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Characterization of Torovirus from Human Fecal Specimens

Lynn Duckmanton,*,† Bo Luan,† John Devenish,† Raymond Tellier,†,‡ and Martin Petric*,†,‡,[1]

*Department of Microbiology and Medical Genetics, The University of Toronto, Toronto, Canada; †Division of Microbiology, The Hospital for Sick Children, Toronto, Canada; and ‡Department of Laboratory Medicine and Pathobiology, The University of Toronto, Toronto, Canada

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The toroviruses, Berne virus (BEV) and Breda virus (BRV), are recognized pathogens of horses and cattle, respectively. Torovirus-like particles (TVLPs) that are immunologically related to BRV have been reported as etiological agents of gastroenteritis in humans. Of the toroviruses, only BEV has been shown to replicate in cell culture. Hence, these agents can be routinely detected only by electron microscopy (EM), although serological testing has been used as well. Our studies have provided supporting evidence that the TVLPs detected in the stool specimens of pediatric patients with gastroenteritis are human toroviruses. By EM, these particles are morphologically similar to BEV and BRV. Thin-section electron microscopy revealed that TVLPs contain toroidal-shaped nucleocapsids. Viruses purified from human fecal specimens agglutinate rabbit erythrocytes. BRV antiserum as well as convalescent sera from patients with gastroenteritis whose stools contain TVLPs were shown to contain antibodies that react with purified TVLPs as demonstrated by hemagglutination inhibition, immunoelectron microscopy, and immunoblotting. RNA extracted from partially purified TVLP preparations is amplifiable by RT-PCR using primers bracketing a 219-base region at the 3′ end of the Berne virus genome. Sequence analysis of amplicons from five isolates showed a high degree of identity with the corresponding BEV sequence.

INTRODUCTION

The torovirus prototype, Berne virus (BEV), was isolated in 1972 from the rectal swab of a diarrheic horse in Berne, Switzerland (Weiss *et al.,* 1983). A decade later, a morphologically similar virus, called Breda virus (BRV), was found in Breda, Iowa, in the stools of neonatal calves with diarrhea (Woode *et al.,* 1982). Other BRV isolates were later found in feces from a colostrum-deprived calf in Iowa and in the stools of diarrheic calves in Ohio (Woode *et al.,* 1985; Weiss and Horzinek, 1987). It was found that the two enteric pathogens, BEV and BRV, are morphologically and antigenically related (Weiss *et al.,* 1983), and as such they were initially proposed to comprise a separate family (Weiss and Horzinek, 1987; Horzinek *et al.,* 1987). However, recent analysis of the genome and replication strategy of BEV indicates that toroviruses are more closely related to the coronaviruses (Snijder *et al.,* 1991, 1990c). Thus, BEV and BRV are now classified as members of the genus *torovirus* in the family *Coronaviridae* (Cavanagh *et al.,* 1994, Cavanagh, 1997).

Morphologically, toroviruses are composed of an elongated tubular nucleocapsid of helical symmetry, surrounded by an envelope bearing peplomers. These particles measure 120 to 140 nm at their largest diameter (Horzinek *et al.,* 1987; Weiss and Horzinek, 1987). In the mature virion, the helical nucleocapsid is torus-shaped, from which the virus gets its name. However, the nucleocapsid may also exist in the form of a rod or crescent in a subset of particles (Weiss *et al.,* 1983).

Purified BRV particles were found to hemagglutinate mouse and rat erythrocytes, whereas BEV particles hemagglutinate human O, rabbit, and guinea pig erythrocytes (Horzinek *et al.,* 1987). These viruses also have cross-reacting antigens as evidenced by immunoelectron microscopy (IEM) (Beards *et al.,* 1986) and enzyme-linked immunosorbant assay (ELISA) (Weiss *et al.,* 1983; Brown *et al.,* 1987; Koopmans *et al.,* 1989). Viral structural proteins include the phosphorylated nucleocapsid protein, N (molecular mass 20 kDa), the envelope protein, M (molecular mass 25 kDa), and the glycoprotein peplomer, S, which migrates between 75 and 100kda because it has variable degrees of N-glycosylation (Horzinek *et al.,* 1986; Weiss and Horzinek, 1987).

The organization of the BEV genome and its replication strategy have been described by Snijder *et al.* (1990a). The BEV genome has been shown to be a single-stranded positive sense RNA of approximately 26–28 kb in length whose organization is similar to that of equine arterivirus (EAV), avian infectious bronchitis virus, and mouse hepatitis virus, with whom there is evidence of common ancestry (Horzinek *et al.,* 1987; Snijder and Horzinek, 1993; Snijder *et al.,* 1994). In addition, the sequence of 269 nt, upstream of the poly(A) tail of the BRV

Human torovirus nucleotide sequence has been deposited with GenBank under Accession No. AF024539.

[1] To whom correspondence and reprint requests should be addressed at Division of Microbiology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8. Canada. Fax: (416) 813-6257.

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genome has been determined and shown to be 93% identical to that of BEV (Koopmans *et al.,* 1991).

Evidence that toroviruses could also be potential human pathogens initially came from Beards *et al.* (1984) who reported the appearance by EM of particles similar to BEV and BRV in the fecal specimens of children with diarrhea. These agents, termed human torovirus-like particles (TVLPs), have since been proposed to be related to BEV and BRV on the basis of morphological and serological similarities. (Beards *et al.,* 1986). Further support for the existence of a human torovirus was obtained when human stool specimens which were found to contain TVLPs by EM were shown to be reactive in a torovirus-specific ELISA based on BRV antiserum (Koopmans *et al.,* 1993).

The above studies have provided good evidence that the TVLPs found in the stools of pediatric patients with gastroenteritis are human toroviruses. However, there remains a need to further characterize the biochemical, serological, and molecular properties of human TVLPs. In this study, we report on the purification and characterization of human torovirus from the fecal specimens of patients with gastroenteritis.

