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Enzyme-Linked Immunosorbent Assay Reactivity of Torovirus-Like Particles in Fecal Specimens from Humans with Diarrhea

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Toroviruses are recognized enteric pathogens of cattle and horses; in humans, similar pleomorphic particles have been described, but doubt has been raised concerning their identity as viruses. We screened fecal samples from humans with diarrhea for the presence of torovirus-like particles (TVLPs) by electron microscopy and subsequently used an enzyme-linked immunosorbent assay (ELISA) with bovine torovirus reference reagents to test for the presence of torovirus antigens. To add another selection criterion to this heterologous ELISA, we enriched the TVLPs from the stool specimens by using sucrose density gradients before testing. The results of ELISA and EM correlated significantly, the ELISA having a sensitivity of 68% and a specificity of 86% (chi-square, $P < 0.0001$). In the gradient, peaks of ELISA reactivity were found at a buoyant density of 1.16 g/ml and were parallel to those found when using bovine torovirus. Furthermore, in 50% of the ELISA-positive gradients, a hemagglutinin for human group O erythrocytes comigrated with the peaks of ELISA reactivity. We were unable to isolate human TVLPs in human colonic tumor or rectal tumor cells. We cloned and sequenced amplification products obtained by low-stringency polymerase chain reaction amplification using consensus primers mapping to the 3′ end of the genome of animal toroviruses, but found no significant homologies with animal torovirus sequences. Rabbits were inoculated with material from the gradient peak fractions of human stool specimens, and their sera were assayed for immunologic comparison with bovine torovirus as a reference. A two-way antigenic cross-reactivity was seen between human TVLP and bovine torovirus reagents when tested by ELISA. The rabbit antisera to human TVLP detected a higher number of electron microscopy-positive stool specimens than did the rabbit antisera to bovine torovirus. The application of these assays and reagents should help to elucidate the roles of TVLPs and toroviruses in diarrheal disease in humans.

Toroviruses are enveloped, positive-stranded RNA viruses that cause enteric infections and diarrhea in animals. The toroviruses of horses (Berne virus; BEV) and cattle (Breda virus; BRV) are the best studied members of this new genus (torovirus) within the family *Coronaviridae* (6, 19). Toroviruses have been characterized at the molecular level by using BEV, which replicates in cell culture (21–23), whereas their pathogenesis and epidemiologic features have been examined mainly in cattle with BRV infections (8, 13, 14, 25, 27, 28). The viruses can be detected in clinical specimens by immune electron microscopy (IEM), enzyme-linked immunosorbent assay (ELISA), hemagglutination assay (HA), and nucleic acid hybridization (8, 12, 13, 26, 28). These methods have been used to establish the fact that toroviruses are associated with diarrhea in cattle (14, 16, 27).

The role of toroviruses as etiologic agents of human disease has remained in question because of the difficulty in establishing a clear diagnosis. Torovirus-like particles (TVLPs) have been found by electron microscopy (EM) in feces from children and adults with diarrhea in Great Britain, France, and The Netherlands (2, 15, 20), but their pleomorphic appearance and the presence of other fringed particles in stool specimens indicate that alternative tests are needed to confirm the identities of these particles. We previously reported on the use of a hybridization assay with BEV cDNA probes to detect toroviral RNA in stool specimens from humans (10), but high background levels and rapid degradation of the RNA in stool specimens made interpretation of these results difficult. Brown et al. (4) attempted antigenic confirmation and described a TVLP-containing stool sample, which had a hemagglutinin for rat erythrocytes that was blocked by BRV type 2 (BRV2) antibodies and reacted weakly in an ELISA for the detection of BRV2 antigen. Antigenic cross-reactivity of human TVLPs and bovine torovirus was further suggested by IEM results showing TVLP aggregates in two stool samples incubated with sera from BRV2-infected calves (1, 2). The extent of this cross-reactivity is unclear, since serosurveys in humans have failed to demonstrate the presence of antibodies when tested by a neutralization test (NT) with the BEV isolate and by BRV2 antibody ELISA (4, 5, 25), suggesting that possible toroviruses of humans are antigenically distinct from torovirus strains from animals or are rare.

