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Bovine Enteroviruses as Indicators of Fecal Contamination

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Surface waters frequently have been contaminated with human enteric viruses, and it is likely that animal enteric viruses have contaminated surface waters also. Bovine enteroviruses (BEV), found in cattle worldwide, usually cause asymptomatic infections and are excreted in the feces of infected animals in large numbers. In this study, the prevalence and genotype of BEV in a closed herd of cattle were evaluated and compared with BEV found in animals in the immediate environment and in environmental specimens. BEV was found in feces from 76% of cattle, 38% of white-tailed deer, and one of three Canada geese sharing the same pastures, as well as the water obtained from animal watering tanks, from the pasture, from streams running from the pasture to an adjacent river, and from the river, which emptied into the Chesapeake Bay. Furthermore, BEV was found in oysters collected from that river downstream from the farm. These findings suggest that BEV could be used as an indicator of fecal pollution originating from animals (cattle and/or deer). Partial sequence analysis of the viral genomes indicates that different viral variants coexist in the same area. The possibility of identifying the viral strains found in the animals and in the contaminated areas by sequencing the RNA genome, could provide a tool to find the origin of the contamination and should be useful for epidemiological and viral molecular evolution studies.

Enteroviruses belong to the family *Picornaviridae*. They are the most common viruses infecting a wide range of mammals. Eighty-nine serotypes have been identified: 62 associated with human infections and 27 associated with animal infections. The latter group includes 22 simian enteroviruses, 2 bovine enteroviruses (BEV), and 3 porcine enteroviruses. The classification of picornavirus genera and species has been ratified by the Executive Committee of the International Committee on Taxonomy of Viruses (http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/Sequence Database/). It is generally accepted that only a small proportion of enteroviruses are known, and every year, several new isolates are identified and the classification is revised. Most virus identification and typing of new enterovirus isolates are for those infecting humans. Considering the diversity of enteroviruses in humans, it is likely that many animal enteroviruses have yet to be described. Like other RNA viruses, their genetic variability is very high, and it is likely that new virus derivatives are frequently generated from the current population (12).

In nature, enteroviruses have many advantages for transmission compared with other viruses. They infect the gastrointestinal tract, frequently causing asymptomatic or mild infections. In infected animals, large numbers of progeny are produced that are shed in the feces. Finally, the virus particles are very stable under a wide range of environmental conditions, such as pH, temperature, and salinity (16, 17, 20, 29, 31). Thus, enteroviruses can remain infective for long periods in soils, biological specimens, and aquatic environments, including marine environments. It is believed that most viruses found in natural waters are cyanophages and other types infecting microalgae (19) and that the concentration of such viral particles exceeds that of bacteria by 5- to 10-fold (14, 42), reaching concentrations as high as 10^6 particles per ml in some locations (9). Many reports describe water contaminated with human enteroviruses, especially in sites close to urban areas and related to outbreaks of human infections (1, 2, 5, 8, 30, 34). However, no reports could be found related to contamination of surface waters by animal feces containing enteroviruses.

BEV are considered endemic in cattle in many regions (3, 21, 45). Infections are typically asymptomatic, with healthy animals as carriers. However, infections can also been associated with diarrhea and abortions. Enteroviruses are shed in large numbers in the feces and transmitted via the fecal-oral route (38). Based on this knowledge, it seemed reasonable to hypothesize that if BEV could be found in the environment, it could serve as an indicator for the presence of feces originating from cattle farms.

Advances in molecular biology techniques have provided highly sensitive and specific reverse transcriptase-PCR (RT-PCR) protocols utilizing a DNA fragment that can be sequenced to detect and type enterovirus isolates (2, 8). Detection generally has been performed with primers for RNA regions with motifs conserved within this group of viruses (11, 15, 32, 33, 35). The enterovirus genome is a single-stranded RNA-positive molecule of approximately 7,400 nucleotides (nt). There is a long open reading frame coding for the capsid and nonstructural proteins, preceded by a noncoding region of about 800 nt, which has an additional region of 110 nt in BEV. This region acquires a secondary structure that is important in the translation and replication of the viral genome and contains a number of motifs conserved in entero- and rhinoviruses (43, 44).

