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The novel hemagglutinin-esterase genes of human torovirus and Breda virus

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Abstract

Human torovirus (HTV) and Breda virus (BRV), members of the genus torovirus in the family Coronaviridae, are established infectious agents of humans and cattle, respectively. The hemagglutinin-esterase (HE) gene of Breda virus serotype 2 (BRV-2) has been identified and the nucleotide sequence for BRV serotype 1 (BRV-1) genome which contains the open reading frames for the viral structural proteins has been reported revealing the presence of a 1.25 kb gene whose nucleotide sequence is identical to that of the BRV-2 HE gene. In this study, we amplified the 1.2kb HE gene from the HTV genome using long RT-PCR and sequenced the amplicon directly. At the nucleotide level, the HTV HE gene manifests 85% sequence identity to the HE genes of BRV-1 and BRV-2 and 89% identity with the X pseudogene sequence of BEV. The 1.25 kb ampiclons which contained the HE genes of BRV-1 and HTV were cloned and expressed in a baculovirus system and the proteins purified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Hyperimmune sera prepared in guinea pigs against these proteins were reactive with both bovine torovirus (BTV) and human torovirus (HTV) antigens. By immunoblot, they reacted specifically with a 65 kDa protein corresponding in size to the torovirus HE protein. Furthermore, the hyperimmune sera but not the preimmune sera reacted with a series of BTV-positive and HTV-positive fecal specimens by immunoblot and dot blot analysis. By immunoelectron microscopy (IEM) torovirus particles from BTV-positive specimens from calves with diarrhea and HTV-positive specimens from patients were aggregated by the hyperimmune sera. Human convalescent sera and gnotobiotic calf post-infection sera reacted by immunoblot with the expressed 65 kDa protein. The expressed HE protein of HTV has important diagnostic potential. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Novel hemagglutinin-esterase genes; Human torovirus; Breda virus

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1. Introduction

Breda virus (BRV), the bovine torovirus prototype and human toroviruses (HTV) are members of the genus torovirus within the family Coronaviridae, which together with the Arteriviridae forms the order Nidovirales (Cavanagh, 1997). They are related to the torovirus prototype Berne virus (BEV) and the newly described porcine torovirus (Kroneman et al., 1998). BRV was first isolated in 1982 from the stools of neonatal calves with diarrhea in Breda, Iowa, and is now an established infectious agent of cattle (Woode et al., 1982). Two serotypes of BRV have been recognized by hemagglutination inhibition (HI) tests, enzyme-linked immunosorbant assays (ELISA), and immunoelectron microscopy (IEM). BRV-1 represents the original isolate from Iowa, and BRV-2 includes isolates from Ohio and Iowa (Woode et al., 1985). Toroviruses were first reported as potential human pathogens with the observation of torovirus-like particles in the fecal specimens of children with diarrhea by electron microscopy EM (Beards et al., 1984). Recently, the morphological, serological, and molecular properties of these particles were elucidated, establishing them as human toroviruses (Duckmanton et al., 1997). A study on the prevalence of torovirus in a pediatric population showed that this agent is definitively associated with gastroenteritis in children (Jamieson et al., 1998).

BRV and HTV are relatively pleomorphic particles that are difficult to recognize by EM, and cannot as yet be grown in cell culture. Aside from EM, diagnostic testing for these agents is limited to serological and molecular methods (Brown et al., 1987; Koopmans et al., 1989, 1993, 1997; Duckmanton et al., 1997, 1998a).

The genes for the structural proteins of BRV-1 were recently characterized using long RT-PCR and sequencing (Duckmanton et al., 1998b). BRV-1 was found to have an open reading frame (ORF) for a 1.25 kb hemagglutinin-esterase gene with an identical nucleotide sequence to the HE gene of BRV-2 with the 3’ end of this gene being 77% identical to the X pseudogene of BEV (Cornelissen et al., 1997; Snijder et al., 1991). However, very little is known about the genome of HTV. To date, only the 3’ non-coding region and the 3’ terminus of the nucleocapsid gene of the HTV genome have been sequenced (Duckmanton et al., 1997). The aims of this study were to determine whether the HE gene is present in the HTV genome, and if it is present, to express the BRV-1 and HTV hemagglutinin-esterase proteins, and investigate them for their immunospecific properties.