Etiologic and epidemiologic studies of TVLP in humans will require improved methods for the detection and confirmation of the presence of these viruses in fecal specimens, which was the subject of the study described here. Since previous reports (1, 2, 4) suggest the presence of common antigens on human TVLPs and BRV2, we used immunoassays based on BRV2 reagents to test fecal specimens from children with diarrhea. The stool specimens were prescreened by EM for TVLPs and were then assayed for torovirus antigen in a blind fashion. To improve the assay, antisera to human TVLPs were prepared in rabbits and their reactivities were compared by ELISA with those of antisera

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to BRV2. In addition, amplification products, obtained by reverse transcriptase polymerase chain reaction (RT-PCR) with primers to equine and bovine torovirus consensus sequences and RNA extracts from human stool specimens as a template, were cloned, sequenced, and analyzed.

MATERIALS AND METHODS

Reference virus and sera. BEV grown in equine dermis cells and BRV2 from an infected gnotobiotic calf (GC68), which have been described previously (9, 24), were used as reference strains. Control antisera included calf antisera to BRV2 (GC76) and *Cryptosporidium* spp. (GC75), as well as rabbit antisera to adenovirus (rabbit anti-adeno) and BRV2 (rabbit anti-BRV2 [RaB2]) (8, 13, 27, 28). Immunoglobulins were precipitated from the serum by salt fractionation (7) and were then dialyzed against phosphate-buffered saline solution (PBSS). To remove nonspecific reactivity with human cells, the immunoglobulin G fractions were absorbed with an equal volume of packed human rectal tumor cells (American Type Culture Collection, Rockville, Md.) for 30 min at 37°C and were then centrifuged at 10,000 × *g* for 30 min. The supernatant was divided into aliquots and stored at −20°C.

Clinical specimens. In June 1991, we alerted electron microscopists from eight EM centers in the United States and Canada to look for TVLPs and provided them with electron micrographs and written descriptions of animal toroviruses. In October 1991, the EM unit at the Hospital for Sick Children in Toronto began identifying TVLPs in stool specimens from children with gastroenteritis. Over the subsequent 6 months, 89 samples with and without TVLPs were coded and sent to the Centers for Disease Control and Prevention in Atlanta for further testing. The hospital is a 493-bed tertiary-care referral center where all stool specimens from patients with gastroenteritis are examined by EM for the presence of enteric viruses within 24 h of collection. Clinical and epidemiologic information was collected from the charts of these patients and will be presented elsewhere (7a).

EM and IEM. Stool specimens were kept in tightly closed plastic transport containers at 4°C. Negative-contrast EM was performed by applying a 20% stool suspension in 1% ammonium acetate to a 400-mesh grid precoated with polyvinyl formal and carbon. Excess fluid was removed with filter paper, and the grid was stained for 1 min with a drop of 2% phosphotungstic acid (pH 7.0). The dried grids were placed under a UV source for 5 min for inactivation of virus and were examined in an electron microscope (Philips EM 300) at a magnification of ×50,000 (17).

For IEM, stool preparations were mixed with a 1:50 dilution of RaB2 for 30 min at 37°C and were stained as described above.

Preparation of rabbit antisera to TVLP. Four female New Zealand White rabbits were screened for the absence (titer, <10) of torovirus antibodies in a BEV NT, since more than 60% have been shown to react positive by this assay (23a). The animals were inoculated with purified human TVLPs (three rabbits; RaH1 to RaH3) or bovine torovirus BRV2 (RaB2a; not to be confused with reference serum RaB2 described above). The three human stool specimens were selected because they had high ELISA titers, were negative for other known viruses, and were available in sufficient quantities. Sucrose gradient fractions with high ELISA reactivities were pooled and sucrose was removed by using microconcentrators (Centricon 100; Amicon, Danvers, Mass.) by following the manufacturer's instructions. Each virus preparation was emulsified with an equal volume of adjuvant (Titermax; Cytrex, Norcross, Ga.) (3). The rabbits were inoculated intramuscularly with two 40-μl emulsions and were bled at 2-week intervals starting at 4 weeks postinoculation. Booster injections were given after 8 weeks; boosters consisted of 50 μl of each antigen with adjuvant. The rabbits were euthanized and exsanguinated after 12 or more weeks. Immunoglobulins were precipitated from the sera as described above.

Preparation of samples for ELISA. Stool samples were diluted 1:5 in PBSS and were clarified by low-speed centrifugation. Virus was pelleted from the supernatant through 15% (wt/wt) sucrose onto a 50% (wt/wt) sucrose cushion. One milliliter of interphase was collected and was either tested directly by ELISA or diluted 1:4 in PBSS and layered onto a linear 10 to 50% (wt/wt) sucrose density gradient. After a 2-h centrifugation at 100,000 × *g*, the gradients were fractionated from the bottom. Stool specimens from a gnotobiotic calf (GC68) infected with BRV2 were purified in the same manner and served as a positive control.

