

Self-Assembly of the Recombinant Capsid Protein of a Bovine Norovirus (BoNV) into Virus-Like Particles and Evaluation of Cross-Reactivity of BoNV with Human Noroviruses

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Received 23 June 2004/Returned for modification 5 August 2004/Accepted 5 September 2004

None of the enteric caliciviruses except Po/Sapo/GIII/Cowden/80/US replicates in cell culture, which complicates efforts to develop control strategies or to study viral replication. To develop serological assays for bovine noroviruses (BoNVs) and to determine the cross-reactivity of BoNV with human noroviruses, we generated two recombinant baculoviruses, rCV186-OH and rJNCV, to express the capsid genes of Bo/CV186-OH/00/US (*Norovirus* genogroup III [GIII], genotype 2 [GIII/2]). rCV186-OH expressed the expected 57-kDa capsid protein, but rJNCV expressed a truncated capsid protein of 35 kDa. Sequence analysis of rJNCV identified a single nucleotide deletion in the P domain of the capsid gene, which introduced a stop codon at amino acid 323. The recombinant capsid protein produced by rCV186-OH but not that produced by rJNCV self-assembled into virus-like particles (VLPs) similar to native BoNV. An antibody-capture enzyme-linked immunosorbent assay (ELISA) and antigen-capture ELISA (Ag-ELISA) detected serum antibody and antigen, respectively, from calves infected with Bo/CV186-OH/00/US but not antibodies or antigens to other enteric viruses. In other tests of the GIII/2 BoNV Ag-ELISA, no cross-reactivity was observed with VLPs from one GI and four GII human noroviruses and porcine sapovirus Cowden strain. Because, like human noroviruses, BoNVs do not grow in cell culture, the BoNV VLPs will be useful in the serological assays described for the detection of BoNV antibody and antigen. Consistent with the phylogenetic analysis of the capsid genes of bovine and human noroviruses (M. G. Han, J. R. Smiley, C. Thomas, and L. J. Saif, *J. Clin. Microbiol.* 42:5214-5224, 2004), the results suggest that GIII/2 BoNV does not share significant antigenic relationships with the five characterized human noroviruses tested.

Members of the family *Caliciviridae* have a linear, positive-sense, single-stranded RNA genome of 7.4 to 8.3 kb that is composed of two or three open reading frames (ORFs) (3). The family *Caliciviridae* is divided into four genera, *Norovirus*, *Sapovirus*, *Vesivirus*, and *Lagovirus*, on the basis of genomic organization and phylogenetic analysis (4, 17, 38). Noroviruses and sapoviruses are also referred to as enteric caliciviruses and cause diarrhea in humans and animals. On the basis of the genetic divergence in the polymerase and capsid genes, noroviruses and sapoviruses are classified into three distinct genogroups, with each genogroup being further defined by branches representing distinct genotypes or genetic clusters (1, 17, 20, 55).

Most genetically characterized bovine enteric caliciviruses (BECVs) are classified as a third genogroup (genogroup III [GIII]) of norovirus, while the recently characterized Nebraska (NB) BECV, Bo/NB/80/US, which is most closely related to sapovirus and lagovirus, remains unclassified; but it has been proposed to represent a new calicivirus genus (2, 10, 35, 43, 50). Bovine noroviruses (BoNVs) are divided into two genotypes in norovirus GIII. Bo/Jena/80/DE is the prototype of

genotype 1. Bo/Newbury agent (NA)-2/76/UK, Bo/CV-186-OH/00/US, and most other BoNVs that have been characterized are classified as genotype 2. The genome of norovirus is composed of three ORFs, and the major single structural capsid protein is encoded by ORF2. The capsid protein of BoNV is composed of 519 to 522 amino acids (aa), which is smaller than the capsid proteins of human noroviruses (35, 43, 51).

Although the vesiviruses, such as feline calicivirus, San Miguel sea lion virus, and vesicular exanthema of swine virus, can replicate in cell culture (39, 52, 57), none of the members of the genus *Lagovirus* or enteric caliciviruses except Po/Sapo/Cowden/80/US grow in cell culture (13, 45). The fastidious growth requirements of these viruses have been an obstacle to the development of control strategies, the study of in vivo and in vitro viral properties, and antigenic characterization and classification. Virus-like particles (VLPs) of noroviruses and sapoviruses can be generated by a baculovirus expression system and are morphologically and antigenically similar to native virus. They have been used to produce the antigens and antibodies needed for diagnostic assays (18, 19, 22, 28, 29, 33). Recently, VLPs of Bo/Jena/80/UK (GIII, genotype 1 [GIII/1]) were reported (12).

An enzyme immunoassay with VLPs of Norwalk virus as the antigen was used as a serotyping assay for the detection of antibodies or antigens to the prototype Norwalk virus as well as those to other human noroviruses (18, 19, 22, 28, 29). In addition, VLPs can be used to determine the antigenic relationships among enteric caliciviruses and to study the relationships between different serotypes or genotypes (3). Antigenic group-

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ing based on the use of VLPs has also been correlated with genotyping by the use of capsid gene sequences (24). In this study, we generated recombinant baculoviruses expressing the capsid gene of the BoNV Bo/CV186-OH/00/US (GIII/2) and confirmed the self-assembly of the proteins expressed into VLPs. The VLPs were used to develop serological assays to detect BECV antigen and antibody and to characterize the capsid protein. We also examined the cross-reactivity of GIII/2 BoNV with human noroviruses, porcine sapovirus Cowden strain, and NB-like BECV.

