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Reverse Transcription-PCR Assays for Detection of Bovine Enteric Caliciviruses (BEC) and Analysis of the Genetic Relationships among BEC and Human Caliciviruses

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Two genetically distinct bovine enteric caliciviruses (BECs) have been identified: the norovirus (NLV) Jena and Newbury Agent-2 (NA-2) BECs, which are genetically related to human noroviruses, and the Nebraska (NB) BECs, which is related to sapoviruses and lagoviruses but may also represent a new calicivirus genus. The prevalence of these two BEC genotypes in cattle is unknown. Although reverse transcription-PCR (RT-PCR) primers for human NLV recognize NLV-BECs, the genetic relationships between NLV from humans and the NLV-BECs commonly circulating in cattle is undefined. In the present study, veal calf fecal samples were assayed for enteric caliciviruses by using six RT-PCR primer sets designed for the detection of human NLVs or BECs. Caliciviruses genetically related to the NLV-BEC Jena and NA-2 strains or to the recently characterized NB BEC strain were identified in three of four and four of four sampled veal herds, respectively. Extended 3′-terminal genome sequences of two NLV-BECs, designated CV95-OH and CV186-OH, encoding the RNA-dependent RNA polymerase (RdRp; open reading frame 1 [ORF-1]), VP1 (ORF-2), and VP2 (ORF-3) genes were determined. Phylogenetic and sequence identity analyses of each genome region demonstrated these viruses to be most closely related to the NLV-BEC Jena and NA-2 strains. In initial testing, the human P289-P290 (P289/290) primer set was found to be the most sensitive for calicivirus detection. However, its failure to identify all positive fecal pools (as determined by other assays) led us to design two new primer sets, CBECU-F/R and NBU-F/R, for the sensitive and specific detection of NLV-BEC (NLV-BEC Jena and NA-2) and BEC-NB-like viruses, respectively. The RT-PCR assays with the new primers were compared against other primer sets, including P289/290. Composite results of the tests completed by using the new assays identified 72% (54 of 75) of veal calf fecal samples as positive, with 21 of 21 sequenced reaction products specific for the target RdRp gene. The same design strategy used for the new BEC assays may also be applicable to the design of similar assays for the detection of human caliciviruses (HuCVs). Our data support the genetic relationship between NLV-BECs and NLV-HuCVs but with the NLV-BECs comprising two clusters within a third NLV genogroup.

Caliciviruses (family *Caliciviridae*) are nonenveloped viruses, 27 to 35 nm in diameter with single-stranded RNA positive-strand genomes of 7 to 8 kb. Calicivirus genomes encode major (VP1) and minor (VP2) structural capsid proteins and nonstructural proteins that include a helicase/ATPase, a cysteine protease, an RNA-dependent RNA polymerase (RdRp), and a 5′ genome-linked VpG protein (12). Phylogenetically, members of the family *Caliciviridae* are placed in four genera. Enteric caliciviruses, including those infecting humans (HuCV), belong to the genera *Sapovirus* (SLV) and *Norovirus* (NLV), whereas the conventional animal caliciviruses belong to the genera *Vesivirus* and *Lagovirus* (18). Many caliciviruses have a distinctive surface morphology composed of cup-shaped depressions, but those with a less-defined, amorphous, surface structure are designated as small round-structured viruses within the NLV genus (12).

HuCVs are emerging pathogens that are a leading cause of acute epidemic gastroenteritis (8, 15). HuCVs are currently estimated to be responsible for >90% of food- and waterborne viral gastroenteritis in humans of all ages (15). In animals, caliciviruses cause a spectrum of diseases that include gastroenteritis (pigs, calves, dogs, and chickens), vesicular lesions and reproductive failure (pigs and sea lions), rhinitis and conjunctivitis (cats and cattle), a fatal hemorrhagic disease (rabbits), and a stunting syndrome (chickens) (23).

Enteropathogenic bovine enteric caliciviruses (BECs) with small round-structured virus morphology, which are similar to human NLV, have also been identified in cattle. In recent years, two such NLV-BEC strains from Europe, Jena (14) and Newbury Agent-2 (NA-2) (4), were genetically characterized and shown to be similar to human NLVs. Similar virus gene sequences were also detected in pooled fecal samples collected from veal calves in The Netherlands (25). These findings have raised questions about the host range of BECs because it was unclear whether these viruses comprised their own distinct

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TABLE 1. RT-PCR primers evaluated for the detection of caliciviruses in calf fecal samples

Primer pair	Sequence (5′→3′)	Sense	Target virus(es)[b]	Source or reference
NV36	5′-ATA AAA GTT GGC ATG AAC A	+	NLV-GI	Wang et al. (27)
NV35	5′-CTT GTT GGT TTG AGG CCA TAT	−	NLV-GI	Wang et al. (27)
P290	5′-GAT TAC TCC AAG TGG GAC TCC AC	+	NLV, SLV	Jiang et al. (11)
P289	5′-TGA CAA TGT AAT CAT CAC CAT A	−	NLV, SLV	Jiang et al. (11)
JV12	5′-ATA CCA CTA TGA TGC AGA TTA	+	BEC, NLV	Vinjé et al. (26)
JV13	5′-TCA TCA TCA CCA TAG AAA GAG	−	BEC, NLV	Vinjé et al. (26)
J11U	5′-CCA TCA ACC ATT GGA TTT TGA C	+	NLV-GIII	H. Tsunemitsu[a]
J11L	5′-ACC TGG CGG CGA AGC TCA ATT G	−	NLV-GIII	H. Tsunemitsu[a]
J21U	5′-AAC CAT TGG ATA TTA ACC CTG TG	+	NLV-GIII	H. Tsunemitsu
J21L	5′-GAC CAT CTA CCT GCT GCT TCA	−	NLV-GIII	H. Tsunemitsu
BEC-POL5′	5′-TAT GAG CCA GCC TAC CTT GG	+	NLV-GIII	K. O. Chang and L. J. Saif, unpublished data
BEC-POL3′	5′-ACC TGG GAC GTG CAT GGG A	−	NLV-GIII	K. O. Chang and L. J. Saif, unpublished data
NBU-F	5′-TTT CTA ACY TAT GGG GAY GAY G	+	BEC-NB-like	J. R. Smiley and L. J. Saif
NBU-R	5′-GTC ACT CAT GTT TCC TTC TCT AAT	−	BEC-NB-like	J. R. Smiley and L. J. Saif
CBECU-F	5′-AGT TAY TTT TCC TTY TAY GGB GA	+	NLV-GIII	J. R. Smiley and L. J. Saif
CBECU-R	5′-AGT GTC TCT GTC AGT CAT CTT CAT	−	NLV-GIII	J. R. Smiley and L. J. Saif

[a] Primer sequences based on BECs identified in Japanese dairy calves genetically related to BEC/Jena and NA-2 were kindly shared by Hiroshi Tsunemitsu, Japanese National Institute of Health-Scichinohe Research Unit, Shichinohe, Aomori, Japan.
[b] NLV-GIII, "classic" NLV-like BECs; BEC-NB-like, NB-related BECs.

genetic lineage or whether they were, potentially, part of a common pool of viruses circulating between animals and humans.

We have also identified BECs, by electron microscopy and reverse transcription-PCR (RT-PCR) assays, that are genetically and morphologically similar to human NLVs in fecal samples collected from cattle in the United States (J. Smiley et al., Abstr. 81st Annu. Meet. Conf. Res. Workers Anim. Dis., abstr. 78, 2000). Moreover, we recently characterized the complete genome of a BEC morphologically similar to NLV-BECs but genetically distinct. This BEC strain, designated Nebraska (NB), is a prototype virus of a new BEC genogroup or a new calicivirus genus; this virus is most closely related to SLVs and lagoviruses (21). To further ascertain information about the prevalence, genetic heterogeneity, and zoonotic risk of caliciviruses circulating in U.S. cattle, six different RT-PCR primer pairs, based on sequences of either human or bovine NLVs (Table 1), were evaluated for their ability to detect caliciviruses in fecal samples collected from Ohio veal calves. The results of these RT-PCR assays and sequence data determined for selected virus strains, including sequences of two NLV-BEC virus strains, CV95-OH and CV186-OH, were used to design new primer sets. These primer pairs, NBU-F and NBU-R (NBU-F/R) and CBECU-F/R (where "U" stands for universal), are used for the specific and sensitive diagnosis of the NB and "classical" NLV-BEC genotypes, respectively. We present here the first description of the genetic composition of NLV-BECs in U.S. cattle and the prevalence of NLV-BECs and NB-BECs in two Ohio veal calf farms.