ELISA. Serial twofold dilutions of the 15 to 50% sucrose interphase or of the gradient fractions were prepared in 0.1 M $Na_2CO_3/NaHCO_3$ coating buffer (pH 9.6), and 50 μl was adsorbed to duplicate wells of a flat-bottom microtiter plate (Immulon II; Dynatech Laboratories, Chantilly, Va.). After overnight incubation at 4°C, the plates were washed with PBSS containing 0.05% Tween 20 by using a Microwash II ELISA plate washer (Skatron/AS, Lier, Norway). Fifty microliters of 1% ovalbumin in coating buffer was added, and the plates were incubated for 30 min at 37°C. After washing, 50 μl of a 1:500 dilution of purified immunoglobulin G from negative control serum (preimmunization serum from rabbits, or rabbit anti-adenovirus) or postimmunization serum RaB2 or RaH in ELISA buffer (PBSS, 0.35 M NaCl, 1 mM EDTA, 0.05% Tween 80) was added to parallel wells, and the plates were incubated for 30 min at 37°C. The plates were washed again, and 50 μl of alkaline phosphatase-labelled goat anti-rabbit immunoglobulin G diluted 1:2,000 in ELISA buffer was added to the wells. After 30 min of incubation at 37°C, the plates were rinsed and 100 μl of the substrate solution was added (*p*-nitrophenyl phosphate in 10% diethanolamine buffer). After 2 h at room temperature, the absorbance values of the reactions were read at 410 nm (V_{max} microplate reader; Molecular Devices Corporation, Palo Alto, Calif.). Stool samples were considered positive if the signals in the presence of anti-BRV serum were at least twice as high as those with the nonimmune serum and the anti-adenovirus serum (positive/negative ratio [P/N], ≥2).

HA. Serial twofold dilutions of the gradient fractions were made in 10-μl volumes of PBSS in V-bottom microtitration plates. Bovine fetal serum was diluted 1:5 and was adsorbed with packed rat erythrocytes or with human group O erythrocytes until all spontaneous agglutination was lost, and 10-μl volumes of a 1:200 dilution in PBSS were added to each well. After 10 μl of a 1% suspension of the rat or human erythrocytes in PBSS containing 0.1% ovalbumin was added, the plates were incubated for 2 h at room temperature and read.

Tissue culture propagation of BEV and NT. BEV was propagated in equine dermis cells as described previously (24). To test the rabbit sera (RaB2a and RaH1 to RaH3 pre- and postimmunization) for neutralizing antibodies to BEV, the serum specimens were heated for 30 min at 56°C, serially diluted in tissue culture medium, and mixed with 10 or 100 50% tissue culture infective doses of BEV. After 30 min at

37°C, the virus-serum mixture was added to triplicate wells of a confluent monolayer of equine dermis cells. Neutralization was scored visually by looking for the absence of cytopathic effect (CPE) at 2 to 5 days postinfection. GC75 and GC76 sera, processed in the same manner, were used as negative and positive controls, respectively (1).

Tissue culture isolation. Gradient fractions that showed reactivity by ELISA were pooled, diluted 1:100 in modified Eagle's medium (MEM) with 10 μg of trypsin per ml, and inoculated onto human rectal tumor cells and human colonic tumor cells (American Type Culture Collection, Rockville, Md.). The cells were grown to confluency in six-well plates in MEM, supplemented with 10% fetal bovine serum–2 mM glutamine–100 U of penicillin per ml–100 μg of streptomycin–20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2). Prior to infection, the cultures were rinsed with TNE (50 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA) and 1 ml of the inoculum was added. After 1 h at 37°C, 9 ml of MEM with 5 μg of trypsin per ml was added. The cultures were incubated for 5 days at 37°C, frozen, and thawed once, and the cell lysate was diluted 1:100 for further passaging. The cells were monitored for CPE and cell lysates of passages 4 and 5 were tested by ELISA and RT-PCR. For the ELISA, lysates from infected and mock-infected cells were coated onto microtiter plates and the plates were incubated overnight at 4°C in a 1:2 dilution in carbonate buffer.

RT-PCR. RNA was extracted from the stool specimens and cell lysates by binding to size-fractionated silicon dioxide particles as described previously (12). RT-PCR was performed as described previously by using primer sequences chosen from the 3'-noncoding region of the torovirus genome that is highly conserved (93% identical nucleotides [12]) between bovine and equine toroviruses (11). The primers (a 21-mer and a 22-mer) were fully conserved and were used at annealing temperatures that were 9°C below the recommended annealing temperature (11). The products were analyzed on a 2% agarose gel in TAE (0.04 M Tris-acetate, 1 mM EDTA) and were stained with 0.5 μg of ethidium bromide per ml in H_2O.