MATERIALS AND METHODS

Cells. *Spodoptera frugiperda* 9 (Sf9) cells were grown in spinner culture bottles and were maintained with Hink's TNM-FH insect medium (JRH Biosciences, Inc., Lenexa, Kans.) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) and 0.1% pluronic acid solution (JRH Biosciences, Inc.) at 27°C with continuous stirring. Cells were passaged when their numbers reached 2.0×10^6 to 2.5×10^6 cells/ml. To infect the cells for VLP production, 3×10^7 cells, which were subcultured 1 day before infection, were plated into a 165-cm² cell culture flask. Prior to the addition of the virus inoculum, the cells were kept at room temperature for 30 min. Only Sf9 cells at passage levels less than 30 were used for VLP production.

Viruses and antisera. The capsid gene of Bo/CV186-OH/00/US was cloned for protein expression. Bo/CV186-OH/00/US was collected from a diarrheic Ohio veal calf and was subsequently shown to cause diarrhea upon passage in gnotobiotic (Gn) calves (51). Bo/CV186-OH/00/US (GenBank accession no. AF542084) was genetically classified as norovirus GIII/2, and the capsid gene shares 67 and 96% amino acid identities with Bo/GIII/Jena/80/DE and Bo/GIII/Newbury agent-2/76/UK, respectively (51).

Bovine rotavirus (BoRV) IND strain (9), cell culture-adapted bovine coronavirus (BoCV) Mebus strain (49), bovine torovirus (BoTV) Ohio strain (25), BECV NB strain (50), and bovine hyperimmune or convalescent-phase antisera to these viruses were used to determine the specificities of the antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA) and antibody-capture ELISA (Ab-ELISA) for BoNV. Bovine (Bo9114) and guinea pig (GP2-9) hyperimmune antisera, produced by using Bo/CV186-OH/00/US and BoNV VLPs, respectively, were used in these studies for immune electron microscopy (IEM), Western blot analysis, and ELISA.

Amplification of Bo/CV186-OH/00/US capsid gene. Bo/CV186-OH/00/US viral RNA was extracted with TRIzol reagent (Invitrogen, San Diego, Calif.) from 100 μ l of infected calf fecal sample supernatants prepared from a 1:10 dilution of feces in phosphate-buffered saline (PBS; 0.01 M; pH 7.4). RNA was eluted in 50 μ l of sterile nuclease-free water. The primers designed for amplification of the Bo/CV186-OH/00/US capsid gene were CvcapF2 (5'-TCCTTCCCGATTGTGTA-3'; positions 1358 to 1375; GenBank accession no. AF542084) and CvcapR2 (5'-AACCCGCAAGAAGTGATGAAAA-3'; positions 2949 to 2972). A one-step reverse transcription-PCR (RT-PCR) was used to amplify the capsid gene of Bo/CV186-OH/00/US. The calculated size of the Bo/CV186-OH/00/US capsid gene RT-PCR product was 1,618 bp. For RT-PCR, 4 μ l of RNA and reverse primer (0.2 mM) was treated at 70°C for 10 min and 4°C for 5 min and was added to the RT-PCR master mixture, which contained PCR buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100), 2.5 mM MgCl₂, 0.5 mM deoxynucleoside triphosphates, 5 U of *Taq* DNA polymerase, 5 U of avian myeloblastosis virus reverse transcriptase, and 10 U of RNasin RNase inhibitor. All reagents were from Promega Corporation (Madison, Wis.). The RT-PCR conditions were as follows; 45°C for 60 min; 94°C for 2 min; and 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min, and 72°C for 10 min. The RT-PCR products were analyzed on 1% agarose gels and were held at 4°C until purification.

Subcloning of capsid genes in baculovirus transfer vectors and capsid gene sequencing. RT-PCR products from two independent RT-PCRs were purified and ligated to pCR2.1 TOPO TA cloning vectors (Invitrogen). Two capsid gene cloning vectors, pCRBCap-186 and pCRJNCap, were generated for subcloning of the Bo/CV186-OH/00/US capsid gene. Plasmid DNAs of pCRBCap-186, pCRJNCap, and pBlueBac 4.5 were digested with *Xba*I-*Hind*III or *Eco*RI for subcloning of the capsid gene into the pBlueBac 4.5 baculovirus transfer vectors (Invitrogen). Gel-purified capsid gene DNAs of pCRBCap-186 and pCRJNCap were ligated into pBlueBac 4.5 vectors with T4 DNA ligase (New England Biolabs, Inc., Beverly, Mass.) to create the transfer vectors pBac-CV186 and pBac-JN, respectively, which contained the Bo/CV186-OH/00/US capsid gene. All RT-PCR products and plasmid DNA were purified with a QIAquick gel extraction kit (Qiagen). All protocols used to generate cloning and baculovirus

transfer vectors were completed by standard procedures (47) or according to the instructions of the manufacturer. One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen) were used for transformations. To confirm the orientation and sequence of the capsid gene in pBac-CV186 and pBac-JN, the capsid gene was sequenced with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, Calif.).

Generation of recombinant baculoviruses expressing the capsid gene. pBac-CV186 and pBac-JN were transfected into Sf9 cells with a Bac-N-Blue Transfection kit (Invitrogen), according to the instructions of the manufacturer. Recombinant baculovirus clones were purified from wild-type (WT) baculoviruses by plaque assays, and the presence of the capsid gene inserts was confirmed by PCR analysis with baculovirus-specific forward and reverse PCR primers (Invitrogen). The cloned recombinant baculoviruses which were generated from pBac-CV186 and pBac-JN, designated rCV186-OH and rJNCV respectively, were propagated in Sf9 cells to make master virus stocks that were stored at 4°C. The titers of the recombinant baculoviruses were determined by plaque assay.