2. Materials and methods

2.1. Specimens and sera

Stool specimens, demonstrated by EM to contain human torovirus or human rotavirus or to be free of detectable virus particles and acute/convalescent paired sera from patients diagnosed positive for human torovirus were obtained from the Virology Laboratory at the Hospital for Sick Children, Toronto, Ont. Bovine torovirus-positive fecal specimens from diarrheic calves, control specimens from asymptomatic calves, and rotavirus-positive specimens from diarrheic calves were obtained from the Animal Health Laboratory in Guelph, Ont. The above human and bovine specimens have previously been studied for the presence of viruses (Duckmanton et al., 1997, 1998a). The stool specimen from a gnotobiotic calf infected with a purified preparation of the Breda virus-1 (code GC-32), and antisera to BRV-1 (bzBRV-1) from experimentally infected calves were obtained from Dr G. Woode, Texas A&M and Dr M. Hardy, Montana State University.

Antibody prepared in guinea pigs to the recombinant N protein of BRV (gpHIzN) has been previously described (Duckmanton et al., 1998b).

2.2. RNA extraction

As described previously (Duckmanton et al., 1998a), all fecal specimens were diluted in an equal volume of phosphate-buffered saline and clarified by differential centrifugation. Viral RNA was extracted from the partially purified superna-
tants of BRV-1-positive samples and HTV-positive samples using TRIzol reagent (Gibco BRL, Gaithersburg, MD) with all mixing steps performed by repeated inversions to prevent RNA shearing.

2.3. Primers

Oligonucleotide primers (ACGT Corp., Toronto, Canada) used to amplify the HTV HE gene were deduced from the sequence upstream and downstream of the BRV-1 HE gene whose sequence was reported previously under accession number AF076621 (Duckmanton et al., 1998b). The sense primer (5’ TCTAGTGTTA AGTTTGAGTA GCACATTAC TC 3’) and the antisense primer (5’ GACATGGCAC AGCATTTGGA TTAAGCATA G 3’) bracketed a genome fragment of approximately 1.4 kb.

Oligonucleotide primers used to amplify the 1.25 kb fragment containing the ORF of the HTV HE gene were designed based on the sequence of the HTV HE gene obtained in this study. The sense primer (5’ GGGCTGCTAG C 6 ATGCTGAGT TTAATACTTT TTTTTCCATC TTTTGCCTTT GCAGT 3’), designed in the 5’ end of the HTV HE gene ORF, contained a NheI restriction site upstream of the start codon for cloning purposes. The antisense primer (5’ GATCCGCTAG C 6 ACAAAAAAA ACTTATAATT ACAAATATTA AAATAACAC CACCACC 3’), designed in the 3’ end of the HE gene ORF, did not contain a stop codon and had a NheI restriction site in its place. Primers used to amplify the 1.25 kb fragment containing the ORF of the BRV-1 HE gene for cloning were identical to those used for the HTV HE gene ORF except that the sense primer had GGC in place of AGT at its 3’ end.

2.4. Long RT-PCR

The RT and PCR reactions were performed using the approach of Tellier et al. (1996a,b) as described previously (Duckmanton et al., 1998b). The following cycling parameters were used to initially amplify the 1.4 kb region containing the HTV HE gene, as well as to amplify the ORFs of the HTV and BRV-1 HE genes for cloning. Denaturation at 99°C for 35 s, annealing at 67°C for 30 s, and elongation at 68°C for 5 min for 35 cycles. Reactions were analyzed by electrophoresis on a 1% agarose gel, subsequently stained with ethidium bromide.