The present study was conducted to determine if BEV could serve as an indicator of bovine fecal contamination in an area associated with animal agriculture. To make this determina-

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tion, a survey was conducted to determine the presence of BEV in a closed herd of cattle located adjacent to the Wye River, a tributary of the Chesapeake Bay in Maryland. In addition, water samples were collected from animal watering tanks, pastures, runoff streams, and the Wye River. Furthermore, fecal specimens were collected from resident deer and geese, and gill and stomach washings from oysters collected from the adjacent river were examined.

MATERIALS AND METHODS

Virus and cells. BEV type 2 (strain PS87) and Madin-Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection (Manassas, Va.). The virus was propagated in MDBK cells grown in Eagle's minimum essential medium (MEM) containing 5% fetal calf serum and antibiotics. Cells were infected at the rate of 1 PFU/cell, and the supernatant containing viral progeny was harvested after 24 to 36 h, when cytopathic effects (CPE) were observed in most (>80%) of the cell monolayer. The supernatant containing the virus was clarified by centrifugation at 5,000 × *g* for 15 min at 4°C. The titer of the virus stock was determined in MDBK cells and stored at 20°C until used. All supernatants that did not exhibit CPE on MDBK cells were seeded on Buffalo (referring to State University of New York at Buffalo) green monkey (BGM) kidney cells, a cell line that is susceptible to infection by most BEV, provided by M. A. Jimenez-Clavero (INIA, Madrid, Spain).

Fecal collection. Fecal specimens were collected directly from the rectum of each of 137 purebred Black Angus cattle at the University of Maryland Wye Research and Education Center, Wye Angus Program, Queenstown, Md., and from 2 purebred Holstein cows from a closed research herd at the USDA Dairy Unit, Beltsville, Md. Feces from 50 white-tailed deer (*Odocoileus virginianus)* and 4 Canada geese (*Branta canadensis)* were collected from pastures at the University of Maryland Wye Research and Education Center, where cattle had not grazed for several months and where cattle feces were not observed. The rationale for examining feces from deer was that they are ruminants that inhabit the same environment as cattle and might serve as hosts to the same flora, fauna, and viruses. The rationale for examining feces from geese came from the observation that geese shared pastures with cattle and that geese picked through cattle feces to remove and eat seeds that passed through the bovine digestive tract.

Approximately 15 g of bovine, deer, or goose feces was suspended in 30 ml of phosphate-buffered saline (PBS) for 12 h with occasional vortexing. The suspension was centrifuged for 15 min at 15,000 × *g*, and the supernatant was transferred to a clean tube. PBS was added to a final volume of 50 ml and further centrifuged for 10 min at 15,000 × *g*. Supernatant was filtered through a 0.45-μm-pore-diameter membrane (Acrodisc 13-mm syringe filter; Pall Corporation, Ann Arbor, Mich.), supplemented with antibiotics, and stored at 2°C until used. Approximately 15 g of deer and geese feces was collected for each specimen, which was processed in the same manner as cattle feces. However, after the first centrifugation, the extracts were treated with 5% chloroform to extract the virus from the organic material and to eliminate bacterial contamination. This treatment does not affect enterovirus viability. The tubes were then centrifuged at 10,000 × *g* for 5 min, and the supernatants were filtered through a 0.4-μm-pore-diameter membrane and transferred to a clean tube containing antibiotics (penicillin-streptomycin) and stored at 20°C.