Production and purification of BoNV VLPs. Sf9 cells were infected with rCV186-OH or rJNCV at a multiplicity of infection of 5 to 10 and were incubated at 27°C. To purify the BoNV VLPs, the infected Sf9 cells and cell culture supernatants were harvested at 7 days postinoculation (dpi). The VLPs were purified from cell culture supernatants and cell lysates by ultracentrifugation through a 40% (wt/vol) sucrose cushion, followed by CsCl density gradient (1.362 g/cm³) ultracentrifugation (31). Purified VLPs were analyzed by Western blotting and IEM as well as for their densities and particle sizes and were used to produce guinea pig hyperimmune antiserum. The sizes and densities of VLPs in CsCl were measured from scanned electron microscopy photographs (Adobe Photoshop, version 6.0) and with a refractometer (Bausch & Lomb, Rochester, N.Y.), respectively. The protein concentrations of the VLPs were determined with a Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, Calif.).

Validation of expression of recombinant capsid protein by immunofluorescence and Western blot analyses. Uninfected control and rCV186-OH- and rJNCV-infected Sf9 cells were fixed with 80% acetone and incubated with Bo9114 hyperimmune antiserum (1:400) and naive Gn calf (negative control) serum (1:400). After the cells were washed with PBS (0.01 M; pH 7.4), they were incubated with fluorescein isothiocyanate-conjugated anti-bovine immunoglobulin G (IgG; heavy and light chains; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). The cells were observed for immunofluorescence with an Olympus IX70 fluorescence microscope (Olympus America, Inc., Melville, N.Y.).

The CsCl density gradient ultracentrifugation-purified VLPs or concentrated recombinant proteins were solubilized with Laemmli buffer (34), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membranes were blocked with 5% normal goat serum in PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T) for 1 h at room temperature and incubated with GP2-9 guinea pig hyperimmune antiserum (1:1,000) or Bo9114 bovine hyperimmune antiserum (1:200) and then with goat anti-guinea pig IgG conjugated with horseradish peroxidase (HRP; 1:3,000; Kirkegaard & Perry Laboratories, Inc.) or mouse anti-bovine IgG conjugated with biotin (1:5,000; Sigma, St. Louis, Mo.) and HRP conjugated with streptavidin (1:3,000; Kirkegaard & Perry Laboratories, Inc.). The blots were developed with tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Inc.). All reaction mixtures were incubated for 1 h at room temperature before they were washed three times with PBS-T (0.05%).

Kinetics of recombinant protein production in Sf9 cells. Sf9 cells infected with rCV186-OH and rJNCV were harvested every 2 days from 1 to 13 dpi. Infected cells and cell culture supernatants were harvested separately. Cells were lysed by three cycles of freezing-thawing or by treatment with lysis buffer containing protease inhibitors (58). The treated cell lysates and supernatants were concentrated by ultracentrifugation at $112,700 \times g$ (SW28Ti rotor; Beckman Coulter, Inc., Fullerton, Calif.) for 2 h at 4°C. The resultant pellets were resuspended in TNC buffer (10 mM Tris-HCl [pH 6.5], 140 mM NaCl, 10 mM CaCl₂) containing 100 mM leupeptin (Sigma) and 10 mM pepstatin A (Sigma). The ELISA titers of the recombinant protein were determined by the Ag-ELISA described below.

IEM and production of hyperimmune antiserum to VLPs. VLPs purified from rCV186-OH-infected Sf9 cell culture supernatants by CsCl density gradient ultracentrifugation were diluted 1:50 and were then incubated at 4°C overnight with hyperimmune antiserum Bo9114 (1:500) or GP2-9 (1:2,000) in TC buffer (0.05 M Tris-HCl, 15 mM CaCl₂ [pH 7.4]). After ultracentrifugation at $112,700 \times g$ (SW41Ti rotor) for 2 h at 4°C, the pellets were collected and stained with 3% phosphotungstic acid (pH 7.0) and examined with an electron microscope (H-7500; Hitachi High Technologies, Inc.).

Preparations of BoNV VLPs whose morphologies were confirmed by IEM

were used to produce guinea pig hyperimmune antiserum by a previously described protocol (21). The antibody titers of the hyperimmune antisera were determined by the Ab-ELISA described below.

ELISA for detection of BoNV antibody and antigen. The sucrose-purified VLPs were used as antigens for the detection of BoNV antibodies by the Ab-ELISA. The VLPs were directly coated onto microplates (Nunc-Immuno plate; Nalgene Nunc International, Rochester, N.Y.) at 100 μ l per well and a final concentration of 5 μ g/ml in carbonate-bicarbonate buffer (0.05 M, pH 9.6) and were incubated at 4°C overnight. The VLP-coated microplate was incubated with 100 μ l of the test serum samples. HRP-labeled goat anti-bovine IgG (Kirkegaard & Perry Laboratories, Inc.) diluted 1:3,000 in PBS-T (0.05%) containing 2% normal goat serum (Invitrogen) was added to each well of the microplate, followed by the addition of 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate (Kirkegaard & Perry Laboratories, Inc.). Each reaction was completed by incubation at 37°C for 1 h. The absorbance at 405 nm was measured by using an Emax microplate reader (Molecular Devices Co., Sunnyvale, Calif.). Serum samples with absorbances two times greater than the ratio of the absorbance for a positive sample to the absorbance for a negative serum sample were considered positive.

To determine the antibody titers in individual samples, the sera were serially diluted twofold in PBS (pH 7.4). Mean ELISA antibody titers were calculated and expressed as the reciprocal of the highest serum dilution with a positive absorbance.

Eighty-seven uninfected Gn calf serum samples and Bo9114 hyperimmune antiserum were used as negative and positive control sera, respectively, to determine the cutoff value. To determine the specificity of the Ab-ELISA, bovine hyperimmune or convalescent-phase sera with antibodies against BoRV IND strain, BoCV Mebus strain, BoTV Ohio strain, and BECV NB strain were used.