2.5. DNA sequencing

PCR products were excised from agarose gel, and purified using the Jetsorb system (Genomed, Frederick, MD) according to the manufacturer’s recommendations. Purified amplicons were then sequenced directly as described previously (Duckmanton et al., 1998b) Sequence data were analyzed using the computer programs GCG version 8 (Genetics Computer Group Inc., Madison, WI), and Gene Runner version 3.04 (Hastings on the Hudson, NY).

2.6. Cloning and expression

PCR products containing the HTV and BRV-1 HE genes were purified using the Jetsorb system (Genomed). Amplicons were ligated to dephosphorylated pETL-EK (His) 6 baculovirus transfer vectors, and transformed into Epicurian coli XL1-blue MRF supercompetent cells (pCR-Script Amp SK+ cloning kit; Stratagene) as per the manufacturer’s recommendations. Plasmids containing the PCR inserts were purified from LB broth cultures using the Wizard Miniprep DNA Purification System (Promega), followed by a standard phenol:chloroform extraction (Sambrook et al., 1989). Positive clones were sequenced as described above and those with inserts intact and in frame were transfected into Spodoptera frugiperda (Sf9) insect cells using the BaculoGold transfection system (PharMingen Canada, Mississauga, Ont.) as per the manufacturer’s recommendations. Supernatants from the transfected cells that contained the recombinant baculovirus were serially diluted, added to Sf9 cells, overlaid with 1% SeaPlaque (FMC BioProducts, Rockland, ME) in serum-free Grace’s medium containing 250 mg/ml Bluo-Gal (Gibco BRL), and incubated
at 27°C for 1 week. Viral DNA was extracted from the supernatants of plugs of agarose containing single blue plaques using the DNAzol system (Gibco BRL) according to the manufacturer’s recommendations, and tested for the presence of the HE gene by PCR as described above. The viral supernatants from clones that contained the HE gene were then amplified in Sf9 cells to obtain high titre stocks. To screen for expression of the HTV and BRV-1 HE proteins the cell pellets were resuspended in 6× SDS sample buffer and subjected to SDS-PAGE on a 12% resolving and 4% stacking gel and transferred electrophoretically to a polyvinylidene fluoride (PVDF) nylon membrane (Millipore, Bedford, MA) for 90 min at 100 V for immunoblotting. The membranes were blocked overnight in 5% skim milk in Tris-buffered saline containing 0.5% Tween-20 (TBST). The membranes were then incubated for 1 h at room temperature in a 1:1000 dilution of mouse-His*Tag Antibody (Babco, Richmond, CA) in TBST, washed and incubated for 2 h at room temperature in a 1:2000 dilution of horseradish peroxidase conjugated-goat anti-mouse IgG in TBST. After washing the membranes were developed in a 50 mM TBS solution containing 10% 4-chloro-1-naphthol in methanol and 0.025% hydrogen peroxide. Color development at room temperature was complete within 5–10 min.

2.7. Preparation of antisera to the HE proteins of BRV-1 and HTV in guinea pigs

The resuspended cell slurries containing the expressed HE proteins of BRV-1 or HTV were subjected to preparative SDS-PAGE. Bands appearing after Coomassie brilliant blue R250 staining which corresponded to the 65 kDa HE proteins of each virus were excised and soaked in distilled water for 2 h and used to immunize adult male guinea pigs as described previously (Duckmanton et al., 1998b). Pre- and hyper-immunization sera to the BRV-1 and HTV HE proteins were respectively designated gpPIzBRV-HE, gpHIzBRV-HE, and gpPIzHTV-HE, gpHIzHTV-HE. The sera were heat inactivated at 56°C for 30 min, aliquoted and stored at −20°C.