Water sample collection. Water samples (200 ml) were collected from different sites on or near the Wye Angus Program Farm at locations adjacent to the Wye River. Samples WI1 and WI2 were taken from runoff streams flowing from cattle fields to the adjacent Wye River. Samples WF1, WF2, and WF5 were taken from standing water in pastures. WF3 and WF4 were taken from cattle watering tanks on the farm. RNA was directly extracted from 140 μl of each of these water samples, without any concentration, as described below. Samples WR1 (Covington Cove), WR2 (Bryan Bar), WR3 (Pintail Point), and WR4 (Bluff Point) consisted of 50 liters of water collected from different sites in the Wye River at approximately 2 km from the shore and about 2 m below the surface (salinity, 14.0 to 15.5 ppt; temperature, 4.7 to 5.8°C). These water samples were filtered through a 3-μm-pore-diameter membrane (MF; 293 mm diameter; Millipore, Bedford, Mass.). The membranes were cut into pieces, and the retained material was recovered in PBS (0.2 ml/cm^2 of filtered material). RNA was extracted from 140 μl of these concentrated water samples. RT-PCR was performed with 1/10 of the final RNA preparation. All samples were stored in sterile tubes at 4°C until used.

Oyster samples. Thirty Eastern oysters (*Crassostrea virginica*) were collected from a bar in the Wye River, approximately 3 km downstream from the farm. Immediately after collection, hemolymph was aspirated via syringe from the adductor muscle, and the stomach and gills were dissected. All specimens were stored in separate polypropylene tubes on ice until arrival at the laboratory. Within 24 h of collection, hemolymph was pooled into three pools of 10 oysters each; stomachs and gills from each oyster were processed individually. Tissues were cut into small pieces and suspended in 5 ml of MEM (1 ml/g of tissue) for 1 h with occasional vortexing. After centrifugation at 1000 × *g* for 10 min, the supernatants were filtered through a 0.4-μm-pore-diameter filter. Antibiotics were added to the filtrates, and the mixtures were transferred to clean tubes. All samples were stored at 20°C until used.

Viral infectivity. Supernatants from fecal extracts and oyster gill washings were added to MDBK cells seeded in 24- or 96-well plates (Falcon, Becton Dickinson, Franklin Lakes, N.J.), at a 1:5 final dilution (e.g., 40 μl of fecal extract–160 μl of culture medium). Cultures were maintained at 37°C and 5% CO_2 for 2 to 6 days. CPE in cell monolayers were observed between days 2 and 4 of incubation. A 96-well plate with duplicates of 42 samples and including a row with 10-fold dilutions of a positive BEV sample was maintained for 12 days. In this assay, after 5 days of culture, new wells with CPE were not observed. Samples of water were diluted 1:3 in culture medium, and the infectivity was assessed by the appearance of CPE as described above. All samples that did not exhibit CPE on MDBK cells were seeded on BGM kidney cells, where they also failed to produce CPE.

RNA extraction. RNA was extracted with the QIAamp Viral RNA Mini kit (Qiagen, Valencia, Calif.). A 140-μl volume of each sample (water, water eluates, oyster washes, fecal extracts, or culture supernatants) was processed following the instructions of the supplier. The extracted RNA was eluted in 60 μl of RNase-free water, and 6 μl from each of these preparations was used for each RT-PCR (equivalent to 14 μl of the original sample).

Virus detection. A 183-bp fragment containing part of the 5′-terminal portion of the RNA molecule was amplified to detect BEV RNA with the Superscript One-Step RT-PCR kit (Gibco BRL, Life Technologies, Grand Island, N.Y.). The oligonucleotides were provided by Gene Probe Technologies, Inc., (Gaithersburg, Md.) and included a forward primer (5′ACGGAGTAGATGGTATTCCC; nt 188 to 207) and a reverse primer (5′CGAGCCCCATCTTCCAGAG; nt 389 to 420); the sequences of both primers are taken from the 5′ nontranslated region (NTR) sequence of BEV PS87 (GenBank accession no. X79368). The amplification reaction was performed according to the manufacturer's instructions. Briefly, reactions were made in 25 μl of reaction buffer containing 0.5 μM each primer and 0.5 μl of RT-DNA-*Taq* polymerase enzyme mix. A total of 6 μl of extracted RNA was tested for each sample. Thermal cycling consisted of 50°C for 30 min; 95°C for 15 min; and 40 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min. A final elongation at 72°C was performed at the end of each reaction. The reaction products (10 μl), stained with 0.5 μg of ethidium bromide per ml, were analyzed by agarose electrophoresis. Special care was taken to avoid RNA contamination and false positives: positive controls were never included in the RNA extraction when the specimens were processed, filter tips were used on pipettes, and all RNA extraction processes were performed in a separate sterile environment.