For the development of the Ag-ELISA for the detection of the BoNV antigen, the microplate was coated with the GP2-9 guinea pig hyperimmune antiserum or pooled serum from uninoculated guinea pigs, each diluted 1:500, by the same method used for the Ab-ELISA. After the microplates were coated, they were blocked with 5% gelatin (Sigma). Fecal samples from calves orally inoculated with Bo/CV186-OH/00/US or other enteric viruses were added to the microplates, followed by the addition of Bo9114 bovine hyperimmune antiserum (1:5,000), mouse anti-bovine IgG conjugated with biotin (1:10,000; Sigma), HRP conjugated with streptavidin (1:4,000; Kirkegaard & Perry Laboratories, Inc.), and ABTS substrate (Kirkegaard & Perry Laboratories, Inc.). Cell culture lysates infected with BoCV Mebus strain and BoRV IND strain and fecal samples from calves infected with BoTV Ohio strain and BECV NB strain were used to determine the specificity of the Ag-ELISA. All fecal samples were diluted 1:10 with PBS (pH 7.4) or Earle's minimum essential medium (EMEM) and were tested in duplicate wells. The mean absorbance of each sample was calculated by subtraction of the mean absorbance of the negative serum-coated well from the mean absorbance of the hyperimmune antiserum-coated wells. Samples with absorbance values greater than the mean absorbance plus three times the standard deviation of the negative fecal samples were classified as positive for BoNV antigen.

Serum antibodies and fecal viral antigens from calves infected with Bo/CV186-OH/00/US were detected by Ab-ELISA and Ag-ELISA, respectively. One- and 2-week-old colostrum-deprived Gn calves were inoculated orally with different stocks of Bo/CV186-OH/00/US, and feces and blood were collected after inoculation. Feces were scored on a scale from 0 to 4 (where 0 is normal), with diarrhea considered a score \geq 3. The virus inocula were prepared by filtration (pore size, 0.2 μ m) of feces from a calf infected with Bo/CV186-OH/US diluted 1:10 (PBS; pH 7.4). The inocula were tested for sterility by culture on blood agar and in thioglycolate broth.

Cross-reactivity of BoNV to human norovirus, porcine sapovirus, and NB-like BECV. GI and GII human norovirus VLPs, porcine sapovirus (Po/Sapo/GIII/Cowden/80/US) VLPs (21), WT and cell culture-adapted (TC) Po/Sapo/Cowden/80/US (13), and the NB-like BECV (Bo/NB/80/US) passaged in Gn calves (50) were tested to determine their cross-reactivities to BoNV in the Ag-ELISA. The human norovirus VLPs tested for cross-reactivity included Hu/Noro/I/Desert Shield/395/90/SA, Hu/Noro/II/Hawaii/71/US, Hu/Noro/II/Toronto24/91/CAN, Hu/Noro/II/Florida/269/93/US, and Hu/Noro/II/MD145/87/US and were kindly provided by K. Green (National Institute of Allergy and Infectious Diseases, National Institutes of Health).

RESULTS

Generation of recombinant baculoviruses and production of VLPs. Two recombinant baculoviruses, rCV186-OH and rJNCV, were cloned from WT baculoviruses by plaque assays. Sf9 cells

infected with both recombinant viruses had specific immunofluorescence by use of the Bo/CV186-OH/00/US hyperimmune antiserum (Bo9114) (data not shown), while control cells showed no immunofluorescence. Both recombinants were analyzed by PCR to confirm the cloning. Two or three clones of the recombinant were selected and amplified for VLP production on the basis of the intensities of their immunofluorescence signals and by the day of appearance of cytopathic effects on infected Sf9 cells. The titers of amplified recombinant clones were 1.8×10^8 to 2.4×10^8 PFU/ml.

BoNV VLPs were produced in Sf9 cells infected with rCV186-OH but not with rJNCV. The recombinant proteins expressed by rJNCV did not self-assemble into VLPs, despite alteration of the experimental conditions, including the virus titers inoculated, the incubation time after inoculation, the insect cells (Sf9 and High Five) used, and the time of analysis after purification by CsCl density gradient or sucrose cushion ultracentrifugation.

Western blot analysis of recombinant capsid proteins expressed by rCV186-OH and rJNCV and kinetics of recombinant capsid protein production in Sf9 cells. Recombinant capsid proteins were analyzed by Western blotting. The recombinant capsid proteins were purified from cell culture supernatants and cell lysates of Sf9 cells infected with rCV186-OH on CsCl density gradients and sucrose cushions and reacted with the hyperimmune antisera Bo9114 (Fig. 1) and GP2-9 (data not shown). Most of the capsid proteins were detected from the supernatants. The antisera did not react with VLPs of Po/Sapo/Cowden/80/US, which was used as a negative control and purified similarly (data not shown). The molecular mass of the recombinant capsid protein was 57 kDa, which agreed with the expected size based on the translated amino acid sequence.

The kinetics of recombinant capsid protein production were determined by Western blotting (Fig. 1) and Ag-ELISA (data not shown). The recombinant proteins purified from the rCV186-OH-infected cell culture supernatants and cell lysates were detected from 1 to 13 dpi by Western blotting and Ag-ELISA (Fig. 1A and B). The band intensities of the capsid proteins harvested from both the supernatants and the cell lysates at 1 dpi were much weaker than those collected at 3 to 13 dpi. Protein bands of the strongest intensity were observed in cell lysates collected at 3 dpi, after which time the band intensity diminished to 13 dpi (Fig. 1A). The highest band intensity of capsid protein purified from supernatants was obtained at 5 and 7 dpi (Fig. 1B). These results were also consistent with those of Ag-ELISA. The ELISA titers of all supernatants except for the supernatants harvested at 3 dpi were higher than those of the cell lysates. The maximal titers of the supernatants were detected from 5 dpi. The highest titer from the cell lysates was detected at 3 dpi, and the titers were reduced after 5 dpi.

