Molecular Characterization of a Bovine Enteric Calicivirus: Relationship to the Norwalk-Like Viruses

B. L. LIU, P. R. LAMBDEN, H. GUNThER, P. OTTO, M. ELSCHNER, and I. N. CLARKE

Molecular Microbiology Group, University Medical School, Southampton General Hospital, Southampton SO16 6YD, United Kingdom, and Federal Institute for Health Protection of Consumers and Veterinary Medicine, Jena Branch, 07743 Jena, Germany

Received 15 July 1998/Accepted 30 September 1998

Jena virus (JV) is a noncultivatable bovine enteric calicivirus associated with diarrhea in calves and was first described in Jena, Germany. The virus was serially passaged 11 times in colostrum-deprived newborn calves and caused diarrheal disease symptoms at each passage. The complete JV genome sequence was determined by using cDNA made from partially purified virus obtained from a single stool sample. JV has a positive-sense single-stranded RNA genome which is 7,338 nucleotides in length, excluding the poly(A) tail. JV genome organization is similar to that of the human Norwalk-like viruses (NLVs), with three separate open reading frames (ORFs) and a 24-nucleotide sequence motif located at the 5’ terminus of the genome and at the start of ORF 2. The polyprotein (ORF 1) consists of 1,680 amino acids and has the characteristic 2C helicase, 3C protease, and 3D RNA polymerase motifs also found in the NLVs. However, comparison of the N-terminal 100 amino acids of the JV polyprotein with those of the group 1 and group 2 NLVs showed a considerable divergence in sequence. The capsid protein (ORF 2) at 519 amino acids is smaller than that of all other caliciviruses. JV ORF 2 was translated in vitro to produce a 55-kDa protein that reacted with postinfection serum but not preinfection serum. Phylogenetic studies based on partial RNA polymerase sequences indicate that within the Caliciviridae JV is most closely related to the group 1 NLVs.

Caliciviruses cause a wide spectrum of diseases and are a major cause of gastroenteritis in humans (7, 11, 29, 30). However, little is known about enteric caliciviruses from other species (4). The prototype strain of human caliciviruses is Norwalk virus, which was first described in 1972 associated with an outbreak of gastroenteritis and vomiting involving children and staff at an elementary school in Norwalk, Ohio (31). Norwalk virus and the subsequently described Norwalk-like caliciviruses (NLVs) have also been collectively described as small, round-structured viruses (6). NLVs are so called because they can be clearly distinguished from other enteric viruses on the basis of virion morphology. These viruses are approximately 30 to 35 nm in diameter and have an amorphous structure with a ragged edge. This morphology contrasts with that of the Sapporo-like viruses (SLVs) which are also associated with human gastroenteritis. The SLVs are predominantly associated with pediatric gastroenteritis and display the distinctive morphology typical of other well-defined animal caliciviruses (37).

Complete nucleotide sequences are available for the prototype Norwalk virus (23, 28) and two NLVs, Southampton virus (33, 35) and Lordsdale virus (10). These viruses have single-stranded (ss) positive-sense RNA genomes of 7,500 to 7,700 nucleotides organized into three major open reading frames (ORFs). ORF 1 encodes a large polyprotein (180 kDa) that undergoes proteolytic cleavage by a 3C-like protease, ORF 2 encodes a single capsid protein (60 kDa), and ORF 3 encodes a small, basic protein (M₁, 20 to 25 kDa) of unknown function. Comparisons of the three complete genomic sequences together with phylogenetic analyses of many partial sequences have shown that the NLVs can be divided in to two genetic groups (34). NLVs do not grow in cell culture, and therefore molecular studies have relied on the availability of stool samples from volunteers or clinical specimens. Phylogenetic analyses also suggest that the NLVs are quite distinct from the SLVs and constitute a separate genus of the family Caliciviridae (3, 43).

Enteric caliciviruses have been described for a number of animal species, including cattle, pigs, cats, dogs, and chickens (2, 4, 16, 26, 27, 39, 47, 48, 50, 51). However, these viruses remain candidate caliciviruses because of the absence of definitive sequence evidence linking them to the Caliciviridae (8). Progress in the molecular characterization of these viruses has been severely hampered by the lack of routine in vitro procedures for their isolation. Only a porcine enteric calicivirus isolate from the United States has been shown to replicate in cell culture (14, 44). Early studies with the NLVs indicated that the likely target cells for virus replication for the human enteric caliciviruses are enterocytes of the small intestine (1, 52, 53). Bovine enteric caliciviruses, like the NLVs also replicate in enterocytes of the small intestine (22). This similarity in tissue tropism between viruses from different host species together with the recent observation that porcine enteric caliciviruses from Japan are related to group 2 NLVs (55) led us to investigate whether the bovine enteric viruses might also be related to the NLVs. The bovine enteric caliciviruses would certainly be useful as a model system for investigating the pathogenesis of enteric calicivirus infection because fresh, healthy bovine small intestine tissue is readily available, whereas surgically removed fresh, undiseased human small intestine tissue is exceedingly difficult to obtain. Thus, the primary purpose of this work was to characterize the complete genome of a bovine enteric calicivirus originally isolated in Jena, Germany, in 1980 (20).