MATERIALS AND METHODS

Sampling procedures. Fecal samples were collected from four veal calf herds located on two farms, designated A and B, in the state of Ohio, in June and July 2000 and March and April 2002. In 2000, randomly selected veal calves (n = 49) on farm A (herd size = 268) were sampled at calf-ages of 3 and 6 weeks, respectively. At farm B (herd size = 360), 62 veal calves were randomly sampled on the day of arrival (day 0, ~1 week-of-age). Surviving members of this group were then serially sampled at 4, 14, and 35 days postarrival, at estimated average ages of 1.5, 3, and 6 weeks. Samples collected in 2000 were assigned to fecal pools (five samples/pool) based on stool consistency scores (21) and fecal suspensions, at a final concentration of 10%, were prepared in sterile phosphate-buffered saline (pH 7.4). Additional samples from calves age matched to the test cohorts were collected on some visits to increase total samples and were similarly pooled. In general, the sampling density was sufficient to detect the presence of calicivirus at an estimated prevalence of 5% with a >95% confidence. (22).

In 2002, additional fecal samples were collected from 2-week-old veal calves (1-week after farm placement) on farms A and B. These samples were collected to estimate the prevalence of BEC shedding in individual calves and to evaluate the sensitivity of the NBU-F/R and CBECU-F/R primer sets for their detection. Sample collection and fecal suspension preparation were done as described above except that the fecal samples from farm A (n = 36) and farm B (n = 39) were not pooled but tested individually to evaluate shedding by individual calves.

For all collections, fecal samples were obtained by digital anal massage, with new latex gloves being used for each animal. Expelled feces were collected directly into sterile specimen cups and were then cataloged, scored for fecal consistency, placed on ice, and then aliquoted and stored frozen at −20°C.

RNA extraction. RNA was extracted from 200-μl starting volumes of centrifuged 10% fecal suspensions (3,000 × g, 10 min) by using the Trizol-LS (Gibco-BRL) procedure. Recovered total RNA was resuspended in 40 to 50 μl of RNase free water and stored at −20 or −70°C until used.

Sample analysis. (i) One-step RT-PCR assay. RT-PCR assays with the different primer sets were completed by using a standard one-step RT-PCR. Concentrations of reaction components in the final 50-μl (47 μl plus 3 μl of RNA) reaction mix consisted of 2.5 mM $MgCl_2$, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], and 0.1% Triton X-100), 0.2 pmol of each primer/μl, 5.0 U of avian myeloblastosis virus reverse transcriptase, 2.5 U of *Taq* DNA polymerase, and 10 U of RNasin-RNase inhibitor (Promega Corp., Madison, Wis.). Annealing and RT-PCR cycling parameters used for each primer set are summarized in Table 2. For reactions completed by using the NV35/36 and P289/290 primer sets, a single-stranded RNA internal control reagent was included in the reaction mix to confirm reaction performance and RNA sample quality (20). Amplification products were analyzed by 1.5 or 2% agarose gel electrophoresis and visualized with UV after ethidium bromide staining.

TABLE 2. Primer annealing temperatures and cycling parameters used for RT-PCR assays of BECs[a]

Primer pair	RT		Initial denaturation		Denaturation		Annealing		Extension		No. of cycles	Final extension	
	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (s)	Temp (°C)	Time (min, s)	Temp (°C)	Time (s)		Temp (°C)	Time (min)
NV35/36	42	60	94	4	94	45	50	1	72	45	38	72	10
P290/289	42	60	94	3	94	50	49	1	72	50	38	72	10
JV12/13	42	60	94	3	94	45	39	1, 15	72	40	38	72	10
J11 U/L	42	60	94	4	94	40	50	1	72	30	38	72	10
J21 U/L	42	60	94	4	94	50	54	1	72	40	38	72	10
BEC-POL	42	60	94	4	94	40	45	1	72	50	38	72	10
NBU-F/R	43	45	94	4	94	25	50	0, 45	72	45	38	72	10
CBECU-F/R	43	45	94	4	94	25	50	0, 45	72	45	38	72	10
J11U/CBECU-R	43	60	94	4	94	30	56	0, 40	72	60	38	72	10

[a] These figures represent the most commonly used cycling conditions used for this study. Minor changes from these parameters may have been used.

(ii) Sequence analysis. RT-PCR products were selected from different test reactions and sequenced to verify reaction specificity and to obtain genomic data for phylogenetic analysis. Sequencing was completed by using an ABI-377 automated sequencer (Applied Biosystems, Foster, Calif.) at the Molecular and Cellular Imaging Center of the Ohio Agricultural Research and Development Center. Sequences were determined both directly, from RT-PCR products, or from gel-purified reaction products cloned in pCR2.1(T/A) cloning vectors (Invitrogen Corp., Carlsbad, Calif.). Editing, alignment, and analyses of sequence data were performed by using the Lasergene software package (DNASTAR, Inc., Madison, Wis.).

(iii) Sequencing of NLV-BEC strains CV95-OH and CV186-OH. The nucleotide sequences of the 3′-terminal genomes of two NLV-BEC veal calf strains, CV95-OH and CV186-OH, were determined for comparison with the genetically similar Jena (Germany) and NA-2 (United Kingdom) strains from Europe (4, 14). Initially, a 3′-terminal ~3.2-kbp fragment of each virus was amplified by using the J11U and $VNdT_{25}$ (5′-T25-A/G/C-A/G/C/T) primers (1, 14). Preceding PCR, viral cDNA was synthesized with the SuperScript First-Strand cDNA kit (Gibco-BRL/Life Technologies, Rockville, Md.) and the $VNdT_{25}$ primer. The prepared cDNA was used in a hot-start PCR with *Taq* polymerase (Promega) with thermocycling conditions consisting of denaturation at 94°C for 3 min; 35 cycles of 94°C for 15 s, 50°C for 30 s, and 69°C for 4 min; and final extension of 69°C for 10 min. The reaction products were gel purified by using the Qiaex gel extraction kit (Qiagen, Inc.), cloned into pCR2.1(T/A) cloning vectors (Invitrogen Corp.), and sequenced by using the vector M13F/R priming sites to obtain the 5′- and 3′-terminal sequences of the amplified fragments. Using a primer-walking strategy, new 5′ and 3′ primers were designed from the obtained sequence data and used in successive rounds of RT-PCR amplification, cloning, and sequencing to determine the 3′-terminal CV95-OH and CV186-OH sequences. The 5′-terminal sequence of the RdRp gene of each virus was further extended by sequencing cloned amplicons prepared by using the BEC-POL 5′(F) and P289(R) primers in one-step RT-PCRs. The consensus CV95-OH and CV186-OH sequences are based on results obtained for two or three cloned or directly sequenced RT-PCR amplicons produced in the same or different RT-PCRs for each section of the genome.

NBU-F/R and CBECU-F/R primer sets for the detection of BECs. Sequence alignments of the CV95-OH, CV186-OH, and Jena viruses and the BEC-NB and CV23-OH strains were used to design two new primer sets, CBECU-F/R and NBU-F/R (Table 1), for the specific detection of each BEC genotype. Each primer set consists of a degenerate forward primer centered on the conserved YGDD amino acid motif of the RdRp, and a genotype-specific reverse primer based on unique conserved sequence regions hypothesized to be recognition sites for RNA transcription located at the junction between the structural and nonstructural viral proteins (13) (Fig. 1). The sensitivity of each new primer pair was compared to that of the P289/290 primer set on RNA extracts of individual veal calf fecal samples collected in March and April of 2002. Further comparisons were performed on new RNA extracts of fecal pools prepared for postarrival day 0 and day 4 calf fecal samples collected on veal farm B in 2000. The primer sequences and cycling parameters for these assays are given in Tables 1 and 2.