The 57-kDa capsid protein was not detected in the cell culture supernatants or the cell lysates of Sf9 cells infected with rJNCV by Western blotting with the Bo9114 or GP2-9 hyperimmune antisera. A 35-kDa protein in place of the 57-kDa protein was detected in the cell culture supernatants (Fig. 1C) and the cell lysates (data not shown) by use of both hyperimmune antisera.

The cell culture supernatants and cell lysates purified from rJNCV-infected Sf9 cells did not contain recombinant proteins detected by Ag-ELISA. To determine the reactivities of the

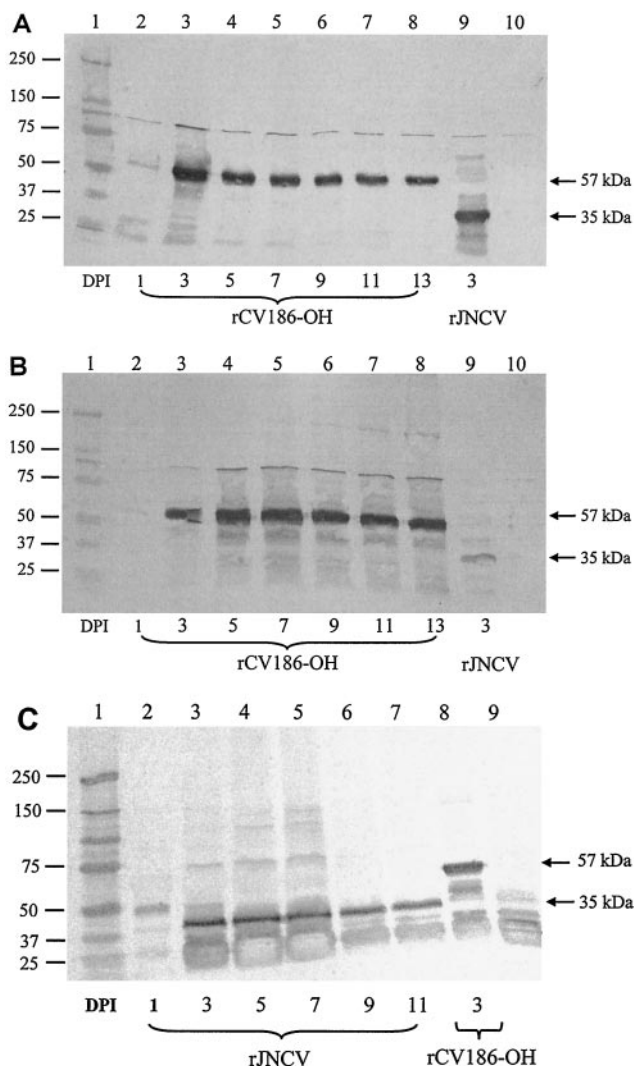


FIG. 1. Western blot analyses of cell lysates (A) and cell culture supernatants (B) of Sf9 cells infected with rCV186-OH and cell culture supernatants (C) infected with rJNCV. The cells were harvested every 2 days from 1 to 13 dpi. The cell lysates and supernatants of Sf9 cells infected with rCV186-OH and the supernatants of rJNCV-infected cells were concentrated by ultracentrifugation at $112,700 \times g$ for 2 h. The cell lysates and supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were detected by using bovine hyperimmune antiserum Bo9114. Cell lysates (A) and supernatants (B) of rCV186-OH infected cells: lane 1, molecular mass marker (numbers on the left are in kilodaltons); lanes 2 to 8, proteins harvested at 1, 3, 5, 7, 9, 11, and 13 dpi, respectively; lane 9, proteins harvested at 3 dpi from Sf9 cells infected with rJNCV; lane 10, uninfected mock Sf9 cell culture lysates (A) and supernatants (B) harvested at 7 days after culture. A recombinant protein of 35 kDa was detected from cell lysates and supernatants of Sf9 cells infected with rJNCV (lane 9). (C) Cell culture supernatants of the rJNCV-infected cells producing a 35-kDa truncated capsid protein: lane 1, molecular mass marker (numbers on the left are in kilodaltons); lanes 2 to 7, supernatants of rJNCV-infected Sf9 cells harvested at 1, 3, 5, 7, 9, and 11 dpi; lane 8, supernatants of rCV186-OH-infected Sf9 cells harvested at 3 dpi; lane 9, uninfected mock Sf9 cell culture supernatants.

recombinant proteins purified from rJNCV, with or without sucrose cushion ultracentrifugation, to bovine hyperimmune antiserum Bo9114, the microplates coated with rJNCV-produced capsid proteins were tested with Bo9114. The proteins

did not react with bovine hyperimmune antiserum Bo9114 by ELISA.

Nucleotide sequence analysis of the capsid gene inserted in transfer vectors. The cloned capsid gene in rJNCV was sequenced to determine the cause of the truncation of the capsid protein expressed by Sf9 cells compared to the sequences of the capsid proteins of Bo/CV186-OH and rCV186-OH. The sequence determined showed that the capsid gene of rJNCV had 2 aa substitutions ($V^9 \rightarrow I$ and $T^{196} \rightarrow S$) in the shell (S) domain and a 1-nucleotide deletion ($^{937}CCCCTTG \rightarrow CCCTTG$ [the deletion is indicated in boldface]) which introduced a stop codon at amino acid position 323. The translated 322-aa sequence had a calculated molecular mass of 35 kDa, in agreement with that observed by Western blotting.