BEV detection sensitivity. The detection limit of the RT-PCR, as determined by adding known amounts of BEV RNA to the RT-PCR, was about 5 to 10 copies of the RNA genome. Before samples were analyzed, the sensitivity of the method was assessed by mixing known amounts of BEV-2 PFU (strain PS87) into fecal extract in which BEV was not previously detected. The protocol for RNA extraction followed by RT-PCR amplification was capable of detecting 0.5 to 2 viral PFU in 14 μl of fecal extract. This estimate refers to the titer of infectious particles and not to the amount of viral RNA molecules, which might be higher, because not all viral particles will infect cells. The viral particles that do not infect cells contribute significantly to the copy number of viral RNA and the limit of detectability by RT-PCR. Each RT-PCR determination corresponds to 14 μl of a 30% suspension of feces in PBS (fecal extract), which is equivalent to 4.2 mg of feces. By extrapolation, all cattle found positive by this method had at least 119 PFU/g of feces (0.5 × 1,000/4.2). To determine the sensitivity of the method to extract RNA from water, followed by RT-PCR, known amounts of BEV-2 were added to RNase-free water, and the RNA extraction protocol was followed as described above. The detection limit of this protocol was about 0.2 PFU in 14 μl of water. Therefore, a positive water sample contained at least 1.4 × 10^4 PFU/liter of water before concentration and at least 37 PFU/liter of water after concentration. The protocol used to detect BEV in the oyster tissue suspensions was found to have the same sensitivity (0.2 PFU/14 μl of tissue suspension). In this case, a positive oyster sample indicated the concentration of BEV was at least 71 PFU per oyster.

Sequencing and sequence analysis. The products from DNA amplifications (15 μl), obtained after RT-PCRs, were purified with a QIAquick PCR purification kit (Qiagen, Valencia, Calif.) and sequenced with the same primers used for

TABLE 1. Detection of BEV in fecal extracts from cattle and deer

CPE result by animal source	No. (%) of extracts	
	± for CPE	+ by RT-PCR
Cattle		
CPE+	56/139 (40.3)	51/56 (78)
CPE−	83/139 (59.7)	21/39 (54)
Total	139	72/95 (76)
Deer		
CPE+	28/50 (56.3)	14/28 (50)
CPE−	27/50 (44.7)	5/27 (18)
Total	50	19/50 (38)

amplification. When more than one DNA band was observed, the band corresponding to the correct size for BEV was excised, and the DNA was extracted and purified with the Qiaquick gel extraction kit (Qiagen). Products of sequencing reactions were analyzed with an ABI PRISM 377 DNA sequencer (Applied Biosystems, Perkin-Elmer Instruments, Shelton, Conn.) at the Beltsville, Md., campus, or at the Servicio de Secuenciacion Centro de Investigaciones Biologicas (Madrid, Spain). Sequence analysis was performed with the EMBL database search and alignment tools Fasta3 and ClustalW, respectively (http://www.ebi.ac.uk/Tools/). The neighbor-joining trees derived from the nucleotide sequence alignments of BEV isolates were made with the tree construction program TREECON for Windows (39).

RESULTS

BEV in cattle. When fecal extracts were examined for infectivity in MDBK cell cultures, infectivity of BEV from Angus cattle was variable; some extracts were highly cytopathogenic, causing complete destruction of the cell monolayer in 2 days, whereas others produced visible CPE after 4 days of culture with a small area of the cell monolayer affected. Still other extracts produced CPE in only one of two wells or produced no CPE at all. The same levels of sensitivity were obtained in fecal specimens from the Holstein cows found negative for BEV and then mixed with known amounts of BEV-2, indicating that the conditions used to obtain BEV in fecal extracts did not inhibit cell infection or cell growth. Approximately 40% of the fecal extract specimens from the Angus cattle produced CPE (doubtful results were not included) (Table 1). All fecal specimens shown to be infectious in MDBK cells ($n = 56$) and 30 fecal specimens that were negative or doubtful were subjected to RNA extraction for the detection of BEV RNA. In this analysis 76% of the specimens were positive (Table 1), giving a DNA fragment of the expected size (183 nt). Of 56 specimens that were infectious for MDBK cells, 51were found positive by RT-PCR by using BEV primers. Of 30 specimens that did not appear infectious for MDBK cells, 21 had a DNA band of the expected size for BEV primers. BEV was not detected in the two fecal specimens from Holstein cows.