A calicivirus has been isolated from cattle (41), but this virus (Tillamook virus; BCV-Bos1) causes respiratory symptoms. Phylogenetic analysis showed that the Tillamook virus is very closely related to the San Miguel sea lion virus and vesicular exanthema of swine virus and can also infect pigs, causing...
vesicular lesions. However, in contrast, Jena virus (JV) (117/80) was discovered by electron microscopic (EM) examination of diarrheic stools from newborn calves (20, 21).

**Molecular analysis of JV.** EM examination of JV showed a typical NLV morphology, with virions of approximately 30 nm in diameter (21). The virus was passaged 11 times in colostrum-deprived, newborn calves, which were transported to the laboratory soon after birth. Each calf received a 5- to 10-ml oral inoculum of fecal supernatant from the previous calf. Inocula were prepared by centrifugation of fecal samples at 3,000 g for 30 min followed by dilution with 4 volumes of phosphate-buffered saline (PBS). Two hours after receiving the inoculum, calves were given 2 liters of colostrum and then fed milk at 500 ml/kg of body weight twice daily. In all passages, the calves became symptomatic with diarrheal disease and the presence of JV was verified by EM.

For molecular studies, the virus was purified from the first diarrheal sample collected at 12.5 h postinfection from newborn calf 319/92 (10th passage). This sample was diluted with 4 volumes of PBS and centrifuged at 3,000 g for 30 min. The supernatant was mixed with 2 volumes of 1.1,2-trichlorotrifluoroethane, and the organic and aqueous phases were separated by centrifugation at 3,000 g for 30 min. The supernatant was extracted with chloroform, and then a sample of the aqueous phase was purified by sedimenting it through a 35% sucrose cushion at 35,000 rpm for 2 hi na Sorvall TH641 rotor.

The virus was passaged 11 times in colostrum-deprived, newborn calves, which were transported to the laboratory soon after birth. Each calf received a 5- to 10-ml oral inoculum of fecal supernatant from the previous calf. Inocula were prepared by centrifugation of fecal samples at 3,000 g for 30 min followed by dilution with 4 volumes of phosphate-buffered saline (PBS). Two hours after receiving the inoculum, calves were given 2 liters of colostrum and then fed milk at 500 ml/kg of body weight twice daily. In all passages, the calves became symptomatic with diarrheal disease and the presence of JV was verified by EM.

For molecular studies, the virus was purified from the first diarrheal sample collected at 12.5 h postinfection from newborn calf 319/92 (10th passage). This sample was diluted with 4 volumes of PBS and centrifuged at 3,000 g for 30 min. The supernatant was mixed with 2 volumes of 1.1,2-trichlorotrifluoroethane, and the organic and aqueous phases were separated by centrifugation at 3,000 g for 30 min. The supernatant was extracted with chloroform, and then a sample of the aqueous phase was purified by sedimenting it through a 35% sucrose cushion at 35,000 rpm for 2 hi na Sorvall TH641 rotor.

This sample was dialyzed against PBS and used for cDNA synthesis. JV (100 μl) was purified through a sucrose cushion was extracted by the rapid endonucleases HaeIII or RsaI (Promega). The DNA fragments were ligated into Smal-digested pSP73 (Promega), and the recombinant plasmids were transformed into Escherichia coli DH5α. Analysis of the inserts from the recombinants allowed the rapid accumulation of sequence data from which a set of custom oligonucleotide primers were synthesized and used to amplify and directly sequence a series of PCR fragments.

**Sequences upstream of the RNA polymerase region as far as the helicase motif were obtained by reverse transcription-PCR, using primers JV2 (5’-AATAGAATTTACGTTGAAA GTG356-3’) and Helicase 3 (5’-1665GGCCMCCCKGGIWKIG GIAA1665-3’).** Primer Helicase 3 was designed from the amino acid sequence motif GXGXXGKT found in the helicase region of all caliciviruses. PCR conditions were the same as those used to amplify the 3’-terminal 3-kb fragment with primers pair JV1 and 5’ T25VN 3’.