MATERIALS AND METHODS

Electron microscopy

Crude diarrheic stool samples suspended in 1% ammonium acetate, or purified TVLP preparations, were stained with 2% phosphotungstic acid and examined by negative contrast electron microscopy (EM) on a Phillips EM 300 at a magnification of 50,000× (Middleton *et al.,* 1977).

Purification of TVLPs

Stool specimens obtained from patients with gastroenteritis whose stools were found to be positive for TVLPs by EM were diluted with an equal volume of 1% ammonium acetate (w/v) and clarified by centrifugation at 9000 *g* for 20 min at 4°. The supernatants were layered on a 4-ml cushion of 25% Histopaque-1077 (Sigma, St. Louis, MO) in 1% ammonium acetate in 12-ml Beckman polyallomer ultracentrifuge tubes. The specimens were then centrifuged in a Beckman SW41Ti rotor at 100,000 *g* for 90 min at 4°. The pellets were resuspended in 1% ammonium acetate and stored at 4°. All control stool specimens used in this study were processed in a similar manner.

Thin-section electron microscopy

Purified TVLP preparations were subjected to ultracentrifugation at 100,000 *g* for 2 h at 4°, and the pellets were fixed in 2% gluteraldehyde for 24 h. After postfixing with osmium tetroxide overnight, the pellets were dehydrated in acetone, infiltrated and embedded in Epon araldite resin, and heat-polymerized overnight. Ultrathin sections (80 nm) were cut on a Reichert Ultracut E ultramicrotome (Leica Canada Inc.), collected on copper grids, and stained with uranyl acetate and Reynolds lead citrate. The sections were examined by EM at a magnification of 70,000× (Quan and Doane, 1983).

Immunoelectron microscopy

Immune electron microscopy was performed using either convalescent serum from BRV-infected calves (baBRV; provided by G. Woode, Texas A&M), or paired acute and convalescent sera from patients with gastroenteritis whose stools were positive for TVLPs by EM (a/c paired sera). The sera were heat inactivated at 56° for 30 min, diluted at 1:50 with 1% ammonium acetate, mixed with an equal volume of purified TVLP preparations or control preparations, and incubated for 2 h at 37°. The preparations were centrifuged at 9000 *g* for 15 min at room temperature. The pellets were resuspended in 1% ammonium acetate and examined by EM at a magnification of 50,000× (Kapikian *et al.,* 1972; Woode *et al.,* 1985). Control specimens included a purified rotavirus-positive fecal sample, and a purified sample from a patient with diarrhea in which no viruses could be detected by EM.

Hemagglutination assay

Serial twofold dilutions of purified TVLP preparations were made in 50-μl volumes of veronal buffered saline (VBS) (Oxoid, England) in V-shaped-bottom microtiter plates. For the hemagglutination assay (HA) 50 μl of a 0.5% suspension of either human O, rabbit, guinea pig, horse, or sheep erythrocytes in VBS was added to each well. The plates were incubated for 2 h at room temperature and examined for hemagglutination (Woode *et al.,* 1985). The reciprocal of the final dilution of the TVLP preparation showing complete hemagglutination was defined as the titer in HA units. A purified fecal specimen documented to be positive for rotavirus by EM and two stool sample preparations from patients with diarrhea in which no viruses could be detected by EM were tested in parallel as controls.

Preparation of guinea pig antisera to TVLPs

Two young adult male guinea pigs were inoculated subcutaneously once a week for 5 successive weeks with a 2:1 suspension of purified TVLPs in Freund's incomplete adjuvant. Pre- and postimmunization sera were collected.

Hemagglutination inhibition

The panel of sera tested for hemagglutination inhibition (HI) included guinea pig preimmune (gpPI) and