Phylogenetic analyses. The translated 3′-terminal RdRp sequences (~155 amino acids [aa]) of the Jena, CV95-OH, CV186-OH, NB, and CV23-OH viruses were aligned with the corresponding regions of prototype viruses of the four calicivirus genera by using the CLUSTALW program (24). Phylogenetic trees were constructed from the sequence alignment by using the maximum-likelihood (ProML) and distance (Prodist) methods of analysis of PHYLIP (V3.6a2.1) (6). For each analysis, 100 bootstrap data sets were created by using the SeqBoot utility of PHYLIP and evaluated by using the default settings of each program, including the following specific test options: (i) selection of JTT (Jones-Taylor-Thornton) AA-substitution matrix for Prodist and ProML analyses, (ii) randomization of sequence input order, (iii) S-option (speedier but rougher) of ProML switched off, and (iv) use of the FITCH program (Fitch-Margolish method) to build final distance trees for the distance analysis. The CONSENSE program of PHYLIP was used to construct a single majority-rule tree of all trees calculated for the bootstrapped data sets, and the tree-drawing program Treeview (V1.6.6) (16) was used to prepare the final tree diagrams.

Nucleotide sequence accession numbers. The sequences of the CV95-OH and CV186-OH viruses have been submitted to the EMBL and GenBank databases under accession numbers AF542083 and AF542084, respectively. Additional 3′-terminal RdRp virus sequences include CV500-OH (AY151258), CV514-OH (AY151259), and CV521-OH (AY151257).

RESULTS

To determine the prevalence and genetic composition of enteric caliciviruses in U.S. cattle, a survey study was completed in which fecal samples were serially collected from two commercial Ohio veal calf herds in the spring of 2000. Samples ($n = 358$) collected from these two farms (farms A and B) were distributed into fecal pools ($n = 75$) of five or fewer members, for which RNA extracts were tested by using six different RT-PCR primer sets (Table 1). This testing was completed to determine the presence and timing of BEC infections on these farms and to differentiate the relative sensitivity of each primer set for calicivirus detection. In all herds studied, there was no apparent association between positive calicivirus test status and the prevalence of diarrhea, but the use of pooled samples made this difficult to assess definitely. The proportion of diarrheic calves is reported for informative purposes and is indicative of the wide range of bacterial, viral, protozoan, and other parasitic agents expected in animals of this age group.

Veal farm A (year 2000). At this farm, a total of 99 fecal samples, distributed into 21 pools, were collected during two farm visits separated by 3 weeks. On the first sampling day (calf age ~3 weeks, 2 weeks postarrival), 34% (17 of 49) of individual calf fecal samples were diarrheic, whereas 16% (8 of 50) of samples were diarrheic on the second visit (calf age ~6 weeks). Results of RT-PCR analyses for the prepared fecal pools showed all of them to be negative for products of the expected size, except for a single fecal pool positive with the P289/290 primer set and four pools with slightly smaller than expected

A. Sequence alignment of various caliciviruses with the CBECU-F and CBECU-R primer sequences.

	S	**Y**	**F**	**S**	**F**	**Y**	**G**	**D**	**Consensus CBECU-F AA-sequence**
5′	TCC	CTC	---	---	--C	--T	--T	-- 3′	NLV-GII/Lordsdale (X86557)
5′	TCT	ATG	-AC	--T	--C	--T	--T	-- 3′	NLV-GII/Mexico (U22498)
5′	TCC	-TA	--C	--T	--C	--T	--T	-- 3′	NLV-GII/Hawaii (U07611)
5′	TCA	--T	--C	--T	--C	--T	--T	-- 3′	NLV-GI/Southampton (LO7418)
5′	TCA	--T	--C	--A	--T	--T	--T	-- 3′	NLV-GI/Norwalk (M87661)
5′	TCC	--C	--C	---	--C	--C	--G	-- 3′	BEC/Jena (AJ011099)
5′	---	--C	---	---	--C	--T	--T	-- 3′	BEC/CV95-OH (AF542083)
5′	--C	--T	---	---	--C	--T	--T	-- 3′	BEC/CV186-OH (AF542084)
5′	**AGT**	**TAY**	**TTT**	**TCC**	**TTY**	**TAY**	**GGB**	**GA 3′**	**CBECU-F**
5′	---	---	---	G-G	TCG	-AT	---	G-C 3′	NLV-GII/Lordsdale (X86557)
5′	---	---	---	G-G	TCG	AAT	CG-	G-- 3′	NLV-GII/Mexico (U22498)
5′	---	---	---	G-G	TCG	-AT	---	G-C 3′	NLV-GII/Hawaii (U07611)
5′	---	-T-	--G	G-G	TCT	-AG	---	G-C 3′	NLV-GI/Southampton (LO7418)
5′	---	-T-	---	G-G	TCT	-AG	---	G-T 3′	NLV-GI/Norwalk (M87661)
5′	---	---	---	---	--C	-AG	--T	GTG 3′	BEC/Jena (AJ011099)
5′	---	---	---	---	---	---	---	--- 3′	BEC/CV95-OH (AF542083)
5′	---	---	---	---	---	---	---	--- 3′	BEC/CV186-OH (AF542084)
5′	**ATG**	**AAG**	**ATG**	**ACT**	**GAC**	**AGA**	**GAC**	**ACT 3′**	**CBECU-R (complementary sequence)**

B. Sequence alignment of various caliciviruses with the NBU-F and NBU-R primer sequences.

	F	**L**	**T**	**Y**	**G**	**D**	**D**	**C**	**Consensus NBU-F Translated AA-Sequence**
5′	**TTT**	**CTA**	**ACY**	**TAT**	**GGG**	**GAY**	**GAY**	**G**	**3′ NBU-F**
5′	--C	--G	--C	---	---	--C	--T	-	3′ BEC/NB (AY082891)
5′	---	---	--T	---	---	--T	--T	-	3′ CV23-OH (AY082890)
5′	--C	TC-	TTT	---	--T	--T	--T	-	3′ NLV/Norwalk (M87661)
5′	--C	TCT	TTC	---	--T	--T	--T	-	3′ NLV/Hawaii (U07611)
5′	---	T-C	TTC	---	--T	--T	--T	-	3′ BEC/CV186-OH (AF542084)
5′	--C	TC-	TTC	--C	---	--T	--T	-	3′ BEC/Jena (AJ011099)
5′	A--	TAC	--C	--C	---	--T	--T	T	3′ PEC/Cowden (AF182760)
5′	G--	-AC	--G	---	--T	--T	--T	T	3′ SLV/Sapporro/82 (U65427)
5′	G--	-AC	--A	---	--T	--T	--T	T	3′ SLV/London/92 (U95645)

	I	**R**	**E**	**G**	**N**	**M**	**S**	**D**	
5′	**ATT**	**AGA**	**GAA**	**GGA**	**AAC**	**ATG**	**AGT**	**GAC 3′**	**NBU-R (complementary sequence)**
5′	---	---	---	---	---	---	---	--- 3′	BEC/NB (AY082891)
5′	---	---	---	---	---	---	---	--- 3′	CV23-OH (AY082890)
5′	TGC	CCG	A-T	TCG	T-A	---	-TG	ATG 3′	NLV/Norwalk (M87661)
5′	C-C	CC-	-TT	TTG	TGA	---	-AG	ATG 3′	NLV/Hawaii (U07611)
5′	T-C	CCG	ATT	TTG	T-A	---	-AG	ATG 3′	BEC/CV186-OH (AF542084)
5′	TCC	CCG	ATT	TTG	T-A	---	-AG	ATG 3′	BEC/Jena (AJ011099)
5′	CCA	GA-	-TG	TTC	GTG	---	GAG	-CG 3′	PEC/Cowden (AF182760)
5′	-AA	-TA	-TG	TTT	G-G	---	GAG	-G- 3′	SLV/Sapporro/82 (U65427)
	No sequence available								SLV/London/92 (U95645)

FIG. 1. Sequence alignments of selected human-NLV and NLV-BEC with BEC-universal primers. (A) Sequence identity of selected human NLVs and NLV-BECs with the CBECU-F and CBECU-R primers. Virus bases sharing identity at degenerate positions of the CBECU-F primer are indicated by underlining. Note that the sequence given for the CBECU-R (reverse primer) is the complementary sequence. (B) Comparison of the sequence identity of different SLV, NLV, and BECs with the NBU-F/R primer set. Underlined virus bases found at degenerate locations of the NBU-F primer indicate positive sequence identities. Note that the sequence given for the NBU-R (reverse primer) is the complementary sequence.

products with the JV12/13 primer set. Sequences of the JV12/13 RT-PCR products showed each to be nearly identical in nature (>99% identity) but nonspecific for calicivirus genes, as determined by using a BLAST search of the GenBank protein database, which identified the closest similarity of the sequences with a "phage-related protein." A similar analysis of the single P289/290-positive fecal pool showed it to share closest similarity with the RdRp of RHDV and SLVs. Subsequent comparison of the P289/290 product sequence with the sequence of the BEC/NB strain showed that the two viruses share 87% nucleotide identity and 99% amino acid identity in a 277-bp virus-specific RdRp sequence. Fecal sample CV23-OH, as determined by trace-back testing, was the single P289/290-positive sample of the original test pool. Immunoelectron microscopy further confirmed the presence of calicivirus particles in the CV23-OH sample (19).