Detection of BEV in deer and geese. To determine if animals that shared the same pastures as the Angus cattle might be infected with or serve as vectors of BEV, 50 fecal extracts from deer and 4 from geese were examined. The specimens were processed and analyzed as done with the cattle specimens. As shown in Table 2, many deer specimens (28 of 59) produced a CPE when added to MDBK cells. However, the correlation between CPE and RT-PCR-positive specimens was poor. Eleven specimens that produced a CPE did not exhibit a band of the size expected for BEV in the RT-PCR analysis. Furthermore, they exhibited other bands. These results could indicate the presence of pathogens different from BEV that can produce a CPE in bovine cells. However, there were five specimens that did not produce CPE, but exhibited a BEV band on the RT-PCR analysis.

TABLE 2. Analysis of BEV in water samples and oysters

Water or oyster sample	Source	Equivalent vol[a]	Result by:	
			CPE	RT-PCR
Water				
WI-1	Runoff stream	1	+	+
WI-2	Runoff stream	1	+	+
WF-1	Pasture pool	1	−	+
WF-2	Pasture pool	1	+	+
WF-3	Animal watering tank	1	−	+
WF-4	Animal watering tank	1	−	−
WF-5	Pasture pool	1	+	+
RW1	Wye River (Covington Cove)	380	−	+
RW2	Wye River (Bryan Bar)	380	−	−
RW3	Wye River (Pintail Point)	380	−	−
RW4	Wye River (Bluff Point)	380	−	+
Oyster pool				
1–10	Oyster gills		+	+
11–20	Oyster gills		+	+
21–30	Oyster gills		+	+
Individual oyster				
1	Oyster stomach		−	−
1	Oyster gills		−	−
3	Oyster stomach		−	−
3	Oyster gills		−	+
5	Oyster stomach		−	−
5	Oyster gills		−	−
6	Oyster stomach		+	+
6	Oyster gills		+	+
8	Oyster stomach		+	+
8	Oyster gills		+	+

[a] Equivalent volume analyzed per sample. A value of 1 corresponds to 14 μl of water.

Of four fecal specimens from geese, one was BEV RT-PCR positive. However, this specimen was not infectious for MDBK cells (data not shown).

Analysis of BEV in aquatic environments potentially contaminated by cattle manure. Of three water samples collected from pastures (WF1, WF2, and WF5) and found positive for BEV by RT-PCR, two were infectious in MDBK cell cultures (WF2 and WF5) (Table 2). One of two samples from animal watering tanks (WF3) was positive for BEV by RT-PCR, but no CPE were observed in cell culture. The two water specimens (WI1 and WI2) from runoff streams leaving the pastures were both positive in MBDK cells and RT-PCR assays.

All water samples from the farm, including runoff, were positive without concentration, indicating that the virus particle concentration was at least 1.4×10^4 PFU/liter. Water samples from the Wye River were negative before concentration, but two (RW1 and RW4) were positive by RT-PCR after concentration.