The 5’ end of the JV genome was sequenced by the random PCR method (10) adapted from the procedures of Froussard (15) and Grothues et al. (19). Briefly, ss cDNA was generated by using specific primers and then converted into double-stranded (ds) cDNA using a random primer, LinkerN7 (5’-TAGTACATAGTGATCCACTGCT8-3’), and Klenow

---

![Diagram](http://example.com/diagram.png)

**FIG. 1.** Diagrammatic representation of reading frame usage in JV. The nucleotide coordinates of the translation products are numbered on the open boxes. The repeat motifs at the 5’ genomic terminus and the predicted 5’ terminus of the subgenomic RNA are aligned beneath the genomic map. Shaded boxes indicate the genomic locations of these motifs.
polymerase. The randomly primed viral ds cDNA was then amplified in two successive, nested PCRs using specific primers and a primer based on the linker component of LinkerN7. Reaction products were sequenced directly to enable the design of new oligonucleotides for repeated rounds of random primer extension toward the 5' terminus. This approach enabled sequence data to be collected to within 80 nucleotides of the authentic genomic 5' terminus.

The genomic 5' terminus of JV was defined by homopolymer tailing and PCR, using a commercial kit (5' RACE; Gibco-BRL) in combination with a number of custom oligonucleotide primers. Separate lots of cDNA were then synthesized from the purified viral RNA and a single primer JV3 (5' -406TGTA

Sequence data obtained from clones were used to synthesize specific primers for direct sequencing of both strands of amplified cDNA with an Applied Biosystems model 373A automated sequencer using Taq cycle dideoxy terminator chemistry. Computer analyses of the sequence data were performed using Lasergene software (DNASTAR Inc., Madison, Wis.). Oligonucleotides were synthesized on a Millipore Expedite 8909 automated synthesizer using β-cyanoethylphosphoramidite chemistry.

JV has a genome of 7,338 nucleotides, excluding the poly(A) tail, and a nucleotide composition of A (20.7%), G (26.2%), T (23.4%), and C (29.6%), with an overall G:C content of 55.8%. The nucleotide sequence contains characteristic motifs located at the 5' terminus of the genome and the start of ORF 2 (Fig. 1). Within the first 24 nucleotides at the 5' terminus of the genome, 18 are conserved when compared to the similar motif at the start of ORF 2. At the 5' terminus is a guanosine residue which is followed immediately by the translation termination codon (TGA). The first translation initiation codon at nucleotide position 5 aligns with the predicted start codon for ORF 2; however, the reading frame terminates after only 17 amino acids. The first initiation codon for a large uninterrupted ORF
that fits the features of ORF 1 from other caliciviruses is located at nucleotide 22 and is situated within a favorable context (GATATGGAT) for translation initiation by the ribosome scanning model (32). Thus, the JV 5′ noncoding region is slightly longer than the 5′ noncoding region of feline calicivirus (19 nt) and rabbit hemorrhagic disease virus (10 nt). This is in striking contrast to those of the human enteric viruses Norwalk, Southampton, and Lordsdale, in which there are just four nucleotides in the 5′ noncoding region. In addition, the first seven amino acids of both ORF 1 and ORF 2 are highly conserved in the human enteric caliciviruses (7). This, together with the location of the first in-frame initiator codon in JV at position 22, strongly suggests that the AUG at nucleotide position 11 in both group 1 and group 2 human NLVs is likely to be the authentic initiator codon, because in both cases it is situated in a favorable context for translation initiation.

As expected, JV ORF 1 encodes the 2C-like helicase, 3C-like protease, and 3D-like RNA polymerase motifs characteristic of the caliciviruses (Fig. 2). At 1,680 amino acids, the ORF 1 polyprotein is similar in size to that of the group 2 polyprotein from Lordsdale virus (1,699 amino acids). The polyprotein sequences from group 1 and group 2 NLVs have been compared (10). This analysis showed a similar overall organization, although there was little identity between group 1 and group 2 polyproteins within the first 150 N-terminal amino acids. JV also has a highly divergent amino terminus for the first 100 amino acid residues, but overall it is more closely related to the group 1 viruses. It is notable that the predicted cleavage dipeptide 526OG in both group 1 and 2 human viruses is also present at the N terminus of the 2C-like helicase and is replaced by a 683QA dipeptide in the corresponding C-terminal cleavage site (38). Interestingly, the genomic RNA of JV contains several polyuridylic tracts that encode highly unusual proline-rich regions in the predicted translation product of the N-terminal region, as previously described for Lordsdale virus (10).