2.8. SDS-PAGE and immunoblotting

Bovine and human stool specimens partially purified by differential centrifugation and positive control Sf9 cells containing either BRV-1 or HTV HE proteins were subjected to immunoblot analysis using the above guinea pig sera. Following SDS-PAGE the proteins were transferred to PVDF membranes as described above. Membranes were incubated for 2 h at room temperature in 1:2000 dilutions of either gpPIzBRV-HE, gpHIzBRV-HE, gpPIzHTV-HE, or gpHIzHTV-HE sera in 1% skim milk in TBST. The membranes were washed and incubated for 1 h at room temperature in a 1:3000 dilution of alkaline phosphatase conjugated-rabbit anti-guinea pig IgG (RzGP; Sigma Chemicals, St. Louis, MO) in 1% skim milk in TBST. Sf9 cells containing the HE proteins were also tested for reactivity with 9 human acute/convalescent paired sera from patients whose stools were diagnosed positive for HTV by EM as described previously (Duckmanton et al., 1997), and bzBRV-1 pre- and post-immune sera from a gnotobiotic calf infected with purified BRV-1. Primary sera were used at dilutions of 1:2000 followed by 1:3000 dilutions of either alkaline phosphatase conjugated-murine anti-human IgG, or alkaline phosphatase conjugated-goat anti-bovine IgG, respectively (Sigma Chemicals). Following further washing, the membranes were developed using a 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP-NBT; SigmaFAST, Sigma Chemicals) dissolved in 10 ml of water.

2.9. Hemagglutination inhibition

A panel of sera were tested by HI against a partially purified TVLP preparation as described previously (Duckmanton et al., 1997). The panel included guinea pig anti-BRV-1 N protein preimmune (gpPIzN) and hyperimmune (gpHIzN) sera, human acute (huA) and convalescent (huC) paired sera, gpPIzBRV-HE and gpHIzBRV-HE sera, and gpPIzHTV-HE and gpHIzHTV-HE sera.
2.10. Immunoelectron microscopy

Immunoelectron microscopy was performed as described previously (Duckmanton et al., 1997, 1998a) using either the gpPlzBRV-HE and gpHIzBRV-HE antisera, or the gpPlzHTV-HE and gpHIzHTV-HE antisera. Control specimens included a purified human rotavirus-positive sample, a purified bovine rotavirus-positive sample, a human virus-negative sample, and a purified sample from a calf with diarrhea in which no viruses could be detected by EM.

2.11. Dot immunoblot

Human and calf stool specimens positive by EM for HTV, BTV, and rotavirus, as well as negative control stools were examined for immunoreactivity with the gpPlzBRV-HE, gpHIzBRV-HE, gpPlzHTV-HE, and gpHIzHTV-HE sera by dot immunoblot analysis as described previously (Duckmanton et al., 1998b).

3. Results

3.1. Long RT-PCR and sequencing of the HTV HE gene

Using primers designed from the genome sequence bracketing the BRV-1 HE gene, an amplicon of 1371 bases was amplified from HTV RNA. The amplicon was excised, purified, and used directly for sequencing. Sequence analysis revealed that the HE gene of HTV contains an ORF of 1251 nts in length, whose nucleotide sequence is 85% identical to that of the BRV-1 HE gene as shown in Fig. 1, and 89% identical to the corresponding 457 base sequence of the X pseudogene of BEV. This ORF, like its homologue of BRV-1, codes for a polypeptide of 416 amino acids which contains domains typical of type I membrane glycoproteins: a 14 residue N-terminal signal sequence, a 24 residue C-terminal transmembrane anchor, and eight potential N-glycosylation sites. Also present in the HTV HE gene is the F-G-D-S-motif which is the putative catalytic site of ICV and coronavirus acetyl esterases. The sequence of the cDNA of the HTV HE gene has been submitted to GenBank under accession number AF 159585.