Characteristics of BoNV VLPs. The VLPs produced from rCV186-OH were morphologically similar to native virus by IEM (Fig. 2). Clumping of the VLPs was observed by IEM, suggesting that the Bo9114 and GP2-9 hyperimmune antisera aggregated the VLPs. The VLPs were 32.4 ± 2.6 nm ($n = 96$) and had a density of 1.31 g/cm³ after CsCl density gradient ultracentrifugation, which are similar to the values for norovirus VLPs reported previously (18, 29). One to 3 mg of VLPs was purified per liter of rCV186-OH-infected cell culture supernatants after CsCl density gradient ultracentrifugation. The mean Ag-ELISA titers were 25,600 to 102,400, which were 10 to 20 times higher than those of VLPs of porcine sapovirus (21). The yield of recombinant capsid proteins purified from the infected cell culture supernatants was greater than that purified from cell lysates, which corresponded to the results obtained by Western blotting and Ag-ELISA. No VLPs were evident for the rJNCV-expressed capsid protein by the CsCl density gradient ultracentrifugation procedures, as described for rCV186-OH.

Antigen and antibody detection by Ag-ELISA and Ab-ELISA from calves infected with Bo/CV186-OH/00/US. BoNV antigen was detected by Ag-ELISA from the feces of Bo/CV186-OH/00/US-infected Gn calves B465 and B524 for up to 7 and 10 dpi, respectively (Fig. 3). The highest absorbance by Ag-ELISA was obtained from fecal samples collected at 2 to 3 dpi, suggestive of higher levels of virus shedding. Calves B465 and B524 had diarrhea for 3 and 4 days after infection, respectively. Ag-ELISA control wells, in which only PBS or EMEM (used as the diluents for fecal samples) was substituted for the fecal samples, had absorbance values of less than 0.08 in wells coated with negative or positive serum. The absorbances of fecal samples from uninfected Gn calves in the positive or negative serum-coated wells were 0.092 ± 0.037 and 0.074 ± 0.021 , respectively. The Ag-ELISA was negative for BoRV, BoCV, BoTV, and BECV NB strain, for which the absorbances were similar to those for the PBS and EMEM control wells. Serum IgG antibodies to BoNV were first detected at 5 dpi. Serum antibody titers subsequently increased from 5 to 10 dpi and reached a maximum at 20 to 25 dpi.

Eighty-seven serum samples from uninfected colostrum-deprived Gn calves were tested as negative controls by the Ab-ELISA. The absorbance was 0.091 ± 0.008 . Like the results of Ag-ELISA, the absorbances of Ab-ELISA for hyperimmune or convalescent-phase serum to BoRV, BoCV, BoTV, and BECV NB strain were similar to those for the PBS controls, which

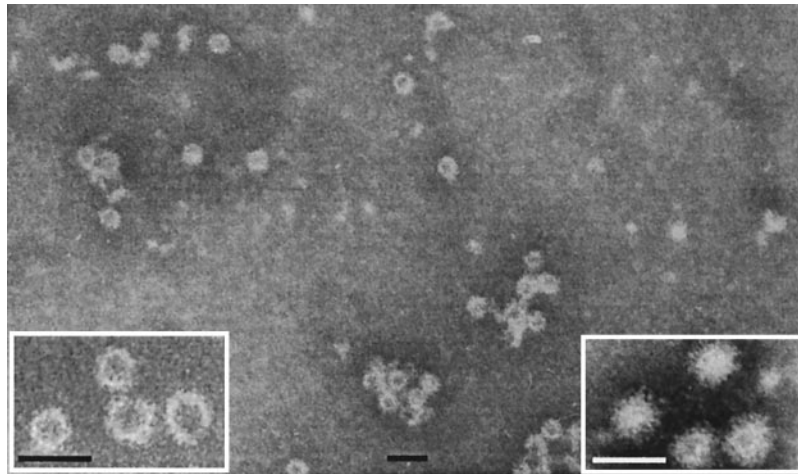


FIG. 2. Immune electron micrograph of BoNV VLPs. The rCV186-OH VLPs were purified by CsCl gradients from the cell culture supernatants of recombinant baculovirus-infected Sf9 cells. The VLPs were incubated with bovine hyperimmune antiserum Bo9114 against Bo/GIII/CV186-OH/00/US and visualized by negative staining with 3% phosphotungstic acid (pH 7.0). The insets show immune electron micrographs of the enlarged BoNV VLPs (left) and native BoNVs (right). Bars, 50 nm.

confirm that there was no cross-reactivity of BoNV/CV186-OH/00/US with these viruses, as detected by Ab-ELISA.

Cross-reactivity of BoNV to human noroviruses and animal enteric caliciviruses. The BoNV Ag-ELISA did not react with the GI or GII human norovirus VLPs, the porcine sapovirus VLPs, or the WT or TC Po/Sapo/Cowden/80/US sapovirus. The protein concentration of each of the human norovirus VLPs and porcine sapovirus VLPs was 0.5 to 1.0 mg/ml. The morphology of these VLPs, confirmed by electron microscopy, was similar to that of the native viruses. The titers of WT and TC Po/Sapo/Cowden/80/US sapoviruses, which were determined by the homologous Ag-ELISA (8), were 2,560 and 1,024, respectively.

DISCUSSION

Human norovirus and sapovirus VLPs have been used to develop serological assays and to determine the cross-reactivities and immunogenicities among these viruses (14–16, 18, 19, 28–30, 32, 33, 42). The VLPs of BoNV Bo/Jena/80/UK (GIII/1) were recently generated (12). In this study, we constructed a recombinant baculovirus to generate VLPs of GIII/2 BoNV and adapted these VLPs for use in the development of an Ab-ELISA and an Ag-ELISA for the detection of BoNV antibody and antigen, respectively.

The capsid protein of Norwalk virus is composed of the S domain and the protruding (P) structural domain. The N-terminal 225 aa of the S domain precedes the adjoining P domain (amino acids 226 to 522) (46). The S domain of the capsid gene is a critical region for self-assembly of the capsid protein into VLPs (6). Although the P domain is not essential for self-assembly of the capsid protein expressed, the morphology, particle size, and stability of particles are dependent on the P domain. Lochridge and Hardy (36) reported that a single amino acid substitution ($^{91}\text{H}\rightarrow\text{R}$) in the S domain of the Snow Mountain norovirus inhibited VLP assembly.