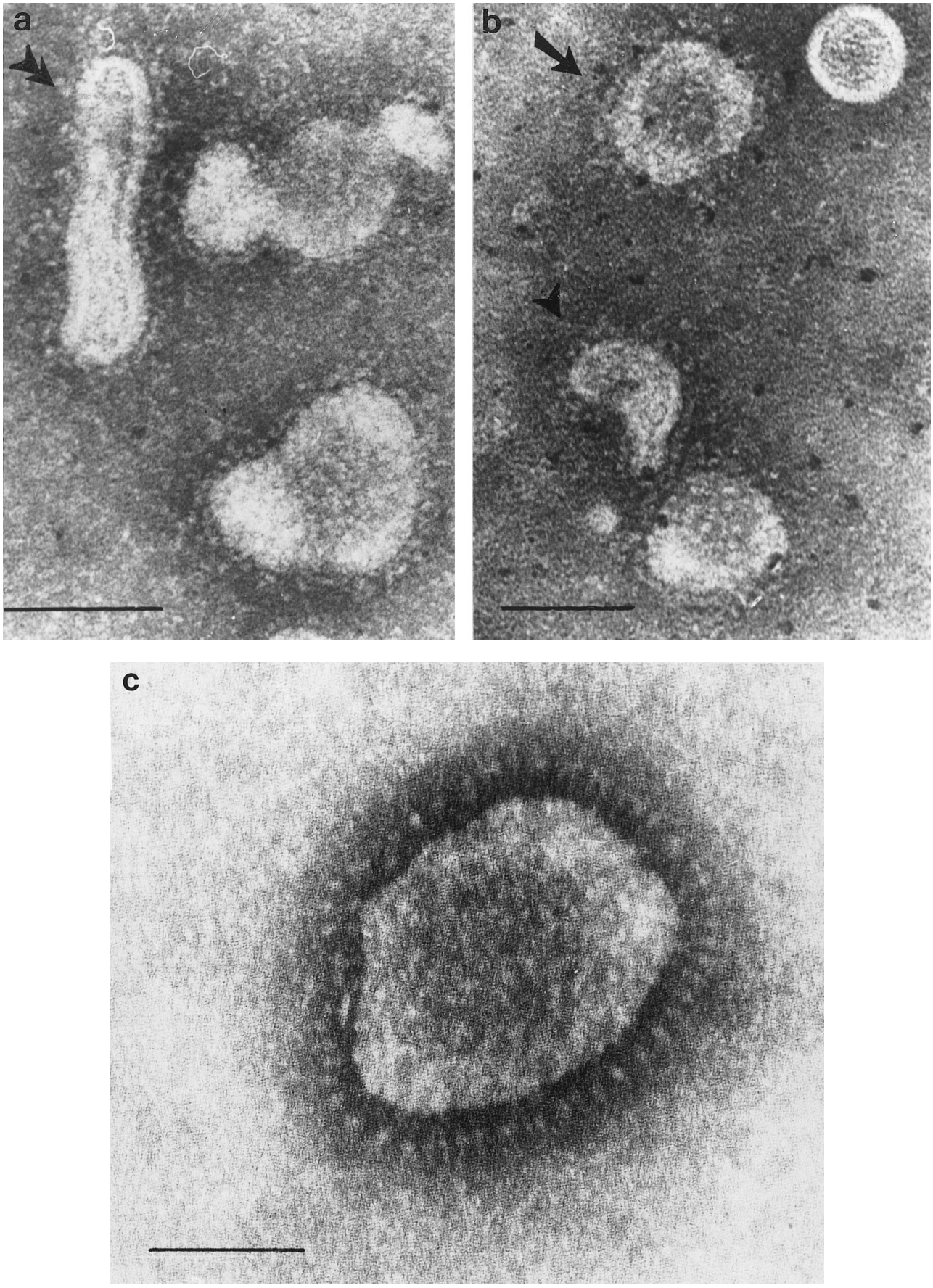

FIG. 1. (a and b) Electron micrographs of a purified fecal specimen showing torus (arrow)-, crescent (arrowhead)-, and rod (double arrowhead)-shaped human TVLPs. (c) TVLPs can be distinguished from enteric coronaviruses whose spikes are more prominent and readily discernable. Bars = 100 nm.

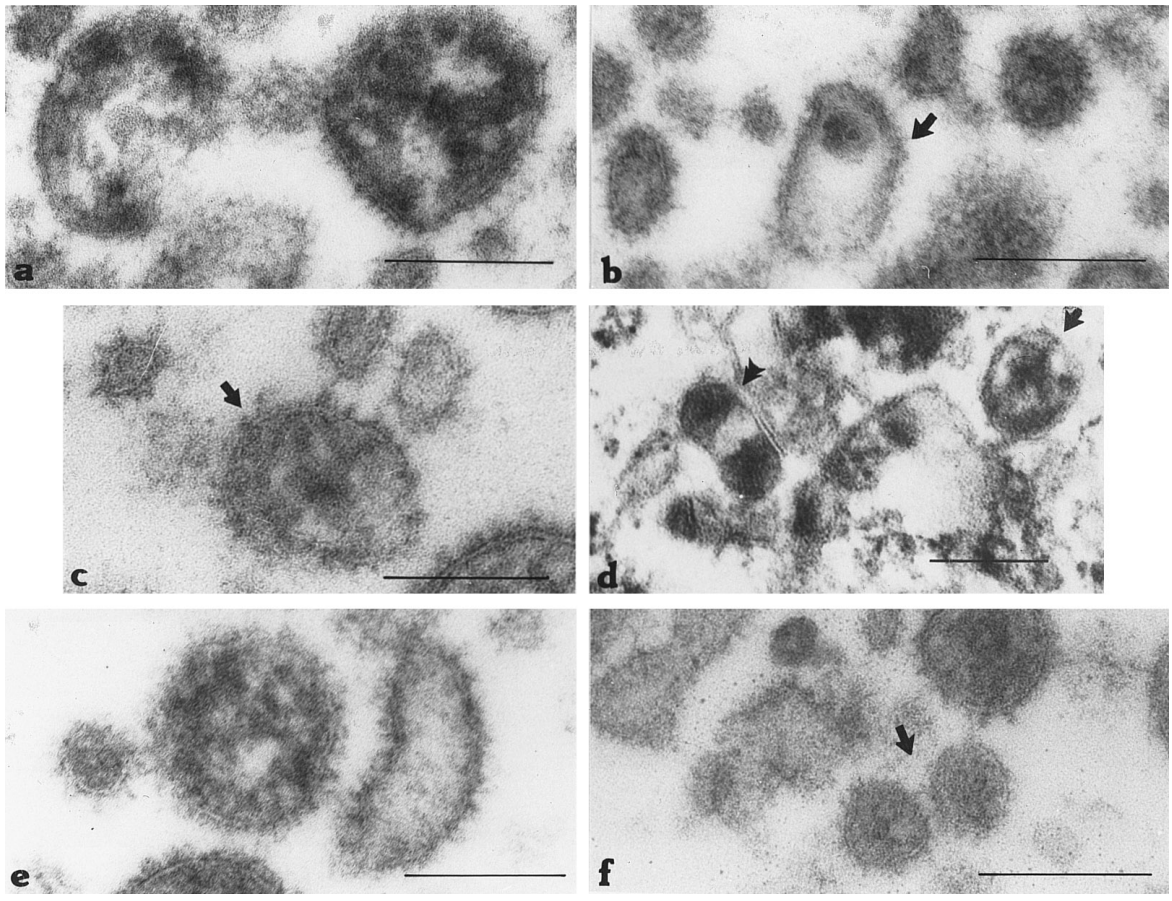

FIG. 2. Electron micrographs of human torovirus particles in ultrathin sections of a purified TVLP preparation. (a) Elliptical and crescent-shaped particles, (b) cross section of torus showing one electron dense area, (c) elliptical particle showing one electron dense area, (d) elliptical particle and cross section of torus showing two electron dense areas, (e) elliptical particle and rod-shaped particle, (f) cross section of crescent-shaped particle. Arrows indicate designated particles and structures. Bars = 100 nm.