3.2. Expression of the BRV-1 and HTV HE genes

Sf9 cells infected with the recombinant baculovirus containing the HE genes of BRV-1 and HTV as confirmed by PCR were subjected to immunoblotting with pre- and post-immune sera from calves infected with BRV-1. The expressed HE proteins (M, 65 kDa) were cell associated and were not secreted into the medium. The HE proteins from infected cells were purified by SDS-PAGE and used to immunize the guinea pigs.

3.3. SDS-PAGE and immunoblotting

As previously described (Duckmanton et al., 1998b), SDS-PAGE analysis of partially purified BTV and HTV preparations revealed a number of bands including bands with an M of 65 kDa for BTV (Fig. 2A) and HTV (Fig. 2C). This 65 kDa band corresponded in size to the HE protein equivalent of BRV-2 that was shown to have an approximate M of 65 kDa in its glycosylated form (Cornelissen et al., 1997).

The guinea pig pre- and hyperimmune sera were tested for their reactivity by immunoblot assay with virus proteins from three BTV-positive stool samples and one BTV-negative control, three HTV-positive stool specimens and one HTV-negative control. The gpHIzBRV-HE and gpHIzHTV-HE sera reacted with the HE proteins in all of the BTV-positive (Fig. 2A) and HTV-positive fecal specimens (Fig. 2C) but not with the virus-negative controls. No reactivity was observed with either of the guinea pig pre-immune sera (Fig. 2B and D).

The gpHIzBRV and gpHIzHTV sera, as well as the bzBRV-1 post-immune serum, and all nine human convalescent sera from patients diagnosed positive for HTV were tested for reactivity with the expressed 65 kDa HE proteins of BRV and HTV. As shown in Fig. 3, the hyperimmune guinea pig sera to BRV and HRV were reactive with both the expressed HE proteins of BRV and HTV. The calf hyperimmune serum and the
Human convalescent sera of which only one of the nine is shown, were also reactive with both proteins. However, the convalescent sera exhibited stronger bands with the homologous proteins. No reactivity was documented with the calf pre-immune sera or the human acute sera.

**Fig. 1.** Alignment of the nucleotide sequence of the cDNA of the HE gene of HTV with that of BRV (brv). Identical nucleotides are shown as dots. The predicted amino acid sequence of the HTV-1 HE protein is also shown.
3.4. Hemagglutination inhibition

To investigate if the HE protein possesses HA activity, the guinea pig antisera to the HE proteins of BRV-1 and HTV were tested for their ability to inhibit hemagglutination using an HI assay as described previously (Duckmanton et al., 1997). Hemagglutination inhibition to a titre of 1:16 was demonstrated with the gpHIzBRV-1-HE and gpHIzHTV-HE sera, and to a titre of 1:32 with the human convalescent serum. In contrast the human acute serum exhibited a titre of 1:2 and the gpHIzN serum and all of the guinea pig preimmune sera showed no hemagglutination inhibition activity.

3.5. Immunoelectron microscopy

The guinea pig antisera to the BRV-1 and HTV HE proteins were tested by IEM for reactivity with three BTV-positive preparations, and three HTV-positive samples. Viral aggregates were observed in all preparations containing the gpHIzBRV-HE serum mixed with the BTV-positive and the HTV-positive specimens (Fig. 4). No aggregates were demonstrated with the gPIzBRV-HE serum. Likewise, viral aggregates were observed in preparations of gpHIzHTV-HE serum mixed with the BTV-positive and HTV-positive specimens, but aggregates were smaller and less frequent than with the gpHIzBRV serum. No

Fig. 2. Immunoblots of fecal specimens of which a set of three are positive for bovine torovirus (BTV +) and human torovirus (HTV +), and single specimens negative for bovine torovirus (BTV –), and human torovirus (HTV –) with (A) guinea pig anti-BRV-1 HE protein hyperimmune serum, (B) guinea pig anti-BRV-1 HE protein preimmune serum, (C) guinea pig anti-HTV HE protein hyperimmune serum, and (D) guinea pig anti-HTV HE protein pre-immune serum. Shown in the left lanes of panels (a) and (c) are Coomassie blue stained SDS-polyacrylamide gels of a BTV + fecal specimen and a HTV + fecal specimen, respectively.
aggregates were observed when the gpPIzHTV-HE serum was mixed with BTV- or HTV-positive specimens. No aggregates of viruses were seen in control specimens reacted with any of the guinea pig sera.