Veal farm B (year 2000). A total of 259 specimens, distributed into 54 pools, were collected during four visits to this farm on 0, 4, 14, and 35 days after calf arrival. At 35 days postarrival, the last day of collection, 4 of 62 calves randomly selected on day 0 for serial sampling had died. Random animals were collected to replace calves that died, while additional samples

TABLE 3. Summary of RT-PCR test results for fecal pools for veal farm B (year 2000)

Days postarrival	No. with diarrhea[a]/total (%)	Fecal pools positive with different RT-PCR primer pairs[b] (no. positive/no. of pools [%])					
		NV35/36	P289/290	JV12/13	J11U/L	J21U/L	BEC-POL
0	9/62 (15)	0/13	5/13	1/13	6/13	3/13	3/13
4	22/62 (35)	0/13	13/13	4/13[c]	5/13	1/13	2/13
14	11/73 (15)	1/15[d]	3/15	0/15	NT[f]	NT	0/15
35	5/63 (8)	0/13	1/13	0/13	NT	NT	0/13
Total	47/260 (18)	1/54 (2)[d]	22/54 (41)	5/54 (9)[c]	11/26 (42)[c]	4/26 (15)[e]	5/54 (9)

[a] Based on fecal scoring system of 0 to 4, with scores of 0 to ≤ 2 being normal and scores of ≥2 being considered diarrheic for individual calf fecal samples.
[b] Primer sequences and reference sources summarized in Table 1.
[c] Sequence analysis of RT-PCR products of pools CVE23-OH and CVE38-OH showed these products to be nonspecific for caliciviruses (related to murine hepatitis virus); other JV12/13 positive fecal pools were not sequenced.
[d] Sequence analysis showed this product to be nonspecific.
[e] Based on samples collected during farm visits on days 0 and 4 postarrival.
[f] NT, not tested.

were also collected on some visits to increase herd sampling density.

The results of RT-PCR tests for the prepared fecal pools (Table 3) showed the P289/290 and J11U/L primers to be the most sensitive of the six sets examined for the detection of BECs. On the day of arrival (day 0) 60% (8 of 13) of fecal pools were RT-PCR positive with one or more primer sets, whereas 14.5% (9 of 62) of individual fecal samples were scored as diarrheic. By day 4 postarrival 100% (13 of 13) of fecal pools were RT-PCR positive, whereas 35% (22 of 62) of collected fecal samples were diarrheic. The number of fecal pools testing positive and the prevalence of diarrhea were reduced on days 14 and 35 postarrival (Table 3).

Sequences of RT-PCR products of fecal pools or individual calf fecal samples, examined for trace-back testing, were completed for P289/290 products of pools CVE-19, -20, and -23 and for individual calf samples CV95-OH (pool CVE-20), CV110-OH (pool CVE-23), CV163-OH (pool CVE-38), and CV186-OH (pool CVE-34). After removal of primer sequences, the 274-bp virus-specific RdRp sequences were aligned together with the corresponding sequences of the HuCV Norwalk, NLV-BEC Jena, NA-2, BEC CV23-OH, and NB viruses, the results of which showed the highest similarity (82 to 100% nucleotide and 92 to 100% amino acid identity) between the Ohio NLV-BECs, NA-2, and Jena viruses (Fig. 2), with the Ohio NLV-BEC and NA-2 viruses being the most closely related. Furthermore, all NLV-BEC isolates shared similar, but lower, nucleotide (73 to 75%) and amino acid (71 to 73%) identities with Norwalk virus. Sequences of the CV23-OH and NB virus shared low nucleotide (27 to 31%) and amino acid (25 to 28%) identities with both NLV-BECs and Norwalk virus (Fig. 2). Sequences determined for JV12/13 RT-PCR products of fecal pools CVE-23 and CVE-38 were both identical but were of a nonspecific nature. A BLAST search of GenBank identified portions of these sequences to have similarity to the murine hepatitis coronavirus.

Sequence of CV95-OH and CV186-OH NLV-BEC strains. Nucleotide sequences of 3,629 (CV95-OH) and 3,633 (CV186-OH) bases encoding the RdRp (open reading frame 1 [ORF-1]), VP1 major capsid (ORF-2), and VP2 minor capsid

CVE-19	CV95-OH	CVE-20	CV110-OH	CVE-23	CV163-OH	CV186-OH	NA2[a]	Jena[a]	Norwalk[a]	NB	CV23-OH	VIRUS
Nucleotide Identities (%)												
	93.8	93.8	92.3	92.3	99.6	92.7	83.2	75.2	53.3	28.8	29.6	CVE-19
100		100.0	95.6	95.6	94.2	94.5	83.2	74.5	52.9	28.1	29.2	CV95-OH
100	100		95.6	95.6	94.2	94.5	83.2	74.5	52.9	28.1	29.2	CVE-20
100	100	100		100.0	92.7	93.1	82.8	75.2	54.7	28.5	29.9	CV110-OH
100	100	100	100		92.7	93.1	82.8	75.2	54.7	28.5	29.9	CVE-23
100	100	100	100	100		93.1	82.8	75.5	53.3	28.8	29.6	CV163
97.8	97.8	97.8	97.8	97.8	97.8		81.8	75.2	51.5	28.8	31.4	CV186-OH
98.9	98.9	98.9	98.9	98.9	98.9	96.7		72.6	53.3	29.2	29.2	NA-2
92.3	92.3	92.3	92.3	92.3	92.3	93.4	92.3		55.5	30.3	29.2	JENA
72.5	72.5	72.5	72.5	72.5	72.5	71.4	72.5	72.5		27.4	26.6	NORWALK
27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	25.3	26.4		86.6	NB
27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	25.3	26.4	98.9		CV23-OH
Amino Acid Identities (%)												

FIG. 2. Nucleotide (upper) and amino acid (lower) identities of various BECs and human Norwalk virus for a 274-bp sequence segment of the RdRp gene. The GenBank accession numbers for NA-2, Jena, and human Norwalk virus are given in Fig. 5.

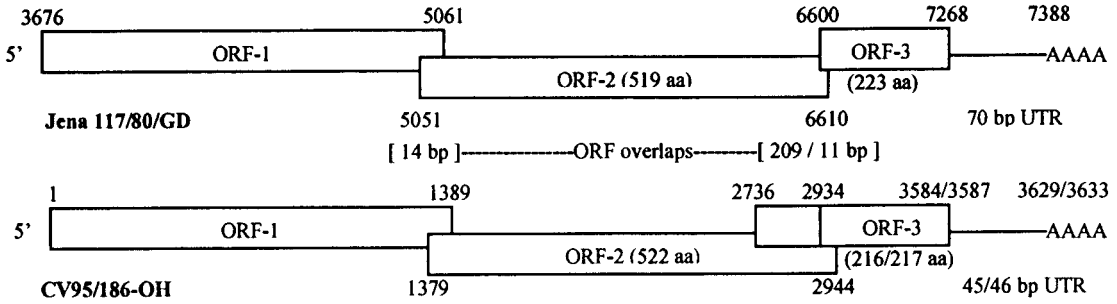


FIG. 3. Diagrammatic representation of the predicted ORFs and overlap regions (including stop codons) of the Jena 117/80/GD and CV95-OH and CV186-OH BECs. Sequence positions indicated for Jena virus correspond to actual positions in the known complete genome sequence (GenBank accession no. AJ011099). The potential extended ORF-3 of the CV95-OH (nt 2736 to 3584, 282 aa) and CV186-OH (nt 2736 to 3587, 283 aa) viruses are shown, along with short ORF-3 gene sequences with potential initiation codons in positions analogous to those of the Jena virus.

