asymptomatic in the data analysis. Because our longitu-

dinal observations suggest that the duration of shedding is quite variable (less than one week to more than six weeks), infants positive for virus-like particles with symptoms more than seven days

before the time of screening may have shed the particles persistently. Additionally, direct electron microscopy is a relatively insensitive method for the detection of viral agents, and the incidence of infected infants may have been greater than that detected by this method.

Most of the infants in our study were premature and thus required intensive care or observation. Our results may reflect the increased susceptibility of the immature infant to colonization and disease associated with virus-like particles or the population bias of our sample. Maass et al. [9] have reported a similar outbreak of gastroenteritis associated with coronavirus in the stools of 15 of 24 symptomatic premature infants, and Moscovici et al. [16] have presented evidence that suggests the association of this agent with neonatal hemorrhagic enterocolitis. None of the infants in our study developed necrotizing enterocolitis as defined by radiographic evidence of intestinal intramural air, although many had symptoms that are associated with the early stages of the disease.

It is known that suckling mice inoculated orally with mouse hepatitis virus (a coronavirus) develop a lethal systemic illness [3]; the virus can be detected in the intestines, liver, and brain, and necrotizing lesions are evident in the intestines, liver, lungs, and lymphoid tissues. In comparison, seven of our infants positive for virus-like particles (four from the initial survey and three from the mother-infant pairs) appeared to be septic, both clinically and hematologically. In each case bacterial cultures were negative as were viral cultures obtained in six of the seven infants. Their symptoms may have reflected a systemic infection due to the virus-like particles; however, the recovery of the particles from involved organs is necessary to establish this relationship. Although we are unable to link the symptoms directly with a definite virus-like particle etiology, the temporal relationship is suggestive.

We have also demonstrated a changing incidence of infants positive for virus-like particles in the NICU over a 10-month observation period. The

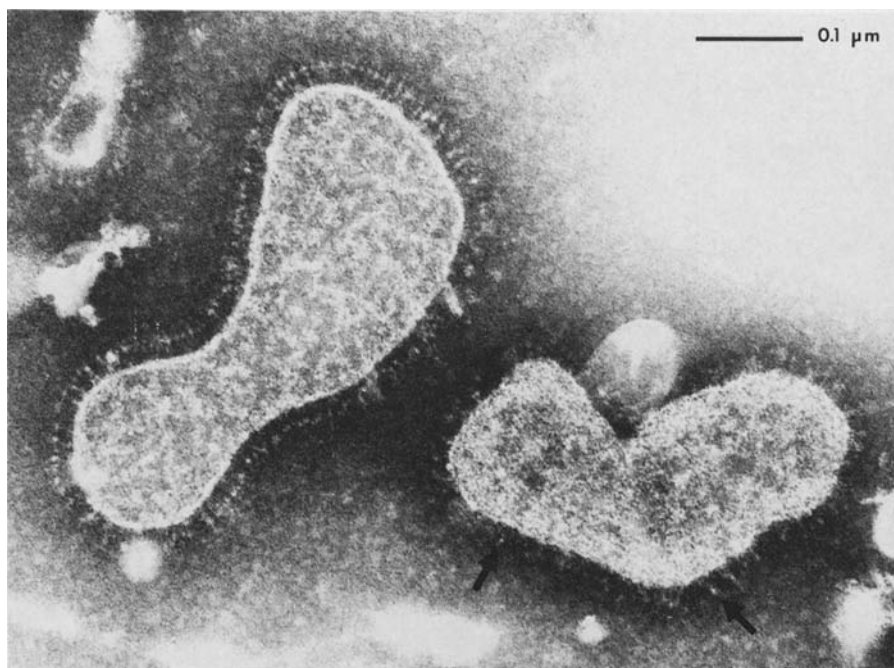


Figure 5. Variation in the fringe morphology of two pleomorphic, enveloped, virus-like particles observed by electron microscopy in the stool of an infant with gastrointestinal illness (negative contrast, phosphotungstic acid). The stool sample was obtained from an infant in a neonatal intensive-care unit after the observation period described elsewhere in the present study. The virus-like particle at the left shows a more typical coronavirus-like appearance with numerous characteristic knob-like and bulbous projections. The virus-like particle on the right shows a less distinct fringe with more widely spaced filaments that have less prominent knobs (arrows) at their distal ends.

high incidence was initially associated with an unusual clustering of infants with severe gastrointestinal disease and occurred at a time when gastroenteritis associated with virus-like particles was also commonly seen in the outside community. The incidence of virus-like particles decreased in the nursery in parallel with the decreased detection of virus-like particles associated with gastroenteritis in the community (authors' unpublished observations).

The present study did not establish the source of the colonization of virus-like particles. However, our observations that the incidence of virus-like particles corresponded to the presence of gastroenteritis associated with virus-like particles in the community, that many infants were positive within the first 72 hr of life, and that the mothers of several of the infants had gastrointestinal symptoms shortly before delivery suggest that colonization or infection may be acquired from the mother before or during delivery.

There has been much controversy over the nature of virus-like particles such as those we observed during this outbreak. Because most of these particles have club-shaped or bulbous instead of the knob-like projections that are characteristic of coronaviruses [17], they have been referred to as myxovirus-like [18]. Dourmashkin et al. [19] even suggest that the "coronavirus-like" particles may represent a yeast-like organism such as *Blastocystis*. Most authors agree, however, that the particles represent viruses and that they are more coronavirus-like than myxovirus-like. The myxoviruses have a narrower, more compact fringe with rigid spikes that tend to be sharp or pointed. The variation in surface projections has already been described for the avian infectious bronchitis virus [20, 21]. In view of this documented evidence of variation, Caul et al. [22] believed that the important criterion for the identification of human enteric coronaviruses in clinical material is the length of the projections in conjunction with their radiating appearance rather than their shape. Nonspecific cell membranes and inner mitochondrial membranes present in fecal material tend to have shorter surface projections and are not usually present in large numbers. Therefore, the possession of classic petal-shaped projections must no longer be considered a prerequisite for coronavirus identification [7, 23]. We think that these pleomorphic particles are somehow related to the cor-

onaviruses because we have consistently observed them in large numbers in stools containing the more classic coronaviruses (figure 5). Naqi et al. [24], in a study of transmissible (coronaviral) enteritis of turkeys, show an electron micrograph similar to figure 5. Whether this variation reflects different morphologic stages in coronavirus development, an effect of intestine-associated enzymes or antibodies on fringe morphology, or strain variations remains to be determined. Our immune electron microscopic studies show definite clumping of envelopes without readily identifiable fringes next to the definite fringed particles. This result may be an indication of the presence of envelope-associated viral antigens and might explain some of the variation in fringe morphology seen in the direct examination of stool specimens.

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