3.6. Dot immunoblot

The above pre- and post-immune sera to the HE proteins of BRV-1 and HTV were examined for their ability to detect torovirus in stool specimens by a dot immunoblot system. The fecal specimens had been previously characterized as torovirus-positive or negative by EM and RT-PCR (Duckmanton et al., 1997, 1998a), and consisted of ten HTV-positive, ten BTV-positive, five human rotavirus-positive, five bovine rotavirus-positive, five human virus-negative, and five bovine virus-negative fecal specimens. All of the ten specimens that were previously shown to be positive for BTV were specifically reactive with the gpHIzBRV-HE and not the gpPIzBRV-HE sera, and eight of these were reactive with the gpHIzHTV-HE and not the gpPIzHTV-HE sera. All of the ten HTV-positive samples were reactive with the gpHIzBRV-HE serum and nine reacted with the gpHIzHTV-HE serum. The HTV-positive specimens did not react with either of the guinea pig preimmune sera. In addition, none of the virus-negative specimens or rotavirus-positive specimens were reactive with any of the guinea pig sera. Fig. 5 illustrates representative dot blots of

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Fig. 3. Representative SDS-PAGE gel of a Sf9 cells expressing BRV-1 HE protein and HTV HE protein and corresponding immunoblots of gpPIzBRV-HE and gpHIzBRV-HE, gpPIzHTV-HE and gpHIzHTV-HE, human acute (huA) and convalescent (huC) paired sera, and bovine anti-BRV-1 pre-immune (bPI) and post-immune (bHI) sera. Shown in the left lanes of panels (A) and (B) are Coomassie blue stained SDS-polyacrylamide gels of Sf9 cells expressing BRV-1 HE protein and HTV HE protein, respectively.
five BTV-positive, five HTV-positive, five bovine rotavirus-positive, five human rotavirus-positive, five virus-negative bovine and five virus-negative human specimens using the guinea pig preimmune and hyperimmune antisera to either the BRV-1 or HTV HE proteins.

4. Discussion

The existence of recombination in RNA virus evolution is exemplified by the presence of hemagglutinin-esterase genes in some coronavirus and torovirus species. The coronavirus and BRV HE proteins are 65 kDa class I membrane proteins that share 30–35% amino acid identity with the HE-1 subunit of the HE fusion protein (HEF) of influenza C virus (ICV). However, the fact that the HE genes of coronaviruses and toroviruses are located at different positions in their genomes suggests that these viruses acquired their HE genes through separate heterologous RNA recombination events (Snijder et al., 1991). Although the origin of the torovirus HE gene is unknown, it has been speculated that coronaviruses captured their HE module from ICV or a related virus during a mixed infection (Luytjes et al., 1988).