Jiang et al. (29) reported that a 34-kDa cleavage protein of the 58-kDa capsid protein of the Norwalk virus was produced by a recombinant baculovirus containing ORF2 and ORF3.

The 34-kDa protein was detected in infected cell lysates but not in cell culture supernatants and was found to be mainly cell associated. In contrast, rJNCV produced a truncated capsid protein of 35 kDa that was detected by Western blotting in both the cell lysates and the supernatants of infected Sf9 cells. Due to the insertion of the stop codon at amino acid position 323, C-terminal amino acid residues 324 to 522 were deleted. Sf9 cells infected with rJNCV displayed immunofluorescence and the supernatants of these cells reacted by Western blotting with hyperimmune antiserum Bo9114 against Bo/CV186-OH/00/US, which suggests the presence of antigenic sites in the N terminus of the capsid protein. However, the concentrated supernatants or cell culture lysates from rJNCV-infected Sf9 cells did not react with hyperimmune antiserum Bo9114 by

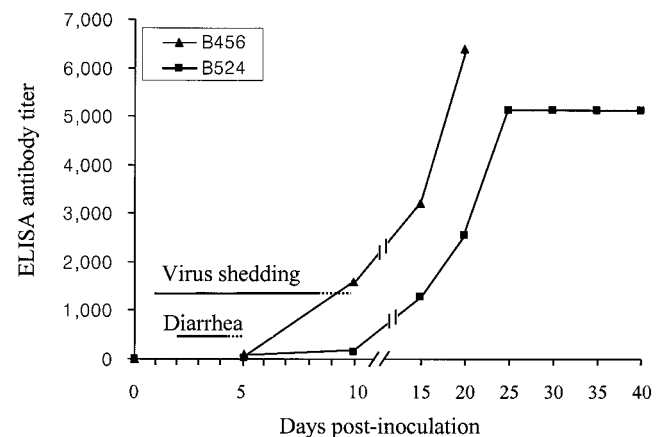


FIG. 3. Serum IgG antibody responses and diarrhea and viral shedding in 1- to 2-week-old Gn calves orally inoculated with GIII bovine norovirus Bo/CV186-OH/00/US. Feces were scored by using a scale from 0 to 4 (where 0 is normal), with diarrhea considered a score ≥ 3 and indicated by a horizontal bar for the average duration (4.5 dpi) and a dotted line for maximum duration (5 dpi). Viral shedding detected by Ag-ELISA is shown by the horizontal bar for average duration (8.5 dpi) and a dotted line for maximum duration (10 dpi).

Ab-ELISA or with hyperimmune antiserum GP2-9 or Bo9114 by Ag-ELISA, indicating that the tertiary structure of the truncated capsid protein was altered or destroyed after ultracentrifugation.

One of the two BoNV/CV186-OH/00/US baculovirus recombinants, rJNCV, failed to express the predicted 57-kDa capsid protein, and no VLPs were obtained. Comparison of the predicted amino acid sequences of rCV186-OH and rJNCV showed two amino acid changes ($V^9 \rightarrow I$ and $T^{196} \rightarrow S$) in the S domain of rJNCV and a 1-nucleotide deletion ($^{937}CCCCTTG \rightarrow CCCTTG$) in the P domain, which introduced a stop codon at amino acid 323. Previously, it was shown that deletion of amino acids 228 to 530 in the P domain of the Norwalk virus capsid protein does not affect the assembly of morphologically smaller VLPs (6). However, rJNCV, which expressed the complete S domain, did not generate VLPs. It is unclear if the loss of expression of the P domain affected the stability of the VLPs or if the amino acid substitutions in the S domain inhibited assembly of the recombinant protein. It is unlikely that the rJNCV VLPs were cleaved by a protease(s), as we examined them immediately after purification. Compared with the capsid sequences of known GIII/2 BoNVs, including Ohio strains, the amino acid at position 9 was variable between strains in which Val, Ile, Thr, and Ala residues were observed (43, 51, 54, 56). However, all known GIII/2 BoNVs had a threonine at amino acid position 196, which suggests that T^{196} may be critical to the self-assembly of recombinant capsid protein into VLPs.

It was previously reported (5) that the Norwalk virus VLPs produced by coexpression of ORF2, ORF3, and the 3' untranslated region (3' UTR) were more stable and produced higher yields than those particles expressed by using ORF2 alone, ORF2 and ORF3, or ORF2 and the 3' UTR. While coexpression of ORF2 with ORF3 and 3'UTR produced high yields of VLPs, recombinant rCV186-OH, which contained only ORF2, yielded 1 to 3 mg of VLPs per liter of cell culture supernatants following CsCl density gradient purification. Expression of recombinant proteins in insect cells is known to depend on factors that include the cell line (Sf9, High Five), the culture medium, the concentration of infected cells, the protein types expressed, the multiplicity of infection, and the time of infection (27, 44). On the basis of our results, rCV186-OH showed increased levels of protein expression in Sf9 cells subcultured 1 day before infection (data not shown). The recombinant capsid protein and VLPs were detected as early as 1 dpi from cell culture lysates and supernatants by Western blotting (Fig. 1) and IEM (data not shown), respectively.