(ORF-3) proteins and 3′ untranslated regions (UTRs) were determined (Fig. 3). ORF analyses of the Ohio and Jena virus strains showed them to have identical 14-bp overlapping regions between the ORF-1/2 start and stop codons (Fig. 3) (14). Between ORF-2 and ORF-3 a potential 209-bp overlap region (CV95/186-OH nucleotides [nt] 2736 to 2944) was observed in both Ohio BEC strains, predicting potential ORF-3 lengths of 849 and 852 bases for the CV95-OH and CV186-OH viruses, respectively. However, closer inspection of this overlap region showed potential initiation codons parallel in position to those predicted for Jena virus in the Ohio BECs, giving alternate ORF-3 coding regions of 651 (nt 2934 to 3584) and 654 (nt 2934 to 3587) bases for the CV95-OH and CV186-OH viruses, respectively (14).

Calculated nucleotide and amino acid sequence identities of the NLV-BEC Ohio, Jena, human Norwalk, and BEC NA-2 (only partial sequence data available GenBank) viruses showed the CV95-OH and CV186-OH strains to be genetically most closely related to the NA-2 strain (ORF-1, 86% nucleotide and 97% amino acid identity) (4). Identities of CV95/186-OH with Jena virus were slightly lower in ORF-2 and -3 compared to ORF-1, whereas all of the BEC strains shared quantitatively similar, but lowest, identities with human Norwalk virus (Table 4). Predicted protein sequence differences between the Ohio and Jena viruses included the insertion of 3 aa in the Ohio viruses at positions corresponding to residues 292 and 427 to 428 in the VP1 capsid protein (522 aa), the deletion of 5 aa (CV186-OH; Jena ORF-3 positions 124 and 132 to 136) or 6 aa (CV95-OH; Jena ORF-3 positions 124, 132 to 136, and 192) in VP2 (assuming shorter ORF-3) and abbreviated 3′ UTRs of 45 and 46 nt in the CV95-OH and CV186-OH viruses, respectively, compared to 70 bases in Jena virus.

Alignment of the translated ORF-2 major capsid sequences of the Ohio, Jena, and Norwalk viruses showed that they have similar organizations of structural domains as defined for the Norwalk virus capsid (17). Consistent with other studies of NLVs, the 5′-amino-terminal of the Ohio BEC capsid proteins, encoding the shell (S) domain, was the most conserved ORF-2 region, whereas the hypervariable (P2) subdomain of the protruding (P) domain was the least conserved (Table 5) (7, 17). Similar comparison of the translated ORF-3 (VP2) proteins showed 94% identity between the CV95-OH and CV186-OH viruses, and 63% and 34 to 36% identity with the Jena and Human Norwalk virus, respectively. As reported for other caliciviruses, the VP2 proteins of the Ohio BECs have calculated isoelectric values predicting them to be basic (pH) in nature.

Veal farm A (year 2002). Samples were collected from Veal farm A in March 2002 at 1-week postarrival of calves on the farm. Diarrhea was recorded for 30% (11 of 36) of collected samples. RT-PCR assays on extracted RNA were completed by using the NBU-F/R, CBECU-F/R, J11U/CBECU-R, and P289/290 primer sets. The CBECU-R primer was substituted in place of the J11L primer after tests that indicated it enhanced assay sensitivity while amplifying a larger portion of the RdRp genomic region.

Composite results of reactions completed with the NBU-F/R and CBECU-F/R primer sets showed a remarkable 70% (25 of

TABLE 4. Percent amino acid and nucleotide identities in ORF-1, ORF-2, and ORF-3 of NLV-BECs and human Norwalk virus

Virus	% Nucleotide and amino acid identity[b]											
	ORF-1				ORF-2				ORF-3[a]			
	CV95	CV186	Jena	NV	CV95	CV186	Jena	NV	CV95	CV186	Jena	NV
CV95-OH		97	77	63		93	63	43		92	56	33
CV186-OH	99		77	64	97		62	42	94		54	32
Jena	90	90		65	68	68		43	63	63		33
Norwalk	74	73	73		45	44	45		36	35	34	

[a] ORF-1, sequence identities were based on 1,386 nucleotide sequences of 3′-terminal RdRp gene; ORF-2, sequence identities for complete ORF-2 (VP1) capsid sequence; ORF-3, sequence identities for complete Norwalk virus (NV), Jena, and short (see text) CV95-OH (nt 2934 to 3584) and CV186-OH (nt 2934 to 3587) ORF-3 sequences.

[b] Nucleotide and amino acid identities are in the upper right and lower left quadrants, respectively, of each section of the table.

TABLE 5. Sequence identities (amino acid) between BEC/CV95-OH ORF-2 (VP1) structural domain coding regions and human Norwalk and NLV-BECs

Virus	S domain (1–225 aa)	Subdomain region of P domain[a]		
		P1-A (226–278 aa)	P2 (279–405 aa)	P1-B (406–520 aa)
BEC/CV186-OH	99.5	98.1	94.0	96.6
BEC/Jena 117/80/GD	87.2	56.6	41.4	66.4
HuCV-Norwalk	59.2	54.7	18.7	44.0

[a] As defined previously (17).

36) of sampled calves shedding caliciviruses (Table 6). Of the 25 positive samples, 20 were found to be positive with the CBECU-F/R primer pair, whereas 10 were positive in tests run with the NBU-F/R primer set. Thus, five samples were found to be positive with both primer pairs, suggesting that these animals were dually infected with both BEC genotypes. Comparison of test results for individual primer sets showed the CBECU-F/R primer pair to have the highest sensitivity for the detection of classical NLV-BECs, with 19 of 36 (53%) of samples testing positive. The J11U/CBECU-R pair was nearly as sensitive (18 of 36, 50%), whereas the P289/290 primer set detected 14 of 36 (39%) samples as positive. There were no instances in which a sample was determined as positive with only the P289/290 HuCV primer set.

Sequences of 12 different RT-PCR products amplified with the J11U/CBECU-R, P289/290, CBECU-F/R, or NBU-F/R primer set from 11 different fecal samples (CV497/499/500/501/505/506/509/510/514/521/523-OH) were all specific for the target RdRp gene. Phylogenetically, 9 of 10 of these products, amplified by using primers targeting NLV-BECs, were most closely related to NLV-BEC NA-2 (data not shown). However, one product, CV521-OH, was most closely related to the Jena strain sharing 82% nucleotide and 96% amino acid identity in the 480-bp virus-specific RdRp gene sequence amplified with the CBECU-F/R primer set. This compares with 76% nucleotide and 88% amino acid identity in the same sequence region between CV521-OH and the NA-2-like CV95-OH and CV186-OH viruses. By a similar comparison, two different NBU-F/R products were found to be most closely related to the CV23-OH strain.

Reanalysis of day 0 and 4 postarrival veal farm B (year 2000) fecal pools. An excellent correlation was found between test results of the CBECU/NBU-F/R assays and those of the multiple primer test panel on the retested veal farm B year 2000 pools. Composite results for each assay group were the finding of 20 of 25 pools positive (Table 6) but included single pools determined to be positive by using only the CBECU-F/R or J21U/L assays, respectively. A total of six day-4-postarrival fecal pools tested positive with both the NBU-F/R and the CBEC UFP/URP primer sets, whereas no NBU-F/R positive day 0 pools were identified.