Cloning and sequencing. PCR products that were in the expected size range were excised from the gel, separated from the agarose by using a 0.45-μm-pore-size cellulose-acetate filter (Spin-X; Costar, Cambridge, Mass.), and further purified by using microconcentrators (Centricon 30; Amicon), according to the manufacturer's instructions. Ten percent of the DNA was loaded onto a 2% agarose gel to estimate the yield. DNA was ligated into a cloning vector for PCR products (PCR1000; TA Cloning Kit; Invitrogen, San Diego, Calif.) at a 1:1 ratio of insert to plasmid, and the ligation mixture was used to transform DH5αME (Bethesda Research Laboratories, Cockeysville, Md.), according to the manufacturer's instructions. Transformants were screened by PCR by using primers to vector sequences outside the cloning site. Colonies containing inserts were grown overnight, and plasmid DNA was extracted by alkaline lysis and was further purified by using Sephacryl S400 spin columns (Miniprep; Pharmacia, Piscataway, N.J.). The plasmid inserts were analyzed by dideoxy-sequencing by using T7 DNA polymerase (Sequenase; USB Chemicals, Cleveland, Ohio) according to the manufacturer's instructions. The sequence information was used to select primers for RT-PCR with RNA extracts from TVLP-positive stool specimens by using the computer program "OLIGO."

Statistical analysis. The results of ELISA were compared with the coded EM results for specimens with and without TVLPs to determine whether the assays were concordant. The data were analyzed by using the Epi Info 5.0 program and the chi-square test with Yates' correction or Fisher's exact test (two-tailed).

RESULTS

EM and IEM. A total of 89 coded stool specimens were screened by EM and were then tested by ELISA. By EM, 60 samples were positive for TVLPs, 21 contained other viruses (14 rotavirus, 3 astrovirus, 1 calicivirus, and 3 Norwalk-like viruses), and 8 were EM negative. Only 10 to 15% of the TVLPs in stool specimens had the typical torovirus shape. The particles were quite pleomorphic (Fig. 1), appearing spherical, kidney shaped, or elongated. They measured 100 to 150 nm in diameter and had peplomers of approximately 10 nm in length. However, a common technical feature was the presence of uneven staining of the particles, with a portion of the particle appearing darker than the remainder. The TVLPs were seen both in unpurified stool specimens and in sucrose gradient fractions with high ELISA reactivity.

The results of IEM were unconclusive, since some stool preparations, incubated with and without RaB2 serum, showed coating of particles with antibody (data not shown). We did not use any of the RaH sera for IEM.

ELISA of stool samples. When the code was broken, ELISA proved to be a good predictor of EM results (Table 1). Of the 60 samples positive for TVLPs by EM, 41 (sensitivity 68%) were positive by ELISA. Of the 29 samples negative for TVLPs by EM, 25 (specificity, 86%) were also negative by ELISA. The overall agreement between the test results was 74% (chi-square, $P < 0.0001$).

Linear 10 to 50% sucrose density gradients were used to further purify 15 to 50% sucrose interphases of human stool specimens and BRV2 (GC68) with high ELISA readings (>0.4; Fig. 2). Peaks of ELISA reactivity were found in gradient fractions 9 to 14 of the TVLP1, TVLP2, and BRV gradients (TVLP1 and TVLP2 indicate two human stool specimens containing TVLPs), indicating a virion density of 1.16 to 1.17 g/ml. When interphase material with low ELISA reactivity was further purified, ELISA peaks were absent or very low.

HA. A peak in HA titer was found to coincide with the ELISA peak for 50% of the gradient-purified TVLP-positive human stool specimens (Fig. 3). Additional HA activity was seen at the top of the gradients. HA activity was observed only for human group O erythrocytes and not for rat erythrocytes.

Tissue culture isolation. Gradient-purified TVLPs ($n = 24$) were diluted in tissue culture medium and were added to confluent monolayers of human rectal tumor and human colonic tumor cells. Scoring for CPE was difficult, because the presence of trypsin in the culture medium resulted in rounding of cells and disruption of the monolayers. After three blind passages, cell lysates from infected and mock-infected cells were tested by ELISA and RT-PCR. No ELISA reactivity above the background level was seen after incubation with RaB2, RaB2a, or RaH1, and RT-PCR amplification of RNA extracts from the cell lysates did not yield detectable products.