hyperimmune (gpHI) sera, baBRV serum, and human a/c paired sera. Prior to use in the HI assay, the serum aliquots were absorbed with an equal volume of packed rabbit erythrocytes for 1 h at 4° to remove nonspecific hemagglutinating activity. Twofold serial dilutions of each serum were made in 25-μl volumes of VBS in V-bottomed microtiter plates. Four HA units of partially purified TVLP preparations in 25 μl of VBS was then added to each well and the plates were incubated for 1 h at room temperature. A 50-μl volume of a 0.5% suspension of rabbit erythrocytes in VBS was added to each well containing TVLP preparations. The plates were incubated for 2 h at room temperature and examined for hemagglutination (Woode *et al.,* 1985). Control hemagglutination inhibition testing was performed using four HA units of a purified rotavirus preparation. Serum aliquots were absorbed with human O erythrocytes. These HI assays were performed as above, except that a 0.5% suspension of human group O erythrocytes in VBS was added to all wells containing rotavirus preparations.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Purified TVLP preparations were subjected to SDS–PAGE on a 15% resolving gel and a 4% stacking gel. The gels were either stained with Coomassie brilliant blue R250 or used to perform an immunoblot procedure (Sambrook *et al.,* 1989). For the immunoblot, the proteins present in the SDS–PAGE gel were transferred electrophoretically to a polyvinylidene fluoride (PVDF) nylon membrane (Millipore, Bedford, MA) for 90 min at 100 V. The membranes were washed and blocked overnight in a solution of 5% skim milk and 5% goat serum in Tris-buffered saline containing 0.5% Tween-20 (TBST) (for blots with a/c paired sera) or 1% pig gelatin in TBST (for blots with baBRV serum). The membranes were then incubated for 4 h at room temperature in a 1:2000 dilution of either human a/c paired sera in 1% skim milk in TBST, or baBRV serum in 1% pig gelatin in TBST. The membranes were washed and incubated for 2 h at room temperature in a 1:1000 dilution of biotinylated murine anti-human IgG

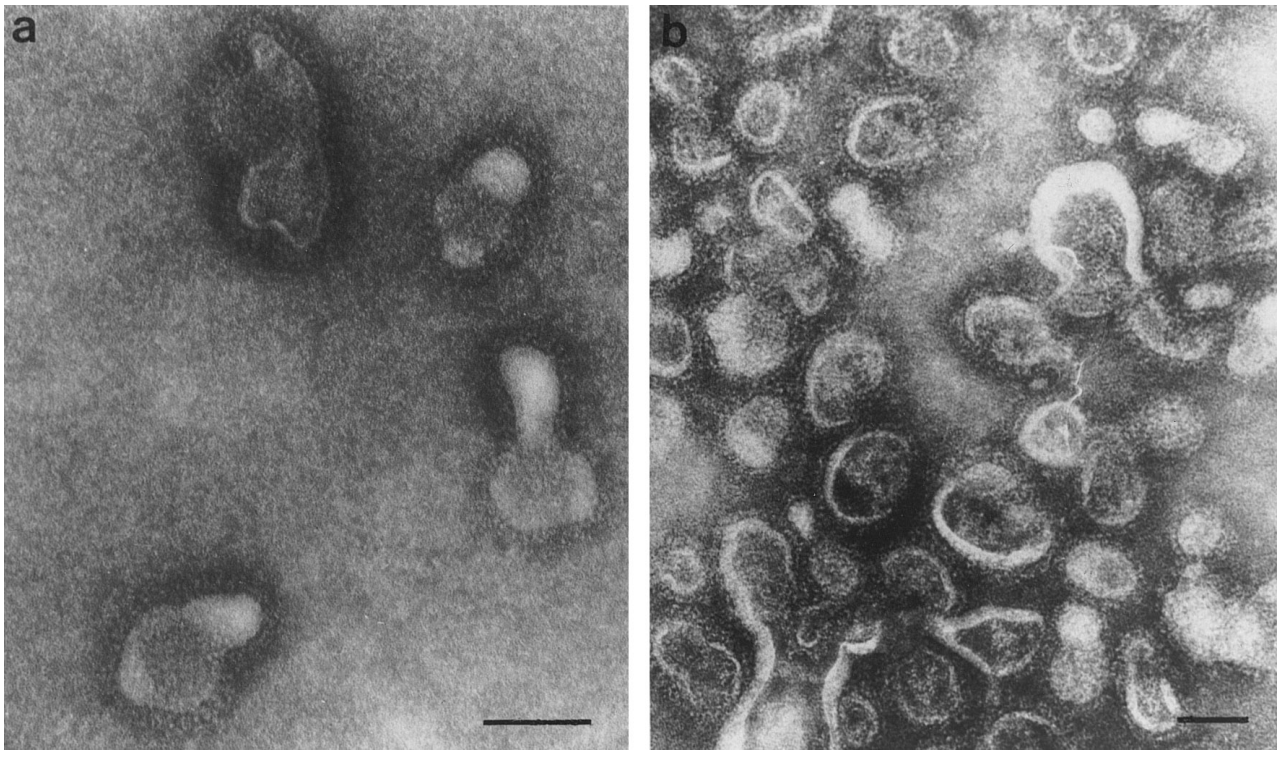

FIG. 3. Immunoelectron microscopy of a purified TVLP preparation with (a) human acute serum and (b) human convalescent serum. Bars = 100 nm.

(Sigma, Mississauga, Ontario, Canada) in 1% skim milk in TBST (for blots with a/c paired sera), or biotinylated goat anti-bovine IgG (Sigma, Mississauga, ON) in 1% pig gelatin in TBST (for blots with baBRV serum). Following further washing, the membranes were incubated for 45 min at room temperature in ImmunoPure ABC peroxidase staining reagents (Pierce, Rockford, IL), treated for 1 min with ECL horseradish peroxidase detection reagent (Amersham Life Science, Oakville, Ontario, Canada), and exposed to Kodak X-Omat AR imaging film for 15–30 s.

RNA extraction

Fecal specimens were diluted in an equal volume of phosphate-buffered saline (w/v) and clarified by centrifugation at 9000 *g* for 15 min at 4°. The supernatant was transferred to a new tube and centrifuged at 12,000 *g* for 15 min at 4°. In a separate room, using dedicated micropipetters and aerosol-resistant tips, viral RNA was extracted from the partially purified supernatant using TRIzol Reagent (Gibco BRL, Burlington, Ontario, Canada) according to the manufacturer's protocol. Each RNA pellet was resuspended in 10 μl of DNase free, RNase free double distilled water (5 prime 3 prime Inc., Boulder, CO) and stored at −80°.