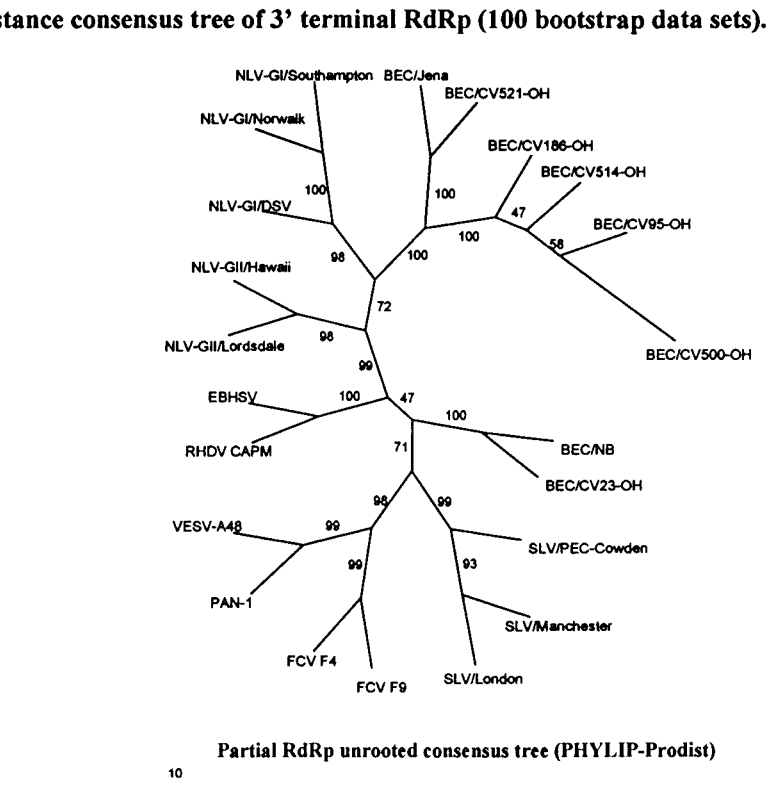
Veal farm B (year 2002). Samples collected from veal farm B in April 2002 were collected from calves averaging 2 weeks of age on day 6 or 7 postarrival at the farm. Feces were scored as diarrheic for 28% (11 of 39) of calves sampled. RT-PCR test results on extracted RNA were completed with the NBU-F/R, CBECU-F/R, J11U/CBECU-R, and P289/290 HuCV primer sets. Composite results of all reactions indicated 74% (29 of 39) of sampled calves to be shedding caliciviruses (Table 6). Of 30 positive samples, 25 and 12 samples were found to be positive with the CBECU-F/R and NBU-F/R assays, respectively. Seven samples were found to be positive with both assays, suggesting that these animals had mixed-genotype infections. Comparison of test results by primer set showed the CBECU-F/R pair to have the highest sensitivity for the detection of NLV-BECs (Jena and NA-2-like) being positive for 62% (24 of 39) of the samples. The J11U/CBECU-R and P289/290 assays detected 15 of 39 (38%) and 19 of 39 (49%) of samples determined as positive, respectively. One sample was positive with only the P289/290 primer set, with a faint gel band present. Sequence and phylogenetic analysis of six partial RdRp products (J11U/CBECU-R or CBECU-F/R) amplified from samples CV-533/540/541/545/555/557-OH showed each virus to be most closely related to NA-2-like NLV-BECs (94 to 95% nucleotide and 99 to 100% amino acid identities with CV-95/186-OH). Similar comparisons of three NBU-F/R assay products of samples CV-526/537-OH and CV 548-OH were all most closely related to the CV23-OH strain.

Phylogenetic analysis. Examination of distance and maximum-likelihood consensus trees constructed for the 3′-terminal RdRp gene (~155 aa) showed the expected branch placement of the prototype caliciviruses as reported in other phylogenetic analyses (2, 3) (Fig. 4). In the distance tree, the NB and CV23-OH viruses were placed on a separate branch rooted between the lagoviruses and SLVs. However, the low 47% branchpoint confidence value indicates variation in this branch designation, which mirrors results of previous analyses for these viruses in other genomic regions (21). All of the compared NLV-BEC strains were placed on a distinct branch rooted with the human NLV-GI viruses, forming two distinct clusters comprised of the Jena and CV521-OH (cluster I) vi-

TABLE 6. Summary of individual calf fecal RT-PCR assay results for veal farms A and B, year 2002, and retested farm B, year 2000, fecal pools

Farm (yr)	Calf fecal samples positive by using different RT-PCR assays[a] (no. positive/total no. of samples [%])				
	P289/290	J11U/CBECU-R	CBECU-F/R	NBU-F/R	Combined NB/CBECU-F/R
A (2002)	14/36 (39%)	18/36 (50)	19/36 (53)	10/36 (28)	25/36 (70)
B (2002)	19/39 (49%)	15/39 (38)	24/39 (62)	12/39 (31)	29/39 (74)
B (2000)[b]	17/25 (68%)	NT	20/25 (80)	6/25 (24)	20/25 (80)[c]

[a] Primer sequences and references provided in Table 1. NT, not tested.
[b] For fecal pools prepared for day 0 and 4 postarrival calves.
[c] No day 0 pools were found to be positive with the NBU-F/R assay.



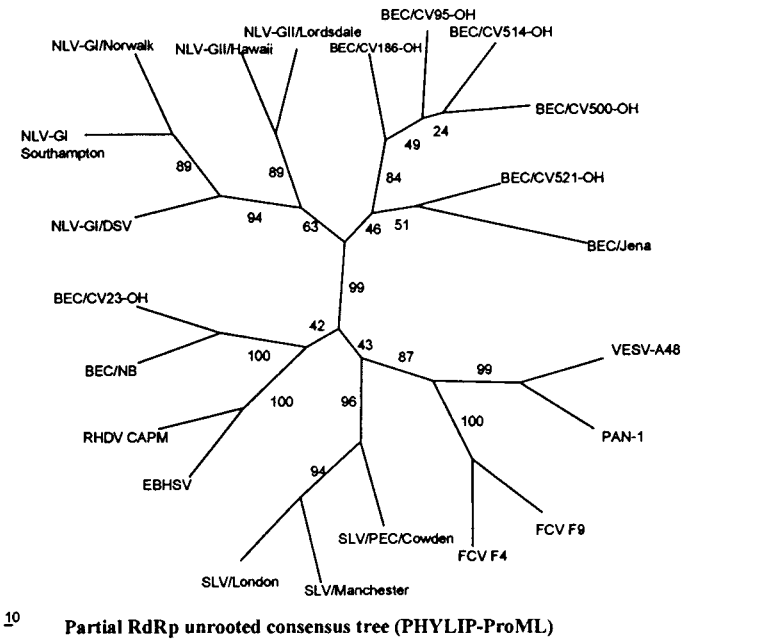


FIG. 4. Distance (A) and maximum-likelihood (B) consensus trees of the 3′-terminal (155-aa) RdRp gene of prototype viruses representing all four calicivirus genera. Trees were prepared from the sequence alignment by using the PHYLIP and Treeview programs and are based on 100 bootstrapped data sets. GenBank accession numbers for most viruses can be found in Fig. 5, while accession numbers of the CV95/186/500/514/521-OH viruses are noted in Materials and Methods.

ruses and the NA-2-like CV95-OH, CV186-OH, CV500-OH, and CV514-OH (cluster II) viruses, respectively (Fig. 4).

In the consensus tree prepared by using the more rigorous maximum-likelihood method, BEC/NB and CV23-OH viruses were grouped as a distinct genetic cluster of the lagoviruses, whereas the NLV-BECs were placed on a distinct branch rooted with the human NLVs (low branchpoint confidence value). As in the distance analysis, the classical NLV-BECs were placed in two genetic clusters with identical group members. However, branchpoint confidence values were lower than those found in the distance tree.

DISCUSSION

After the publication of reports on the genetic characterizations of the BEC/Jena 117/80/DE (4) and Newbury-2/76/UK viruses (14), we initiated work to characterize morphologically similar BEC strains of US origin. These efforts led to the characterization of the BEC-NB strain, a prototype virus of a new BEC genogroup or possibly calicivirus genus (21), and the partial characterization (RdRp) of a second BEC designated "Ohio" strain, phylogenetically related to the BEC-NLV NA-2 virus (K. O. Chang and L. J. Saif, unpublished data). These findings, along with RT-PCR, sequence, and phylogenetic analyses of the year 2000 veal calf data characterizing the NB-like CV23-OH strain, and the NA-2-like CV95-OH, CV110-OH, CV163-OH, and CV186-OH BEC strains, shown to be genetically related to European (4, 14, 25) and Japanese (H. Tsunemitsu, Abstr. 81st Annu. Meet. Conf. Res. Workers Anim. Dis, abstr. 49P, 2000) NLV-BEC field strains, led us to believe that these virus strains were most likely species restricted to cattle. The results of the extended ORF-1, -2, and -3 sequence identity comparisons of the CV95-OH and CV186-OH viruses with characterized bovine and human NLVs further support this conclusion, as do phylogenetic comparisons of the translated partial ORF-1 (RdRp) (Fig. 4) and complete ORF-2 and -3 sequences (not shown) of the NLV-BEC veal calf strains, which demonstrate them to form a third genogroup within the NLV genera that is distinct from characterized human NLVs. Importantly, viruses related to these NLV-BECs have never been reported from human diarrhea outbreak investigations with several of these same human NLV primer sets.