Immunization of rabbits. Sera from all rabbits showed an increase in ELISA reactivity to TVLP1 and BRV, expressed as the absorbance of the postimmunization serum after subtraction of that of the preimmunization serum (P − N; Fig. 4). When using P/N > 2 as a cutoff, only RaB2a and

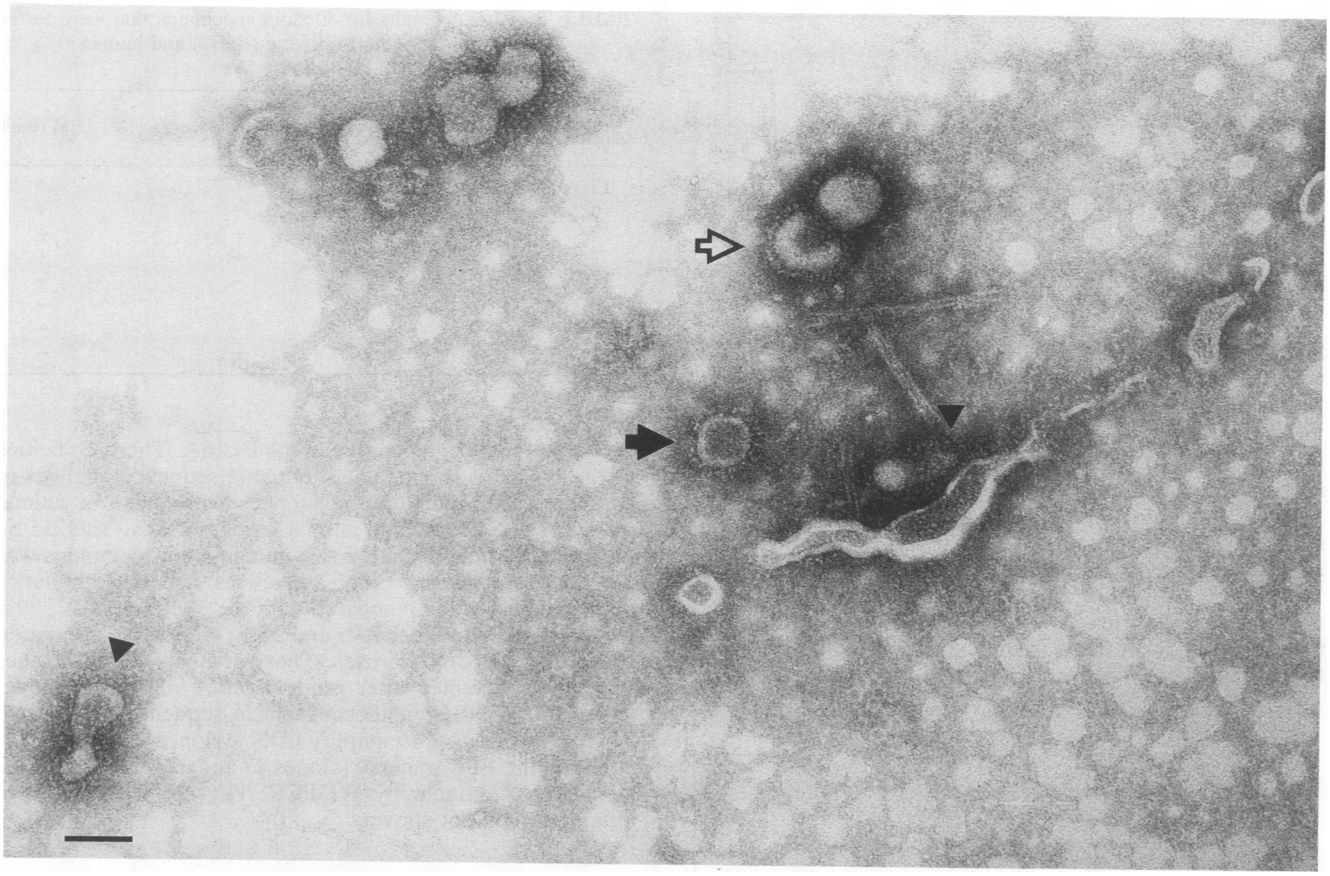

FIG. 1. Electron micrograph of a stool sample containing spherical (filled arrow), kidney-shaped (open arrow), or elongated (arrowhead) human torovirus-like particles. Bar, 100 nm.