RT-PCR

A total of 18 stool samples positive for TVLPs by EM were tested for the presence of torovirus RNA by RT-PCR. For each TVLP-positive sample assayed, we included a negative control (ddH_2O) and a second control consisting of RNA extracted from either a stool sample without any detectable gastroenteritis viruses by EM (8 assays) or RNA extracted from samples containing rotavirus (6 assays) or enteric coronavirus (4 assays). Oligonucleotide primers (General Synthesis and Diagnostics, Toronto, Ontario, Canada) were designed from the 3′ end of the BEV genome (DDBJ Accession No. D00563). The sense primer (5′TAATGGCACTGAAGACTC3′) and the antisense primer (5′ACATAACATCTTACATGG3′) bracketed a genome fragment of 219 nucleotides, which included the 3′ end of the N protein coding region and most of the 3′ noncoding region upstream of the poly(A) tail.

The RT and PCR reaction mixtures were set up in another isolated room, using dedicated micropipetters and aerosol-resistant tips. Reactions were then performed in a third room designated for PCR amplification. For the RT reaction, an RNA aliquot was thawed on ice, and incubated for 5 min at 65°. The RNA was added to 10 μl of the RT mixture containing 5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.25 mM (each) dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI), 2.6 μM random hexamer primers, 20 U RNase Guard (Gibco, BRL), and 50 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The reaction mixture was overlaid with sterile mineral oil and incubated at room

TABLE 1

Immunoelectron Microscopy (IEM) and Hemagglutination Inhibition (HI) Results for Two Human Fecal Specimens Found to be Positive for Torovirus-Like Particles by Electron Microscopy (TVLP 1 and 2)

	IEM				HI		
Sera	TVLP1	TVLP2	Rota	Neg	TVLP1	TVLP2	Rota
hu1 a	−	−	−	−	1:8	1:8	1:8
c	+++	++	−	−	1:128	1:64	1:8
hu2 a	−/+	−	−	−	1:16	1:8	1:8
c	+	+	−	−	1:32	1:32	1:8
hu3 a	−	−	−	−	1:8	1:8	1:4
c	+	+	−	−	1:32	1:32	1:8
hu4 a	−	−	−	−	1:8	1:4	1:8
c	+++	+++	−	−	1:64	1:64	1:16
hu5 a	−	−	−	−	1:16	1:8	1:8
c	++	+	−	−	1:128	1:64	1:8
hu6 a	−	−	−	−	1:8	1:8	1:4
c	++	++	−	−	1:64	1:64	1:4
hu7 a	−	−	−	−	1:8	1:16	1:8
c	+	++	−	−	1:32	1:64	1:8
hu8 a	−	−	−	−	1:8	1:8	1:4
c	++	++	−	−	1:64	1:64	1:8
hu9 a	−	−	−	−	1:16	1:8	1:8
c	++	+	−	−	1:64	1:32	1:8
hu10 a	−	−/+	−	−	1:8	1:4	1:8
a	+	+	−	−	1:32	1:8	1:4
hu11 a	−	−	−	−	1:16	1:8	1:4
c	+++	+++	−	−	1:128	1:128	1:8
hu12 a	−	−	−	−	1:8	1:8	1:8
c	+	++	−	−	1:32	1:64	1:8
baBRV	+++	++	−	−	1:32	1:16	1:8
gp1 PI					1:8	1:8	1:8
HI		ND			1:64	1:64	1:16
gp2 PI					1:8	1:8	1:8
HI		ND			1:64	1:32	1:16

Note. Controls included one rotavirus-positive specimen (Rota), and one patient specimen in which no virus was found by electron microscopy (Neg). Sera used in these assays included 12 human acute and convalescent paired sera (hu a or hu c), one bovine anti-Breda virus serum (baBRV), and two guinea pig preimmune (gpPI) and hyperimmune (gpHI) antisera to TVLPs. −, no aggregates per field; +, 2–3 clumped particles per field: ++, 2–3 aggregates per field: +++, many aggregates per field, ND: not determined. For HI, a four-fold or more increase in titre between the acute and convalescent sera was considered to be a positive HI result, representing sero conversion.

temperature for 10 min. The RT reaction was performed in a Perkin-Elmer (Mississauga, Ontario, Canada) thermal cycler at 42° for 30 min, 99° for 5 min, and then held at 5° for 5 min.

For the PCR reaction, 10 ml of the RT reaction was added to the PCR mixture containing 1 mM $MgCl_2$, 8 mM Tris–HCl (pH 8.3), 40 mM KCl, 2.5 U Amplitaq DNA polymerase (Perkin-Elmer Cetus and Applied Biosystems Inc., Mississauga, Ontario, Canada), and 50 pmol of each primer. The total volume of the PCR reaction was 50 μl. The reaction was overlaid with sterile mineral oil and amplified in a Perkin-Elmer thermal cycler. After denaturation at 94° for 2 min, the reaction was subjected to 35 cycles consisting of denaturation at 95° for 40 s, annealing at 50° for 1 min, and extension at 72° for 1 min 30 s. Reactions were then incubated at 72° for 10 min and held at 4°. Reactions were analyzed by electrophoresis in a 1.2% agarose gel, subsequently stained with ethidium bromide, and viewed under a UV transilluminator.

Cloning and DNA sequencing

PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Amplicons were then cloned into a pCR-Script Amp SK^+ cloning vector, and transformed into Epicurian coli XL1-blue MRF′ Kan supercompetent cells using the pCR-Script Amp SK^+ cloning kit (Stratagene, La Jolla, CA) as per the manufacturer's recommendations. Clones were screened by PCR using the same primers as above. Plasmids containing the PCR insert were purified from LB broth cultures using the Wizard Miniprep DNA purification system (Promega) and sequenced using the protocol for direct label incorporation in the *fmol* DNA sequencing system (Promega) according to the manufacturer's recommendations. Sequence data were analyzed using the computer program GCG version 8 (Genetics Computer Group Inc., Madison, WI). As an additional precaution against plasmid contamination of PCR reactions, all cloning and sequencing assays were undertaken only after the RT-PCR experiments on stool samples had been completed.