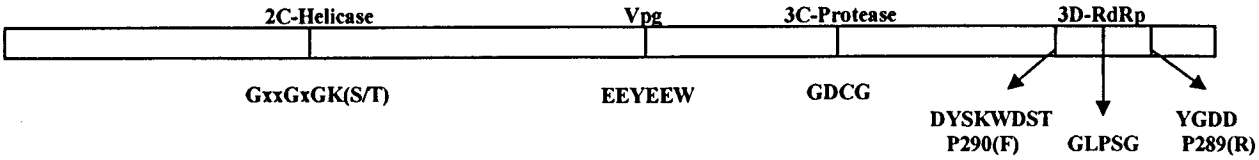
Our results finding a high proportion of nonspecific products with the JV12/13 primer set is not entirely unexpected considering the low primer annealing temperature (39°C) used for the assay. Similarly, in the study by van der Poel et al. with this same primer set, 48% of "target" products were nonspecific in nature (25). Comparison of the JV12/13 primers and the CV95-OH and CV186-OH virus sequences indicate that multiple (JV12) and a 3′-terminal (JV13) base-pair mismatches between the primers and their virus target sequences is the likely cause of their low sensitivity for detecting Ohio NLV-BEC strains.

Our results demonstrate the high sensitivity of the P289/290 assay for the detection of NLV-BECs and partial sensitivity for NB-BECs. Examination of the P289/290 genomic target sequences of prototype viruses representing all four calicivirus genera shows a high degree of sequence conservation between the prototype viruses and the 3′ primer sequence ends, thus making it theoretically possible for viruses of all four genera to be detected with this primer set (Fig. 5). Indeed, we have previously used the P289/290 primer set to amplify SLV-like porcine enteric caliciviruses (9), NB-like BECs (21), and a mink vesivirus and mink SLV enteric calicivirus (10). It is probable that broad calicivirus reactivity may be an inherent characteristic of any primer set designed to target highly conserved genomic regions shared by caliciviruses of all genera, such as the GLPSG and YGDD sequence motifs in the RdRp gene. While such a primer design strategy can enhance assay sensitivity, inattention to cross-genera reactivity can impose limitations on the use of such nonspecific assays for the testing of food, water, or environmental samples unless used in conjunction with other confirmatory assays.

In recognition of this problem, the CBECU-F/R and NBU-F/R primer sets were designed to target sequences centered on the conserved RdRp gene YGDD motif (forward primer) and the BEC genotype-specific sequence element, speculated to be a recognition-promoter site for the transcription of a subgenomic RNA species, located at the junction between the structural and nonstructural proteins (Fig. 1). The primary means of test specificity for each assay is based on the reverse primers, which capitalize on differences between the unique promoter sequences characteristic of each virus genera. The results obtained for the 2002 veal calf samples illustrate the apparent high sensitivity of each assay, while our sequence results show excellent specificity, as indicated by the absence of any nonspecific amplification products. This specificity was also demonstrated in tests with seven different HuCV strains (both NLV-GI/GII and SLV), which showed no cross-genus reactivity between the BEC-universal primer pairs and the HuCV strains (data not shown). Based on sequence alignments of prototype caliciviruses of all four genera, it is probable that this same primer design strategy could be used to create sensitive and specific assays for the diagnosis of other calicivirus genus/genogroups. Also, the present NBU-F/R and CBECU-F/R RT-PCR assays could be used to screen human fecal samples to further ascertain the role of NLV-BECs in human diarrhea outbreaks, with a particular emphasis on human diarrhea cases from developing countries where cattle-human contact is more common.

Data from these studies also demonstrate a high prevalence of calicivirus shedding in young veal calves (72%, 54 of 75 individual calf 2002 samples). This finding is similar, although based on a different sampling strategy, to the findings reported by van der Poel (25) that identified 44% of pooled veal farm herd specimens to be positive for NA-2-like BECs by using a combination RT-PCR (JV12/13 primers)-Southern hybridization assay. Of note, the average calf age in the later study was 12 weeks, whereas calves sampled in our study did not exceed 7 weeks of age. Interestingly, we found low or no virus shedding in pooled samples collected from older calves (3 to 6 weeks of age) in 2000, whereas a high prevalence of virus shedding was detected in younger calves (<3 weeks of age). However, conclusions regarding differences in susceptibility related to calf age cannot be drawn from these two studies, since the ages of calves contributing to BEC-positive fecal pools and the timing of the sampling in relation to introduction of new calves to the herds was not reported by van der Poel et al. (25). The BEC shedding detectable by RT-PCR waned

A. Conserved calicivirus ORF-1 non-structural protein sequence motifs



B. P290 (F)/P289(R) Primer identity with selected human NLV/SLV and BECs.

```
   D   Y   S   K   W   D   S   T Consensus Calicivirus AA sequence
5'--- --T A-A GCA --- --- --A -- 3'  NLV/Norwalk      (M87661)
5'--- --T --T CGA --- --- --A -- 3'  NLV/Hawaii       (U07611)
5'--- --- --A GC- --- --- AG- -- 3'  BEC/CV186-OH     (AF542084)
5'--C --T --A GCC --- --- AG- -- 3'  BEC/Jena         (AJ011099)
5'--C --- AG- --- --- --T --- -- 3'  PEC/Cowden       (AF182760)
5'--- --T --- --A --- --T --- -- 3'  SLV/Sapporro/82  (U65427)
5'--C --- AG- --- --- --T --- -- 3'  SLV/London/92    (U95645)
5'--C --T --- --- --- --- --- -- 3'  BEC/NB           (AY082891)
5'GAT TAC TCC AAG TGG GAC TCC AC 3'  P290(F) primer sequence

5'TAT GGT GAT GAT TAC ATT GTC    3'  P289(R) complementary sequence
5'--- --- --- --- G-G --- --G    3'  NLV/Norwalk      (M87661)
5'--- --- --- --- G-A --- --G    3'  NLV/Hawaii       (U07611)
5'--- --- --- --- G-A --A --G    3'  BEC/CV186-OH     (Smiley,2002)
5'--C --G --- --- G-G --A ---    3'  BEC/Jena         (AJ011099)
5'--C --G --- --- -GT G-G TAT    3'  PEC/Cowden       (AF182760)
5'--- --- --- --- -G- --G TA-    3'  SLV/Sapporro/82  (U65427)
5'--- --- --- --- -G- --A TA-    3'  SLV/London/92    (U95645)
5'--- --G --C --- GGG C-- TA-    3'  BEC/NB           (AY082891)
   Y   G   D   D   G   I   Y     Consensus Calicivirus AA sequence
```

C. Amino acid sequences of caliciviruses in genome regions targeted by the P290/289 primers.

Virus	DYSKWDST	YGDDGIY	Product	Genbank
Manchester	--------.....	.....YGDDCM-	331	X86560
PEC/Cowden	--------.....	.....----CV-	331	AF182760
Jena	---A----.....	.....----E-V	319	AJ011099
NV8Fiia	--TA----.....	.....----E-V	319	M87661
Southampton	--TA----.....	.....----E-V	319	L07418
Hawaii	---R----.....	.....----E-V	319	U07611
Lordsdale	---R----.....	.....----E-V	319	X86557
VESV-A48	--------.....	.....-----V-	331	AF181082
SMSV-1	--------.....	.....-----V-	331	AF181081
PAN-1	--------.....	.....-----V-	331	AF019736
Walrus CV	--------.....	.....-----V-	331	AF321298
FCV-F4	-F-N----.....	.....-----V-	331	D31836
FCV-F9	--------.....	.....-----V-	331	M86739
EBHSV	--------.....	.....-----V-	328	Z69620
RHDV-GDR	--------.....	.....-----V-	328	M67473
RHDV-IA	--------.....	.....-----V-	328	AF258618
BEC-NB	--------.....	.....-----L-	322	AY082891