To determine if oysters might serve as a useful indicator of BEV in an estuarine environment, 30 oysters were collected from the site at which a water sample was obtained (RW1),

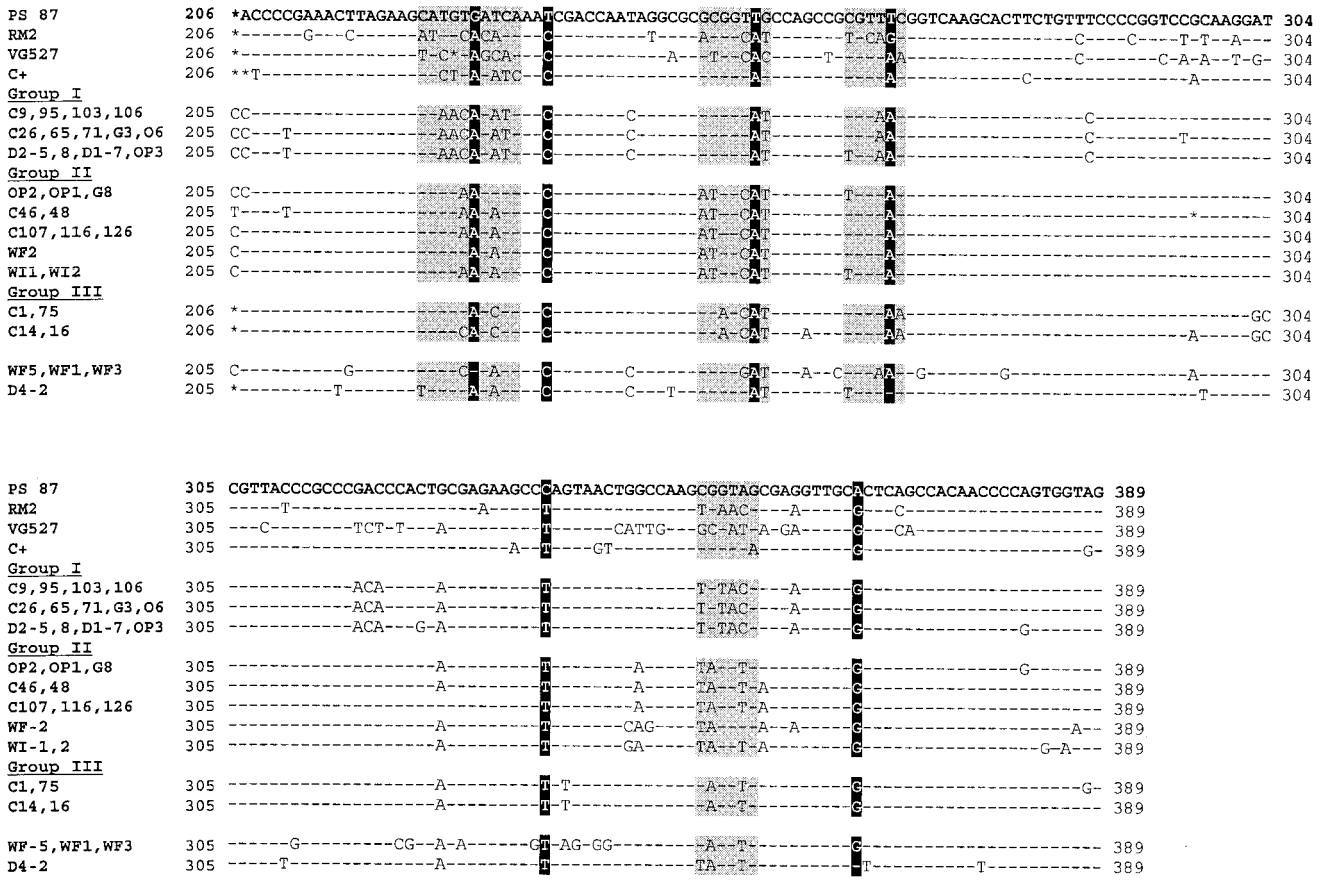

FIG. 1. PS87 (BEV-2) is the sequence deposited in the EMBL database (accession no. X79368). RM2 is a BEV serotype 2 (accession no. X79369). VG 527 is a BEV serotype 1 (accession no. D00214). C+ corresponds to the BEV obtained from American Type Culture Collection and used in this study as positive control. C corresponds to cattle fecal extracts. WF and WI correspond to water samples from the Wye Angus Farm and runoff streams from Wye Island, respectively. D corresponds to deer fecal samples, and G corresponds to goose fecal sample. O corresponds to oyster (stomach), and OP corresponds to a pool of oyster gill washes. Sequences were aligned with the ClustalW program and grouped according to their homology. Regions of high variability are highlighted in gray. Nucleotide changes that are shared by all the isolates in this study are highlighted in black. An asterisk means a nucleotide deletion.