RESULTS

Electron microscopy and thin-section electron microscopy

Human TVLPs detected by negative contrast electron microscopy were morphologically similar to BEV and BRV in that they exhibited crescent-, torus-, and rod-shaped conformations (Figs. 1a and 1b). The particles measured between 100 and 120 nm at their largest diameter. The envelope and peplomers on these particles remained intact throughout the purification process, and these features were readily discernible. The peplomers measured approximately 10 nm in length. TVLPs can be readily distinguished from enteric coronaviruses which are somewhat larger in size and whose peplomer spikes are longer and more prominent (Fig. 1c).

When examined by EM, ultrathin sections of purified virus preparations demonstrated a number of elliptical particles enclosing hollow torus-shaped cores (Figs. 2a, 2d, and 2e), structures corresponding to cross sections of these particles showing either one or two electron dense areas bound by the envelope (Figs. 2b–2d, and 2f), as well as sections showing full rod or crescent shapes (Figs. 2a and 2e). The particles representing the

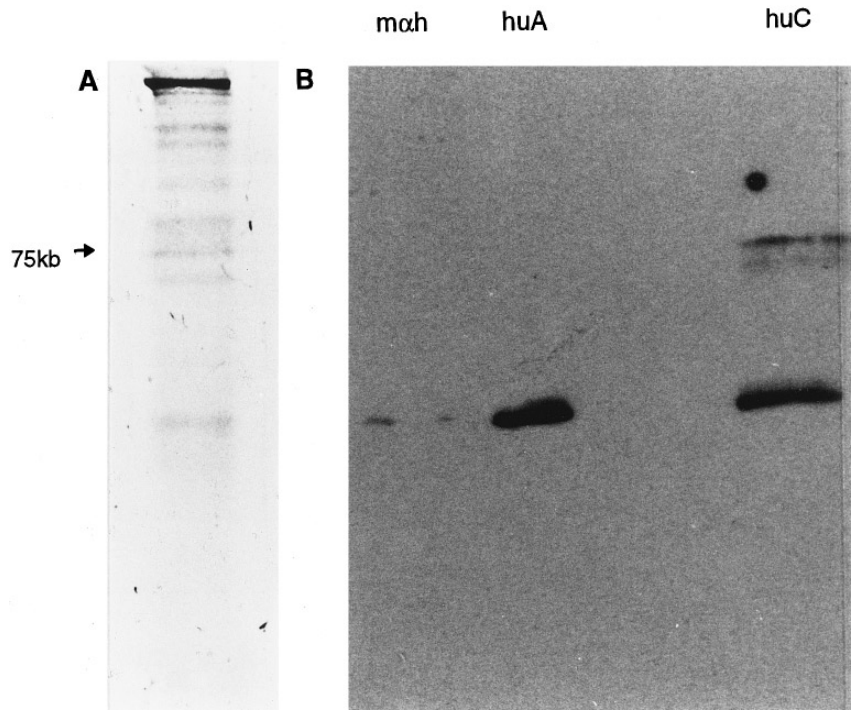

FIG. 4. (a) SDS–polyacrylamide gel electrophoresis and (b) corresponding immunoblot of a purified TVLP preparation using human acute (huA) and convalescent (huC) serum and murine anti-human antiserum (mαh).

whole virion measured 100 to 120 nm in diameter and the nucleocapsid cross sections measured approximately 25 nm in diameter.

Immunoelectron microscopy

A total of 12 human a/c paired sera and one baBRV convalescent serum were tested for their reactivity with two purified TVLP preparations, a preparation containing rotavirus, and a virus-negative preparation. Immunospecific reactivity was defined by the formation of viral aggregates by the convalescent but not the acute serum (Fig. 3). No acute serum was available for comparison with the baBRV serum. In all cases it was found that the convalescent sera were immunoreactive with the TVLPs while the acute sera showed no reactivity (Table 1). The baBRV serum formed numerous aggregates with both TVLP-positive specimens. In all cases, the human acute and convalescent sera were not reactive with the control specimens.

Hemagglutination and hemagglutination inhibition

When purified TVLP preparations were examined for their ability to hemagglutinate the erythrocytes of five different species, rabbit erythrocytes were agglutinated to the highest dilution (1:1280) of virus. Hemagglutination was also observed to a lesser extent with human O erythrocytes (1:40). Minimal activity was observed with the guinea pig, horse, and sheep erythrocytes. To determine the consistency of this reaction, 12 partially purified TVLP preparations from patients with gastroenteritis were tested for HA activity with rabbit erythrocytes. Nine of these had HA titers of 1:640 or greater, and the remainder had titers of 1:40, 1:160, and 1:320. The rotavirus-positive control specimen was found to agglutinate human group O erythrocytes (1:320) as previously demonstrated by Spence *et al.* (1978). Hemagglutination could not be demonstrated with the virus-negative control samples (data not shown).

A number of sera were tested for their ability to inhibit the hemagglutination of rabbit erythrocytes by purified TVLP preparations, or of human group O erythrocytes by a rotavirus preparation. Hemagglutination inhibition of rabbit erythrocytes by purified TVLPs was shown with both of the gpHI sera, and to a lesser extent with the baBRV serum. The gpPI serum showed negligible activity. The human convalescent sera manifested fourfold or greater antibody rises in all but two cases compared to the acute sera (Table 1). The rotavirus control antigen sustained minimal hemagglutination inhibition with all sera tested.