In ICV and coronaviruses, the HE protein displays acetylesterase activity specific for N-acetyl-9-O-acetylated neuraminic acid, and the ICV HEF has been shown to serve as both a receptor-binding and receptor-destroying protein during viral entry (Vlasak et al., 1987; Herrler et al., 1988). Although, receptor binding and membrane fusion in coronaviruses has been shown to be mediated by the S protein (Cavanagh, 1995), it has been suggested that the coronavirus HE may serve as an additional membrane-binding protein (Vlasak et al., 1988; Parker et al., 1989). However, it has been shown from studies on mouse hepatitis virus that infection cannot be mediated by HE alone as it requires the interaction of S with its receptor (Gagneten et al., 1995). Instead, it has been postulated that the HE protein may play a role in the early stages of infection where it mediates viral adherence to the intestinal wall of the host by specifically yet reversibly binding the mucopolysaccharides in the mucus layer that protects the epithelial cells of the enteric tract. The process of binding 9-O-acetylated receptors, followed by cleavage and rebinding intact receptors, may result in virus migration through the mucus layer, thereby facilitating infection (Cornelissen et al., 1997).
As described by Duckmanton et al. (1998b), the nucleotide sequence of the BRV-1 HE gene was shown to be 77% identical at its 3’ end to the X pseudogene of BEV, and 100% identical to the HE gene of BRV-2 (Cornelissen et al., 1997). In this study we have identified a HE gene homologe in the human torovirus. Sequence analysis revealed that the HTV HE gene is 85% identical to the BRV-1 and BRV-2 HE genes at the nucleotide level and 76% at the amino acid level. The corresponding 3’ end of the human HE gene is 89% identical to the BEV X gene.

This is considered a very important finding since we have previously shown that the untranslated 3’ end of the human torovirus genome manifests 99% nucleotide sequence identity with BEV in a 219 base region that was sequenced (Duckmanton et al., 1997). To ensure that this sequence that was reported was derived from the human torovirus, RNA from five virus positive specimens over a 5-year period was amplified by RT-PCR and the amplicons were shown to have the same high level of homology but differed from each other in one to two bases. Likewise, five representative sequences of the same 3’ end region of bovine toroviruses were 97% identical to the BRV sequence and 96% identical to the BEV sequence (Duckmanton et al., 1998a). It is therefore evident that the homology of the torovirus genomes at the 3’ end is very high. In contrast, the existence of a complete HE gene in HTV demonstrates it is a distinct virus from BEV which has only the pseudogene X in place of the HE gene. With the sequence information currently available it would be speculative to further comment on the relatedness of HTV to the previously characterized BEV isolate. It is therefore essential that the S gene of HTV be sequenced and expressed since it is the most likely candidate to code for the epitopes involved in virus neutralization.

The encoded 65 kDa HE protein of HTV contains domains that are characteristic of type I membrane glycoproteins, and a putative acetylenic esterase catalytic site. This indicates that this protein has the same function on both the human and bovine viruses, though it is lacking in the BEV prototype.

By immunoblot, the gpHIzBRV-HE and gpPIzHTV-HE sera reacted specifically with the 65 kDa bands in the BTV-positive fecal specimens from calves with diarrhea and the HTV-positive fecal specimens from patients with gastroenteritis, as well as with the expressed HE proteins. These expressed proteins also reacted by immunoblot with the bzBRV-1 antiserum and all nine human acute/convalescent paired sera from patients whose stools were diagnosed positive for HTV by EM, and who had been shown to have experienced seroconversion by HI and IEM. This type of cross-reactivity among torovirus species has

![Representative dot blot of five human torovirus-positive (HTV +), five bovine torovirus-positive (BTV +), five human rotavirus-positive (HRoV +), five bovine rotavirus-positive (BRoV +), five human virus-negative (HV –), and five bovine virus-negative (BV –) fecal specimens using gpHIzBRV-HE and gpPIzBRV-HE, and gpHIzHTV-HE and gpPIzHTV-HE. Reactive dots were a dark blue colour. Dots corresponding to control specimens and the pre-immune sera had the residual brown colour of the stool specimen.](image-url)
previously been reported between BRV antigens and BEV antibodies by immunofluorescence microscopy assays (IFA) and ELISA (Weiss and Horzinek, 1987), and human stools documented to contain HTV particles by EM have been shown to be reactive in a torovirus-specific ELISA using BRV antiserum (Koopmans et al., 1993). Thus, as was shown for the BRV-2 HE protein (Cornelissen et al., 1997), the HE proteins of BRV-1 and HTV are expressed during natural toroviral infections and the immune response to these is a marker of viral infection. In marked contrast, the expressed nucleocapsid protein of BRV-1 did not react with convalescent human sera from torovirus patients (Duckmanton et al., 1998b). This could be interpreted as evidence that the nucleocapsid proteins of BRV-1 and HTV are antigenically less closely related and that the overall immune response to the nucleocapsid protein is low and hence only antibodies to the homologous virus can be detected.