RaH1 showed significant titer rises at 8 weeks postinoculation. ELISA readings for RaH1 to RaH3 were highest when homologous TVLPs were used as the ELISA antigen. ELISA results were similar when six different TVLP-positive stool specimens instead of BRV or TVLP1 were used as antigen (data not shown). When tested in a BEV NT, RaB2a showed an increase in titer from <10 to >320. None of the sera from rabbits immunized with TVLP neutralized BEV, but the CPE was delayed by 1 day with RaH1 postinoculation serum diluted up to 1:160.

Interphase material of a subset (n = 40) of stool samples was retested by ELISA by using homologous RaH1 pre- and postinoculation sera instead of the heterologous RaB antisera as detector antibodies (Table 2). Of the 15 samples that had been positive by ELISA with RaB2 (RaB2-positive), 13 were positive when RaH1 was used as the detector antibody (RaH1 positive). Seventeen of 25 RaB2-negative specimens were also negative with RaH1. RaH1 was positive with six more stool specimens than was RaB2, five of which were positive by EM for TVLP. The sensitivity of ELISA detection (compared with that of EM) was higher when RaH1

TABLE 1. EM and ELISA results for 89 stool specimens from children with diarrhea[a]

ELISA result	No. of specimens with the following TVLP-EM result:		
	Positive	Negative	Total
Positive	41	4	45
Negative	19	25	44
Total	60	29	89

[a] Samples were screened by EM for the presence (EM positive) or absence (EM negative) of TVLPs, coded, and tested by the ELISA. ELISA was done with rabbit sera to bovine torovirus after partial purification of the stool specimens by sucrose gradients.

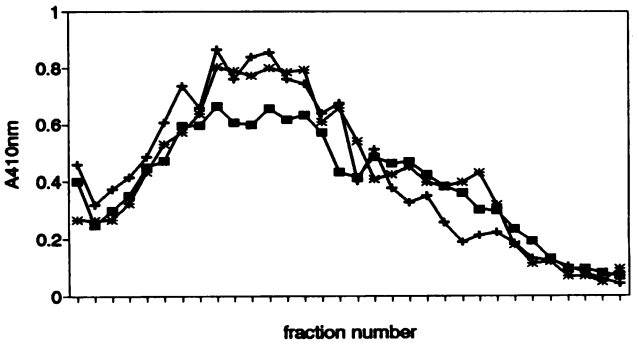

FIG. 2. ELISA results of gradient fractions from a linear 10 to 50% sucrose gradient of two human stool specimens containing TVLPs (TVLP1 [+] and TVLP2 [*]). The gradients were fractionated from the bottom to the top. Bovine reference torovirus was treated in the same manner as a positive control (BRV [■]).

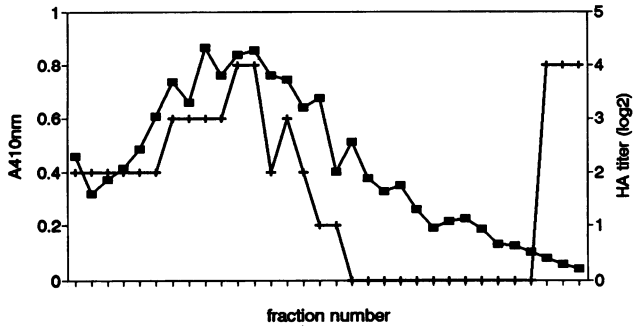

FIG. 3. ELISA (■) and HA (+) results of gradient fractions from a linear 10 to 50% sucrose gradient of TVLP1.

TABLE 2. ELISA results for 40 stool specimens that were tested with rabbit antisera to bovine (RaB2) and human (RaH1) toroviruses

No. of samples	ELISA result		EM result
	RaH1	RaB2	
12	+	+	+
6	−	−	+
7	+	−	+
2	−	+	+
11	−	−	−
0	−	+	−
1	+	−	−
1	+	+	−

serum was used, but RaH1 gave one extra false-positive reaction, thus decreasing the specificity.

RT-PCR, cloning, and sequencing. RT-PCR of some of the stool extracts, using consensus sequence primers for equine and bovine toroviruses, yielded multiple bands (Fig. 5, lanes 1, 2, and 4). A total of nine TVLP-positive and five TVLP-negative stool specimens were tested. The two extracts that resulted in PCR products in the expected size range (Fig. 5, lanes 2 and 4, arrow) were from TVLP-positive stool specimens that had been positive in the ELISA. The specificities of the bands were examined by using sequence analysis to compare their nucleotide sequences with those of animal torovirus and human and animal coronaviruses. The 215-bp fragment obtained from the two human stool specimens was distinct from all the other sequences and had similarity scores of 38% when compared with the 3′ end of BEV, 39% when compared with BRV, and 35 to 40% when compared with different coronaviruses. These scores were not higher than those obtained after randomization of the sequence. Primers derived from the consensus sequence of the two products were used to amplify cDNA clones containing the 3′ end of the BEV genome (clones 27 and 31 [22]) and TVLP stool RNA extracts by RT-PCR, but no products were obtained (data not shown).