SDS–PAGE and immunoblotting

When the TVLP preparations were analyzed by SDS–PAGE, bands with molecular masses of 32, 75, 88, 100, 115, 134, and 141 kDa were detected as shown in Fig. 4. The bands between 75 and 100 kDa are of interest as

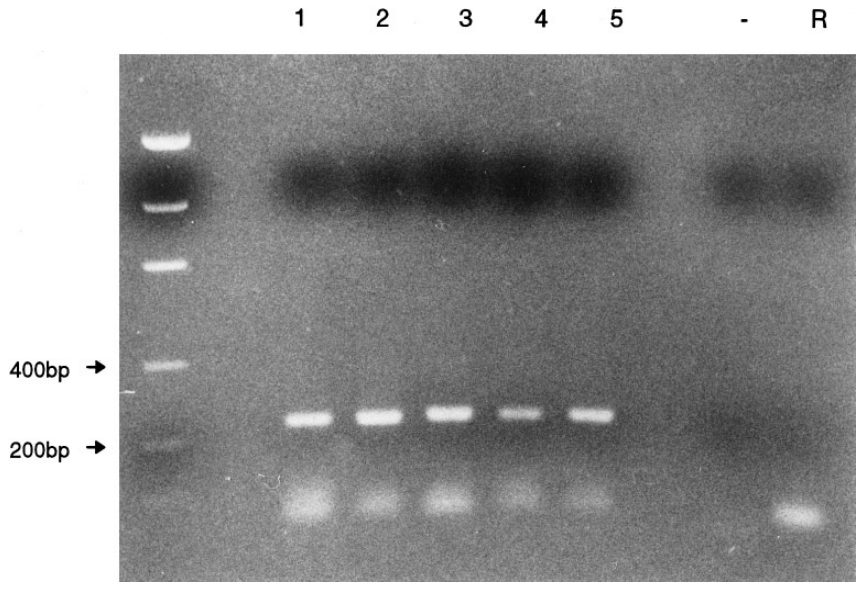

FIG. 5. Gel electrophoresis of RT-PCR products amplified using RNA extracted from five TVLP-positive fecal specimens and primers specific for the 3′ noncoding region of the BEV genome. The fecal specimens were collected over a 4-year period: lane 1, 1993; lane 2, 1994; lane 3, 1995; lanes 4 and 5, 1996. Lanes marked − and R represent a negative control (ddH_2O) and a rotavirus sample, respectively. DNA mass ladder was used as the molecular size marker.

they are expected to correspond to the S protein equivalents of BRV and BEV. To establish whether these bands are specific for toroviruses, the gels were further analyzed by immunoblot using one baBRV serum and two of the a/c paired patient sera whose titers to TVLPs were previously determined by HI and IEM. The human convalescent serum but not the acute serum reacted consistently with bands between 78 and 90 kDa. The 32-kDa band was reactive with both the acute and convalescent sera, as well as with the secondary antibody control, and was therefore considered to be nonspecific. This 32-kDa band was also present in immunoblots using the baBRV serum, and only limited reactivity of the 78-kDa band with this convalescent serum could be demonstrated (data not shown).

RT-PCR

Using primers designed from the genome sequence of BEV, an amplicon of 219 bases was detected in all of the 18 TVLP-positive samples tested. Of the 8 control specimens that were negative for any virus by EM, only 1 was positive by RT-PCR. Of the remaining 10 control specimens that contained either rotavirus or enteric coronavirus, one sample containing coronavirus particles gave a positive result by RT-PCR. Shown in Fig. 5 are representative amplification products after agarose gel electrophoresis.

Cloning and sequencing of torovirus-positive RT-PCR products

To assess the nature of the 219-base product obtained in TVLP-positive specimens by RT-PCR, amplicons obtained from five different samples (Fig. 5) were cloned and sequenced. The nucleotide sequences were compared to that of BEV. These five products were amplified from fecal specimens taken in 4 different years (1993–1996) in order to test for the possibility of genetic variation among the specimens. Clones were screened by PCR using the same primers, and 11 of them (at least 2 clones per sample) were found to contain the 219-base fragment. These 11 clones were sequenced, and a comparison was made with the 3′ region of the BEV genome, which includes the 3′ end of the N protein gene (Fig. 6). The nucleotide sequences of each of the five TVLP isolates were found to be 99% identical to BEV in this area. For each isolate, there were no discrepancies between the sequences of its clones. However, each isolate had at least one, but no more than two nucleotide substitutions compared to BEV. All of these substitutions were situated in the 3′ end of the N gene between nucleotides 49 and 61, and none of these mutations caused changes in the predicted amino acid sequence of the N protein.

DISCUSSION

Berne virus propagated in cell cultures and Breda virus purified from stool specimens of infected calves have been well characterized and are established pathogens of horses and cattle, respectively (Weiss and Horzinek, 1987). Particles with morphological features similar to those of BEV and BRV have been reported in the fecal specimens of children with diarrhea (Beards *et al.,* 1984). However, the status of these torovirus-like particles as human pathogens remained un-

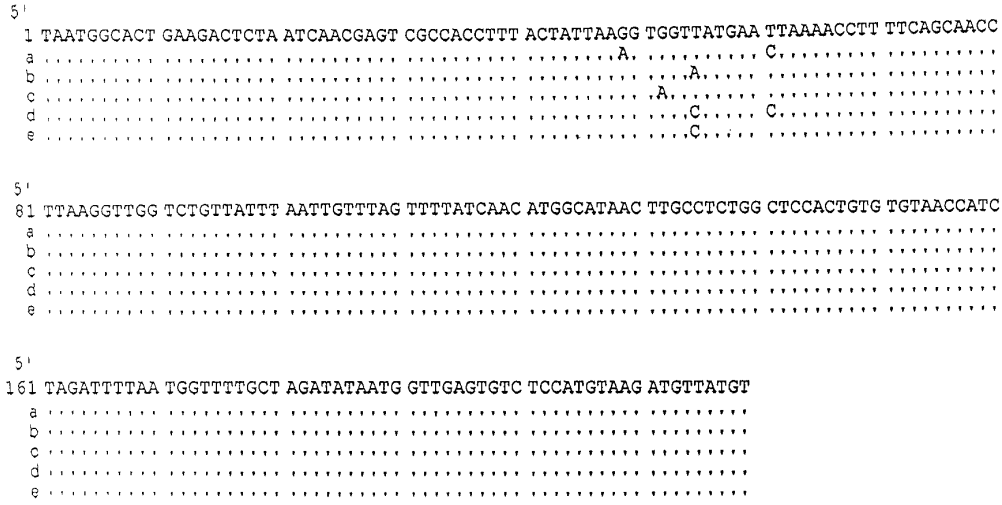

FIG. 6. Alignment of the nucleotide sequence from the 3′ end of the Berne virus genome (consensus) with that of five human TVLP-positive samples collected over a 4-year period (a, 1993; b, 1994; c, 1995; d and e, 1996). Nucleotides identical to the consensus sequence are shown as dots.