By hemagglutination inhibition, the guinea pig hyperimmune sera to the BRV-1 and HTV HE proteins were found to inhibit the hemagglutination of rabbit erythrocytes by a purified human torovirus preparation. This indicates that, in addition to the predicted acetylesterase activity as demonstrated for the HE of BRV-2, the HE proteins of BRV-1 and HTV possess hemagglutinating properties. This does not preclude the possibility that the toroviral peplomer protein may also function as a hemagglutinin because the BEV, which is considered not to possess a functional HE gene, is still capable of hemagglutinating human group O, rabbit, and guinea pig erythrocytes (Horzinek et al., 1987).

By dot immunoblot analysis the gpHIzBRV-HE and gpHIzHTV-HE sera were shown to react specifically with BTV-positive and HTV-positive fecal specimens that had been immobilized on a nylon membrane. Using the dot blot assay in a previous study, we demonstrated that guinea pig antisera to the BRV-1 nucleocapsid protein could detect both BTV- and HTV-positive fecal specimens (Duckmanton et al., 1998b). This shows that there is good potential to apply these reagents to the immuno-specific diagnosis of these viruses from human and bovine fecal specimens. IEM studies demonstrated that both BTV and HTV particles could be aggregated by the gpHIzBRV-HE and gpHIzHTV-HE sera, whereas, virus-negative controls, and the gpPIzBRV-HE and gpPIzHTV-HE sera showed no reactivity. This is consistent with IEM studies performed using bovine antiserum to the expressed BRV-2 HE gene, whereby the HE was identified as a structural protein of toroviruses, reacting consistently with convalescent-phase serum (Cornelissen et al., 1997). Close examination of electron micrographs of BRV and HTV reveal, in addition to the 7–9 nm peplomer proteins, the presence of shorter 4–6 nm surface projections, resembling those formed by the HE proteins of coronavirus (Sugiyama and Amano, 1981). This second ring of small peplomers has previously been observed by Woode et al. (1982) in BRV and by Beards et al. (1984) in human torovirus-like particles. However, the nature of these projections remained unknown. It has been postulated that these smaller surface projections, which are absent from BEV virions, represent the HE proteins of BRV (Cornelissen et al., 1997), and we propose that these are also present on HTV as they are visible by EM.

A comparison of the IEM aggregates resulting from reacting toroviruses with the gpHIzBRV-HE serum with those resulting from using guinea pig hyperimmune serum to the BRV-1 nucleocapsid protein (Duckmanton et al., 1998b) showed marked differences. Most of the aggregates from the antiserum to the HE protein consisted of intact viruses whereas the antiserum to the nucleocapsid protein formed aggregates of broken particles. This is consistent with the HE being a surface protein and the nucleocapsid being an internal protein.

In summary, the HE genes of BRV-1 and HTV that have been amplified using long RT-PCR and sequenced were shown by sequence analysis to be related in part to the torovirus prototype, BEV (Snijder et al., 1991), and to the HE gene of BRV-2 (Cornelissen et al., 1997). Expression of these genes in a baculovirus system resulted in proteins that served as antigens in the development of specific antisera to the HE proteins of BRV-1 and HTV. These sera were used in a
number of serological assays to demonstrate the immunoreactivity of the HE proteins to specific antisera, and to show cross-reactivity among torovirus species. These findings have the potential of being exploited for the design of diagnostic assays for human and bovine toroviruses and have provided us with important tests with which to further study torovirus infections both in experimental systems and in populations.

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