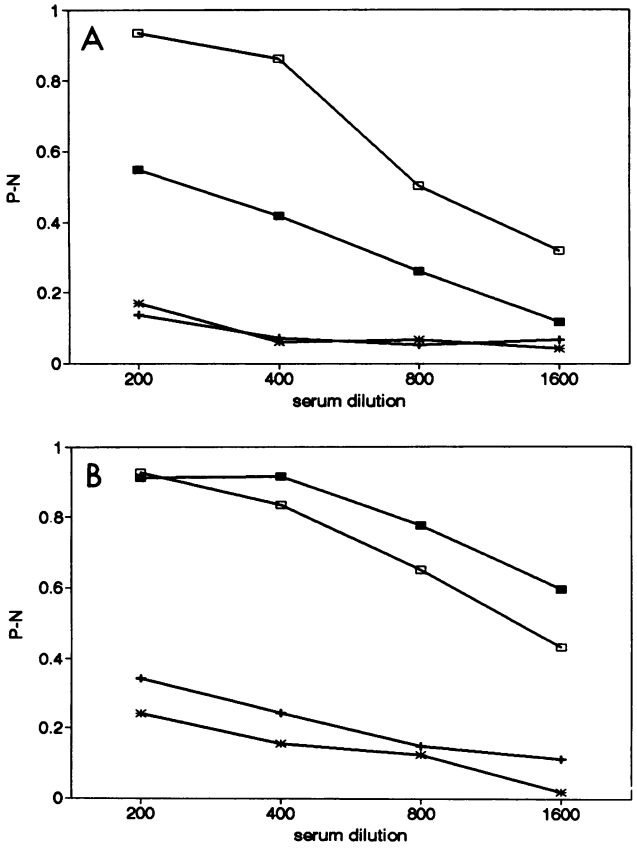

FIG. 4. Reactivity of sera from rabbits inoculated with BRV2 (RaB2a) or TVLP purified from human stool. The sera were tested in an ELISA with either BRV2 (A) or TVLP1 (RaH1; B) as an antigen. ELISA reactivity is expressed as A_{410} of postimmunization serum after subtraction of that of preimmunization serum (P − N). ■, RaH1; +, RaH2; *, RaH3; □, RaB2a.

DISCUSSION

The presence of TVLPs in human stool specimens has been reported by electron microscopists, but the identities of these particles as well as their role in human disease have remained unclear. Diagnosis by EM is difficult because of the pleomorphic appearance of torovirus particles; thus, the use of confirmatory assays is essential. In the present study, the presence of TVLPs in a collection of stool specimens from humans with diarrhea was confirmed by using partial purification and concentration of particles in sucrose gradi-

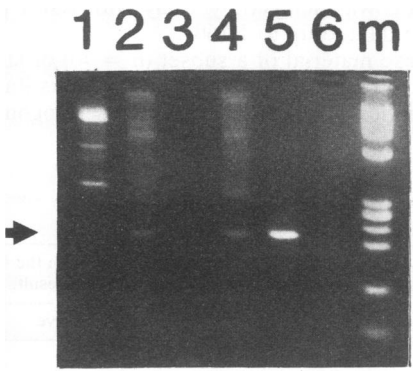

FIG. 5. RT-PCR products obtained by using torovirus consensus primers and RNA extracted from ELISA-negative (lane 1) and ELISA-positive (lanes 2 to 4) stool specimens from humans. RNAs extracted from lysates of equine dermis cells infected with equine torovirus (lane 5) or mock infected (lane 6) were used as positive and negative controls, respectively. The arrowhead indicates the expected product. lane m, molecular weight markers.

ents and subsequent ELISA testing based on bovine reference reagents. When we compared the EM results with the ELISA reactivity of a set of coded stool specimens from humans with diarrhea, we found a highly significant association between the test results, indicating that the ELISA detected antigens that are cross-reactive with animal torovirus proteins in the stool specimens that contained TVLPs. Our findings were confirmed by ELISA, using sera from rabbits that had been inoculated with purified virus preparations from human TVLPs. Antigenic differences between BRV and TVLP are suggested by the finding that the homologous ELISA reactivity of the sera was always higher than the heterologous reactivity with the bovine virus.