clear because they lacked the definitive morphology of other iscosahedral gastroenteritis viruses, they could not be propagated in conventional cell culture, and they could not be identified by readily available immunospecific reagents. Nevertheless, the observation that the TVLPs detected by EM in human stool specimens were reactive in an ELISA based on BRV antiserum provided immunospecific evidence that these agents were toroviruses (Koopmans *et al.,* 1993).

The investigation of TVLPs from patient specimens was initially compromised by difficulties in obtaining adequate preparations of intact virions. While ultracentrifugation through sucrose gradients allowed for the purification of immunoreactive particles (Koopmans *et al.,* 1993), such preparations could not be consistently obtained. This procedure also appeared to alter the morphology of some TVLPs and disrupt the peplomer fringes, thereby making any biochemical analyses of these particles difficult to interpret. Thus, an alternative method was designed to purify TVLPs by sedimentation through a cushion of Ficoll in 1% ammonium acetate. This procedure resulted in highly enriched preparations of intact TVLPs whose peplomer fringes were well preserved. Although these TVLPs should be deemed to be only partially purified, their preserved morphology suggests that they are likely to contain immunoreactive structural proteins.

The morphology of the TVLPs purified from human fecal specimens provides evidence that these agents are toroviruses. Human TVLPs closely resembled BEV and BRV when examined by negative contrast electron microscopy. These purified particles have the same pleomorphic nature as those detected directly in stool specimens. Whether these forms are part of the virus population or are simply due to drying and staining during EM examination is as of yet unresolved. Furthermore, the ultrastructure of the virus, as visualized by thin section EM, was consistent with that found in BEV infected cells and BRV infected gut epithelium (Weiss *et al.,* 1983; Weiss and Horzinek, 1987). These structures included particles with enveloped, crescent-shaped cores, as well as cross-sections through these cores showing the tubular nucleocapsid surrounded by the envelope.

Human TVLPs are morphologically pleomorphic and could not be propagated in cell culture. Therefore, several independent immunospecific approaches, such as IEM, HI, and immunoblotting, were used to characterize these particles. Beards *et al.* (1986) observed a serological relationship between BEV, BRV, and TVLPs on the basis of IEM studies. Using this method, we demonstrated instances of seroconversions to human TVLPs following infection. Our finding that purified TVLP preparations manifested strong hemagglutination activity with rabbit erythrocytes led to the design of a hemagglutination inhibition assay. Seroconversions following torovirus infection were readily documented using acute/convalescent paired patient sera. HI titers were found to be reproducible using TVLP preparations that were either homologous or heterologous to the patient sera. These results were consistent with those found by IEM for the respective sera.

The immunoblot assays showed that the human convalescent sera, but not the acute sera were reactive to a 78- to 90-kDa protein that is present in partially purified virus preparations. Based on its molecular weight, this protein most likely represents the viral glycoprotein peplomer, S, which in the case of BEV and BRV migrates as a band between 75 and 100 kDa due to variable degrees of glycosylation (Snijder *et al.,* 1990b). The dem-

onstration of immunoreactivity to only the S protein in the immunoblot is consistent with reports on coronavirus infections in which the spike protein elicits the major immune response. (Battaglia *et al.,* 1987; MacNaughton *et al.,* 1981; Schmidt and Kenny, 1981). Specific immunoreactivity with the S protein is also in concordance with the detection of immune responses by IEM and HI since both of these assays are likely to involve the peplomer protein. The strong immunoreactivity of the 32-kDa protein cannot be explained at present, but it appears to be relatively nonspecific as it is present with the human acute serum and even to some extent with the secondary antibody control.

It has previously been shown that the 3′ region of the BEV genome, spanning the C terminus of the N gene and the 3′ noncoding region, is 93% identical to the corresponding region of BRV (Koopmans *et al.,* 1991). We designed an RT-PCR assay targeting this region and found that all the samples that were TVLP-positive by EM, were also positive by RT-PCR, whereas other enteric viruses were not detected by this assay. We also obtained a positive PCR result with two samples in which we did not detect TVLPs by EM. Such a result is not unexpected given the presumed higher sensitivity of our RT-PCR assay compared to that of EM.

Sequence analysis of a subset of these amplicons confirmed that TVLPs are related to the torovirus prototype, BEV, since their sequences demonstrated 99% sequence identity in the 219-base region amplified. As the possibility of contamination of PCR products was of great concern to us, exhaustive precautions were taken throughout the procedures. We are confident that these amplicons are not the result of PCR contamination because none of our negative controls ever demonstrated contamination, we have never had the Berne virus in our laboratory, and small but real sequence differences were noted among the five TVLP isolates and with BEV.

In summary, we achieved a purification method for TVLPs that preserved the morphology of these agents. Their morphological features and ultrastructure are similar to previously recognized toroviruses. These particles hemagglutinate rabbit erythrocytes, elicit an immune response, and show immune cross-reactivity with BRV. Finally, sequence analysis of amplicons obtained with an RT-PCR assay targeted at the 3′ end of the torovirus genome demonstrated that TVLPs are related to BEV and BRV.

Thus, the findings reported above have provided several lines of evidence that the torovirus-like particles detected in human fecal specimens are indeed toroviruses. Furthermore, these analyses have identified the peplomer protein as the primary immunoreactive component of human toroviruses and have established immunospecific and molecular approaches for the investigation of torovirus infections in the human population.

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