and tissues were analyzed for the presence of BEV (Table 2). All pools of gill washings were infectious for MDBK cells and were RT-PCR positive. BEV was not detected in pools of hemolymph. Of five oysters that were analyzed individually, BEV was detected in both the stomach and gills in two oysters, and both (as well as gill washing from another oyster) were infectious for MBDK cells. The finding of BEV in gill washes and stomachs, but not in hemolymph, mirrors studies in which the major viral accumulation in shellfish is in the stomach and diverticula (4, 6). Some investigators have reported optimized protocols for detection of viruses in shellfish (4, 7, 18, 25, 36, 37). In the present study, gills and stomach washes were examined as sources of viruses because the methods were relatively easy to perform, the samples did not have inhibitory substances, and the sensitivity was similar to that of the optimized protocols (data not shown). However, in epidemiological studies, optimized methods to detect viruses in water samples should be used (1, 2, 8, 11, 32, 34).

Analysis of sequences. The results presented above show that infectivity for MDBK and the amplification by RT-PCR did not always correlate and that DNA fragments from some specimens that amplified with BEV primers were not the correct size. Amplification of fragments of different sizes was relatively frequent in water samples and extracts from deer feces (data not shown). These findings might have been due to one or more of the following possibilities: (i) the presence of non-BEV infectious and cytopathic viruses, (ii) the greater sensitivity of the RT-PCR method than the sensitivity of the method used to determine infectivity, or (iii) the presence of other enteroviruses with an RT-PCR product of similar size to BEV. To further investigate these possibilities, we sequenced the amplified DNA fragment of a representative number (n = 16) of the 72 cattle fecal specimens that were positive for BEV by RT-PCR. We also sequenced the fragments from water and oyster samples that had a band of the expected size after amplification, as well as those from positive deer and goose feces. When more than one amplicon was observed in agarose gels, the DNA band of the correct size was extracted and used as a template for dye terminator sequencing. The RT-PCR product was sequenced with the same primers used for amplification. The sequences were compared with those of the published sequences for other BEV and aligned by using the