The sensitivity of the ELISA for TVLPs with antisera to bovine torovirus was relatively low, a result which may be explained by the use of heterologous sera, loss of virus particles during the ultracentrifugation steps, or the higher sensitivity of EM detection. Alternatively, since diagnosis of torovirus infection by EM alone is difficult (1, 16), some of the EM results may have been false positive. Beards et al. (1) suggested that at least three of the following five criteria should be met to report the presence of TVLPs in a sample: (i) the mean diameter is 100 nm; (ii) peplomers approximately 10 nm long are present on the surface; (iii) an internal toroidal structure is visible; (iv) the buoyant density in sucrose is 1.14 to 1.16 g/ml, and (v) particles are agglutinated by antisera to BEV or BRV. In our study, these criteria were used throughout, although an internal torus was rarely seen. Beards et al. (1), however, reported that the toroidal inner structure usually was not visible at the initial examination of the grids, but was often found at reexamination a few days later, a procedure which was not done in our study.

The ELISA was very specific (86%), and only one of the four ELISA-positive, EM-negative samples had high absorbance values. As an additional control for the specificity of the ELISA, interphase material of stool specimens that showed high ELISA reactivities was further purified in parallel with a reference torovirus. The ELISA-reactive fractions from human stool specimens banded in the same density range as those of BEV and BRV (1.16 to 1.17 g/ml [9, 24]). In addition, a hemagglutinin for human blood group O erythrocytes was found at the same density, as has been demonstrated for BEV (29). BRV hemagglutinates rat and mouse erythrocytes, but not human erythrocytes (28). The HA activity at the top of the gradients may represent free-floating peplomers, as has been seen with BRV and BEV, or nonspecific HA that is present in many stool specimens (9, 28). These findings suggest that the TVLPs in human stool specimens show antigenic cross-reactivity to BRV. Since attempts to isolate human TVLPs in cell culture were unsuccessful and TVLP antigen had to be prepared from stool specimens, the possibility that a common contaminant in human and bovine stools (from which BRV antigen was prepared) caused the antigenic cross-reactivity cannot completely be excluded. We tried to minimize that possibility by using reagents from gnotobiotic calves that had been screened thoroughly for the absence of known pathogens and by partial purification of the human stool specimens prior to ELISA testing.

This investigation was designed to determine whether a proportion of the "fringed particles" that are frequently seen by electron microscopists might be toroviruses. Our data support preliminary evidence for this possibility that was obtained by IEM studies of Beards et al. (2) and by hybridization tests with equine torovirus cDNA probes (10). In contrast to our findings, Brown et al. (4), when using a BRV ELISA to test 400 human stool specimens, found only one weak positive reaction which banded in sucrose gradients at a density that was different from those of BEV and BRV. Possible explanations for this discrepancy might be the enrichment by sucrose density gradients that we used before ELISA testing was done, the use of an antigen capture step in the protocol of Brown et al. (4), which might be unsuccessful if the antisera had low avidity to heterologous toroviruses, or a higher prevalence of TVLPs in the population of our study.

We previously reported possible amplification of RNA from human stool specimens by using consensus primers to bovine and equine toroviruses (10). We repeatedly obtained amplification products in the expected size range (Fig. 5), along with several other bands. We chose to use cloning and sequencing of isolated products to determine their specificities. The sequence similarities obtained were too low to signify relatedness with animal torovirus sequences, indicating that the amplification products resulted from nonspecific annealing. Furthermore, amplification was obtained neither with reference torovirus cDNA clones or RNA nor with ELISA-positive human TVLP extracts by using primers derived from the cloned PCR products.

In conclusion, TVLPs are found in human stool specimens and can be detected by ELISA with bovine torovirus or human TVLP reference reagents after a purification step is done. EM screening alone is insufficient, but the use of stringent criteria (1) increases the probability of differentiating TVLPs from other fringed particles. Our rabbit antiserum to human TVLPs used in the ELISA increases the sensitivity of the assay, making it a more useful tool in epidemiologic studies of the clinical consequences of infections with TVLP.

TVLPs may be more common in fecal specimens from humans with diarrhea than was previously appreciated. During the course of the study, they were found as frequently as rotaviruses (18a). Since all the stool specimens that we tested were from children with diarrhea, we cannot draw any conclusion about the pathogenic relevance of these viruses in humans. A prospective epidemiologic study is under way to address this issue.

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