The BoNV Ab-ELISA developed with rCV186-OH VLPs detected a serum antibody specific for Bo/CV186-OH/00/US but not for other enteric viral pathogens and Bo/NB/80/US. Deng et al. (12) used a similar Ab-ELISA incorporating baculovirus-expressed GIII BoNV (Bo/Jena/80/DE) VLPs to conduct an epidemiological study of British dairy herds and showed that 99.1% of 824 cattle (25 dairy herds) in Thuringia, Germany, were seropositive for the Jena strain. The capsid gene of Bo/CV186-OH/00/US shares 87 to 96% amino acid identities with known GII/2 BoNVs. However, the amino acid identity of the capsid gene of Bo/CV186-OH/00/US to that of Bo/Jena/80/DE is only 70%. The cross-reactivity of the recombinant capsid proteins between GIII/1 (Jena-like) and GIII/2

(Newbury agent-like) BoNVs is unknown. Previously (51), we identified a Jena-like BoNV strain (Bo/CV521-OH/02/US) in an Ohio veal calf on the basis of genotyping using a partial sequence of the capsid gene of Bo/CV521-OH/02/US revealed that it had higher degrees of nucleotide and amino acid identities to GIII/2 BoNVs, indicative of a recombinant (23). Comparison of the results obtained by the two Ab-ELISAs in future studies should clarify the cross-reactivity between GIII/1 and GIII/2 BoNVs.

The kinetics of the antibody response and viral shedding of BoNV in calves are not well defined, and antigen detection and serologic tests are lacking. In human norovirus infections, the manifestations include short-term immunity, sudden onset of diarrhea for a few days following a 1- to 2-day incubation period, and a short period of viral shedding (37, 48, 53). However, viral shedding was detected at 1 to 13 dpi from volunteers challenged with Norwalk virus (42). The period of BoNV shedding in experimentally infected Gn calves is short (7). The BoNV shedding was detected by solid-phase IEM in experimentally infected Gn calves at 2 to 5 dpi (7, 10, 11). Two Gn calves infected with Bo/CV186-OH/00/US shed viruses for 6 to 9 dpi when the length of viral shedding was determined by Ag-ELISA, whereas the calves shed viruses for 4 to 5 dpi when the length of viral shedding was determined by IEM. BoNV was detected in fecal samples of one calf infected with Bo/CV186-OH/00/US for up to 35 dpi by RT-PCR (data not shown), indicative of a higher sensitivity of RT-PCR for the detection of viral RNA. The sensitivity of Ag-ELISA for the detection of Norwalk virus was reported to be greater or the same as that of RT-PCR (14). A prolonged period of human norovirus shedding (2 years), as detected by IEM and RT-PCR, has also been reported in an immunosuppressed human patient (40).

The antibody responses induced by BoNVs in calves were similar to those induced by human noroviruses in humans (37, 48, 53). Serum IgG antibody against BoNV was first detected (at low titers) in experimentally infected Gn calves at 5 dpi. In the experimental calves tested in this study, IgG antibody titers reached a maximum about 3 weeks after inoculation and remained high until 45 dpi, at which time the calves were euthanized due to size restrictions for calves housed in the Gn calf isolators. Further studies are required to discern the kinetics of the mucosal and systemic immune responses of calves to BoNV.

The BoNVs are genetically classified as *Norovirus* GIII and are distinct from other animal caliciviruses (2, 10, 35, 43). This finding raises concerns about whether BoNVs could be involved in human norovirus outbreaks on the basis of their genetic relationships to human noroviruses. However, on the basis of the results obtained with the capsid gene sequences of BoNVs, BoNVs are genetically distinct from human noroviruses GI and GII and may present a low risk of infection for humans (23, 43). However, as a caveat to this finding, there have been few sequence analyses of BoNVs and counterpart human noroviruses from developing countries, where closer contact between cattle and humans is expected. Bovine rotaviruses were thought to be distinct from human rotaviruses until strains collected from cattle and children in India were compared and found to share common serotypes (26). The antigenic relatedness of noroviruses can be determined by

their reactivities with VLPs and hyperimmune antisera against VLPs (3). In the Ag-ELISA for the detection of BoNVs, VLPs of human noroviruses GI and GII, including Hu/Noro/I/Desert hield395/90/SA, Hu/Noro/II/Hawaii/71/US, Hu/Noro/II/Toronto24/91/CAN, Hu/Noro/II/Florida/269/93/US, and Hu/Noro/II/MD145/87/US, did not react with the BoNV GIII/2 reagents. Noel et al. (41) reported homologous immune responses to Norwalk virus in patients infected with other norovirus GI strains with as much as 38.5% amino acid divergence in the N-terminal sequence of the capsid gene. However, homologous immune responses to the Toronto virus and Hawaii virus, both GII noroviruses, were detected only after infections with noroviruses that had less than 6.5% amino acid divergence from these viruses. The capsid gene of Bo/CV186-OH/00/US shared 55, 53, 46, 45, and 47% amino acid sequence identities with the capsid genes of Hu/Noro/I/Desert Shield395/90/SA, Hu/Noro/II/Hawaii/71/US, Hu/Noro/II/Toronto24/91/CAN, Hu/Noro/II/Florida/269/93/US, and Hu/Noro/II/MD145/87/US, respectively. The GIII/2 BoNVs had 45 to 50% amino acid sequence identity (29 to 57% nucleotide identity) and 43 to 46% amino acid sequence identity (21 to 54% nucleotide sequence identity) to GI and GII human noroviruses, respectively (23). These lower levels of amino acid sequence identity and the lack of antigenic cross-reactivity of GIII BoNVs with GI and GII human noroviruses suggest that BoNVs are antigenically and genetically distinct from the GI and GII human noroviruses. The antigenic relationships between the GIII BoNVs of genotype 1 (Jena-like) and genotype 2 (Newbury-like) require further study. The Ag-ELISA and Ab-ELISA developed in this study are essential for such comparative studies and for epidemiological studies of the prevalence and impact of BoNV-infected cattle.

ACKNOWLEDGMENTS

We thank the Molecular and Cellular Imaging Center of the Ohio Agricultural Research and Development Center for DNA sequencing.

This work was supported by grant R01 AI49742 from the National Institutes of Allergy and Infectious Diseases, NIH.

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