FIG. 5. Comparison of P289/290 RT-PCR assay products for different caliciviruses. (A) Conserved amino acid sequence motifs found in the nonstructural proteins of calicivirus ORF-1. The position of the DYSKWDST and YGDD motifs found in the RdRp are shown with their corresponding P289/290 target primer underlying. (B) Comparison of the nucleotide identities of various NLV, SLV, and BECs with sequences of the P290(F) and P289(R) primers. Note that the sequences given for the P289(R) primer is the complementary sequence. (C) Comparison of corresponding amino acid sequence identities of different caliciviruses with the P290 (DYSKWDST) and P289 (YGDDGIY) translated primer sequences. The expected product size of amplified products and the GenBank accession numbers of the different caliciviruses are also given in the right-hand columns.

rapidly after introduction of calves into the all-in–all-out management systems used on the farms in our study. This finding is consistent with the highly contagious nature of enteric caliciviruses (12), underlining the importance of sampling timing. Also, data from our experimental studies of gnotobiotic calves indicate that calves 3 to 6 weeks old, without specific immunity, are at least as susceptible as calves less than 1 week old (M. Tråvén and L. J. Saif, unpublished). Interestingly, pooled herd samples of 43 commercial Dutch dairies were all RT-PCR negative for BECs (25), suggesting that infections in adult cattle occur more sporadically. Information is lacking on the BEC infection patterns in cattle farm operations other than

veal farms, the occurrence of reinfections in the face of declining active immunity or exposure to new antigenically distinct virus strains, and the importance of passive immunity, indicating a need for more detailed epidemiological studies.

The single Jena-like virus strain (CV521-OH) detected in our study suggests that this may be a minor circulating BEC cluster. Alternatively, the low detection rate may also be due to poor assay sensitivity for this virus group. The results of the phylogenetic analyses of the CV521-OH strain also provides further evidence that NLV-BECs form two distinct genetic clusters, as previously suggested by Ando et al. (2). Although additional sequence data for the CV521-OH virus is not yet available, it would be of interest, for comparative purposes, to ascertain whether the differences in ORF-2, ORF-3, and 3′ UTRs noted between the Jena virus and the NA-2, CV95-OH, and CV186-OH NLV-BEC are characteristic features of each NLV-BEC genetic cluster. Also, the NBU-F/R RT-PCR assay could be used to investigate whether NB-like BECs occur in cattle outside of the United States or in humans. Interestingly, the enteropathogenic BEC NA-1 that was originally reported along with the NA-2 strain has, to date, failed to be genetically characterized (5, 28).

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REFERENCES

1. **Ando, T., S. S. Monroe, J. S. Noel, and R. I. Glass.** 1997. A one-tube method of reverse transcription-PCR to efficiently amplify a 3-kilobase region from the RNA polymerase gene to the poly(A) tail of small round-structured viruses (Norwalk-like viruses). J. Clin. Microbiol. **35:**570–577.
2. **Ando, T., J. S. Noel, and R. L. Fankhauser.** 2000. Genetic classification of "Norwalk-like viruses." J. Infect. Dis. **181**(Suppl. 2)**:**S336–S348.
3. **Berke, T., B. Golding, X. Jiang, D. W. Cubitt, M. Wolfaardt, A. W. Smith, and D. O. Matson.** 1997. Phylogenetic analysis of the caliciviruses. J. Med. Virol. **52:**419–424.
4. **Dastjerdi, A. M., J. Green, C. I. Gallimore, D. W. Brown, and J. C. Bridger.** 1999. The bovine Newbury Agent-2 is genetically more closely related to human SRSVs than to animal caliciviruses. Virology **254:**1–5.
5. **Dastjerdi, A. M., D. R. Snodgrass, and J. C. Bridger.** 2000. Characterization of the bovine enteric calici-like virus, Newbury Agent 1. FEMS Microbiol. Lett. **192:**125–131.
6. **Felsenstein, J.** 1989. PHYLIP–phylogeny inference package (version 3.2). Cladistics **5:**164–166.
7. **Green, J., J. Vinje, C. Gallimore, M. Koopmans, A. Hale, and D. W. G. Brown.** 2000. Capsid protein diversity among Norwalk-like viruses. Virus Genes **20:**227–236.
8. **Green, K. Y.** 1997. The role of human caliciviruses in epidemic gastroenteritis. Arch. Virol. Suppl. **13:**153–165.
9. **Guo, M., K. O. Chang, M. E. Hardy, Q. Zhang, A. V. Parwani, and L. J. Saif.** 1999. Molecular characterization of a porcine enteric calicivirus genetically related to Sapporo-like human caliciviruses. J. Virol. **73:**9625–9631.
10. **Guo, M., J. F. Evermann, and L. J. Saif.** 2002. Detection and molecular characterization of cultivable caliciviruses from clinically normal mink and enteric caliciviruses associated with diarrhea in mink. Arch. Virol. **146:**479–493.
11. **Jiang, X., P. Huang, W. Zhong, T. Farkas, D. Cubitt, and D. O. Matson.** 1999. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT/PCR. J. Virol. Methods **83:**145–154.
12. **Kapikian, A. Z., M. Estes, and R. M. Chanock.** 1996. Norwalk group of viruses, p. 783–810. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 2nd ed. Lippincott-Raven, Philadelphia, Pa.
13. **Lambden, P. R., and I. N. Clarke.** 1995. Genome organization of the caliciviridae. Trends Microbiol. **3:**261–265.
14. **Liu, B. L., P. R. Lambden, H. Gunther, P. Otto, M. Elschner, and I. N. Clarke.** 1999. Molecular characterization of a bovine enteric calicivirus: relationship to the Norwalk-like viruses. J. Virol. **73:**819–825.
15. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
16. **Page, R. D.** 1996. Treeview: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:**357–358.
17. **Prasad, B. V., M. E. Hardy, T. Dokland, J. Bella, M. Rossmann, and M. Estes.** 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science **286:**287–290.
18. **Pringle, C. R.** 1999. Virus taxonomy—1999. Arch. Virol. **144:**421–429.
19. **Saif, L. J., E. H. Bohl, E. M. Kohler, and J. H. Hughes.** 1977. Immune electron microscopy of transmissible gastroenteritis virus and rotavirus (reovirus-like agent) of swine. Am. J. Vet. Res. **38:**13–20.
20. **Schwab, K. J., M. K. Estes, F. H. Neill, and R. L. Atmar.** 1997. Use of heat release and an internal RNA standard control in reverse transcription-PCR detection of Norwalk virus from stool samples. J. Clin. Microbiol. **35:**511–514.
21. **Smiley, J. R., K. O. Chang, J. Hayes, J. Vinjé, and L. J. Saif.** 2002. Characterization of an enteropathogenic bovine calicivirus representing a potentially new genus. J. Virol. **76:**10089–10098.
22. **Smith, R. D.** 1991. Veterinary clinical epidemiology: a problem-oriented approach, p. 130–131. Butterworth-Heinemann, Boston, Mass.
23. **Thiel, H. J., and M. Konig.** 1999. Caliciviruses: an overview. Vet. Microbiol. **69:**55–62.
24. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
25. **van der Poel, W. H. M., J. Vinje, R. van der Heide, M. I. Herrera, A. Vivo, and M. P. Koopmans.** 2000. Norwalk-like calicivirus genes in farm animals. Emerg. Infect. Dis. **6:**36–41.
26. **Vinje, J., and M. P. Koopmans.** 1996. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in The Netherlands. J. Infect. Dis. **174:**610–615.
27. **Wang, J., X. Jiang, H. P. Madore, J. Gray, U. Desselberger, T. Ando, Y. Seto, I. Oishi, J. F. Lew, K. Y. Green, et al.** 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J. Virol. **8:**5982–5990.
28. **Woode, G., and J. C. Bridger.** 1978. Isolation of small viruses resembling astroviruses and caliciviruses from acute enteritis of calves. J. Med. Microbiol. **11:**441–452.