The predicted molecular size of the JV capsid (56 kDa) is in close accordance with the estimated size of the capsid protein from partially purified virions of the candidate bovine enteric calicivirus, Newbury agent 2, described in a recent preliminary report (9) and the capsid of a porcine calicivirus from the United States (45). Alignment of the JV ORF 2 translation product with representative examples of group 1 and group 2 NLVs shows that the capsid protein can be divided into the same three discrete regions as previously reported for the NLVs (11, 17, 36). This observation is consistent with the domain organization proposed for the primary sequence of the Norwalk virus capsid protein (46). The region of most significant homology with the NLVs occurs within the N-terminal 274-amino-acid residues of JV (55.1 to 60.5% amino acid identity). This part of the protein has been predicted to form a β-barrel structure comprising the lower shell domain (S) of the capsid dimer. The C-terminal region of the JV capsid (395 to 519 amino acids) is slightly more variable (48.4 to 53.8%). A central hypervariable region predicted to encode the project-
zymes and cloned into pSP73 (Promega). This recombinant plasmid (pJVC1) was resequenced to ensure that it was an authentic copy of the JV consensus for ORF 2 under the control of the T7 promoter. Purified plasmid pJVC1 was used as a template for in vitro protein synthesis, using a T7 RNA polymerase-coupled reticulocyte lysate system (TNT; Promega) in accordance with the manufacturer’s instructions. The reaction mixture (total volume, 25 μl) was incubated at 30°C, and the reaction was stopped after 1 h for immunoprecipitation and analysis of reaction products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Gels were stained and prepared for autoradiography by treatment with 1 M sodium salicylate–50% methanol for 30 min at room temperature. Gels were then dried under vacuum and exposed to Kodak XAR-5 film at −70°C.

Calf 321/93 (11th passage) was infected with a fecal suspension from calf 319/92 (10th passage) as described above. Blood samples for serum analysis were collected from calf 321/93 before infection with JV and then at weekly intervals. Calf 321/93 was reinfected orally with aliquots of the same inoculum at 1, 2, and 4 weeks after the first infection. Translation products from the TNT-coupled transcription-translation reaction of pJVC1 (5 μl) were incubated with 2 μl of undiluted bovine antisera in 600 μl of radioimmune precipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.15 mM NaCl, 0.1% SDS, 0.5% Empigen BB, 0.1 mM phenylmethylsulfonyl fluoride) and incubated at 37°C for 1 h. Immune complexes formed with bovine antisera were captured by using protein G immobilized on Sepharose 4B Fast Flow beads (Sigma, Poole, United Kingdom). The beads were washed three

FIG. 4. Unrooted phylogenetic tree constructed for a region of the 3D RNA-dependent RNA polymerase gene showing the relationship of JV to other caliciviruses. Shaded ellipses have been added to highlight the distinction between the group I and group 2 NLVs. Accession numbers (in parentheses) for caliciviruses are as follows; swine (AB009412); Mexico (U22498); Toronto (L23831); Lordsdale (X86557); Hawaii (U07611); Melksham (X81879); Norwalk (M87661); Southampton (L07418); Desert Shield virus (DSV) (U04469); bovine Jena (AJ011099); Sapporo (S77903); human calicivirus (HuCV) DCC (U67856); Manchester (X86559); Parkville (U73124); HuCV 27 (U67859); HuCV Lon (U67858); rabbit hemorrhagic disease virus (RHDV) (M87473); European brown hare syndrome virus (EBHSV) (Z69620); Pan 1 (U52086); cetacean (U52091); reptile (U52092); San Miguel sea lion virus (SMSV 1) (M87481); feline calicivirus (FCV) Urbana (L40021); FCV F9 (M86379).
times with RIPA buffer and with a final wash in PBS before derivitization in sample-dissociating buffer and separation by SDS-PAGE. pJVCl used as a template in a coupled transcription-translation reaction gave a single product of 55 kDa when analyzed by SDS-PAGE, indicating that in this system only ORF 2 is translated. Preimmune serum obtained from colostrum-deprived, newborn calf c321/93 before experimental infection with JV was used in an immunoprecipitation reaction with labelled JV capsid. This serum showed that antibodies to the JV capsid protein were not present prior to infection. However, postinfection antisera (4 weeks) was able to immuno precipitation the JV capsid protein (Fig. 3). All 11 calves used in the serial passage of JV developed diarrheal symptoms following oral administration of the JV inoculum. The presence of JV in the postinfection diarrheal stool samples of each calf and the seroconversion of calf c321/93 (11th passage) to the JV capsid protein indicates the highly infectious and immunogenic nature of this agent.

**Phylogenetic analysis.** Unrooted phylogenetic trees were constructed for a region of the 3D RNA polymerase for caliciviruses as previously defined (3). Multiple alignments were performed with CLUSTAL X (56), and unrooted trees were generated using the neighbor-joining method (49). Trees were performed with CLUSTAL X (56), and unrooted trees were subjected to a bootstrap analysis (12) using 1,000 data sets and generated using the neighbor-joining method (49). Trees were

**REFERENCES**