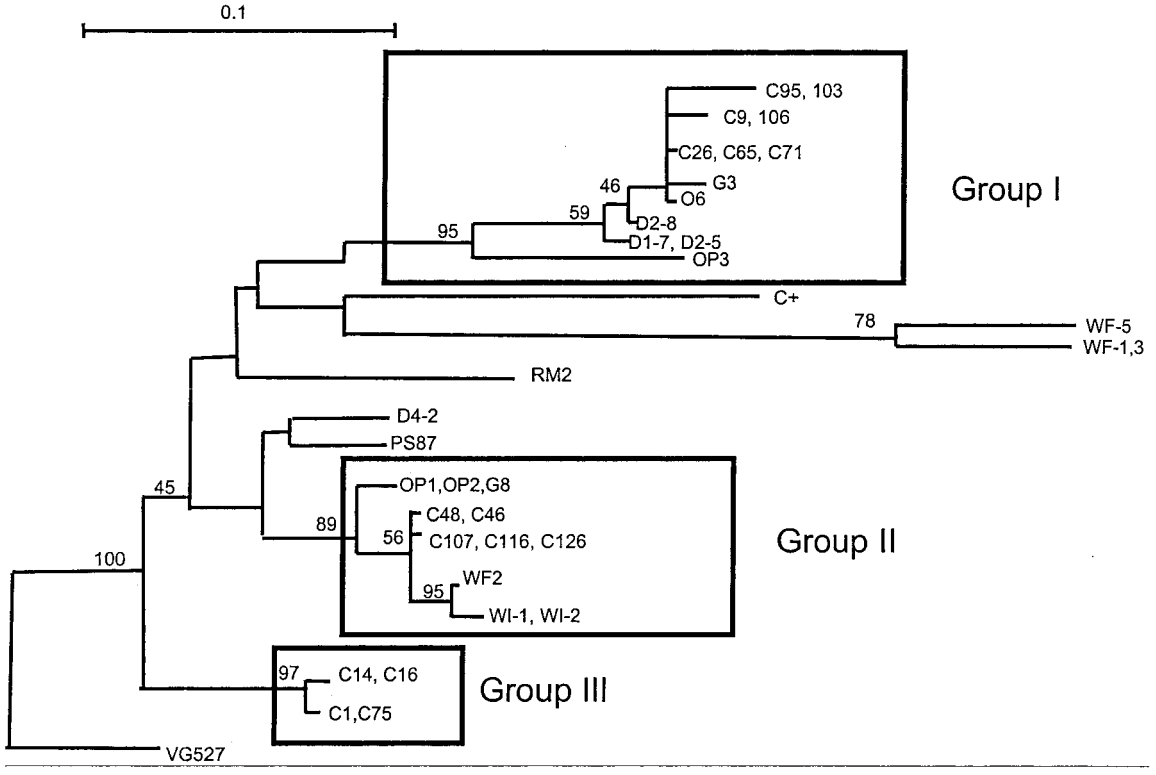

FIG. 2. Phylogenetic analyses of BEV isolates. The figure shows the neighbor-joining tree of the BEV isolates derived from their nucleotide sequence alignment shown in Fig. 1. The numbers in the segments of the trees indicate the percentage of trees in which the branch appears in programs for constructing bootstrap neighbor-joining trees. The length of the branch is proportional to the distance. Phylogenetic analyses were performed with the tree-constructing program TREECOM for Windows (39).

ClustalW program. As shown in Fig. 1, for cattle feces, all sequences corresponded to known BEV, even those that did not produce CPE in MDBK cells. This result suggests that the RT-PCR method was more sensitive than the cell culture infectivity method and that the amount of infectious virus in some feces was not enough to produce detectable CPE in the cell monolayer. As discussed in Materials and Methods ("BEV detection sensitivity"), the ratio of viral particles to PFU can be high, and even totally inactivated viruses would give a positive RT-PCR band, since their RNA can be intact.

All sequences were more closely related to BEV strain PS87 (GenBank accession no. X79368), which has been typed as BEV-2, than to other BEV serotypes. However, all sequences had several nucleotide changes that differed from the PS87 sequence. The same changes were found in specimens from several cattle, indicating that they did not correspond to individual mutations, but most likely were from a variant original virus. Considering these nucleotide differences, the cattle specimens could be divided into three groups, all having at least 84% nucleotide identity compared with PS87.

To determine if BEV was contaminating water and oysters as well as being present in deer and goose feces, and to identify the viral strain, we sequenced the fragments amplified in the RT-PCRs of RNA extracted from these sources. All sequences were compared among themselves and with those of BEV deposited in the EMBL and GenBank databases. Figure 1 shows the alignment of the sequences from each group of cattle and from deer and goose feces, oysters, and water. To simplify the analysis, the samples with similar sequences are presented in one line. Sequences of the same genomic region from the serotypes PS87 (BEV-2), RM2 (BEV-2), and VG527 (BEV-1) are included. As with the cattle specimens, all goose, deer, oyster, and water sequences corresponded to BEV-2. There are four regions that show high variability (nt 224 to 231, 249 to 256, 267 to 269, and 349 to 358). In addition, all isolates found in the present study had 6-nt changes not found in PS87, but shared by both RM2 and VG527 isolates. Most of the isolates found in water taken from Wye Island and in the oyster samples were similar to those from cattle 46, 48, 107, 116, and 126 (group II), while most of the isolates from deer and goose samples and some oyster samples were similar to those of cattle 9, 26, 65, 71, 95, 103, and 106 (group I). These results suggest that probably both cattle and deer contributed to the contamination of the water and estuarine environment with BEV.

To study the relationship among the BEV isolates, sequences of all BEV isolated in this study and those of PS87 and the positive control (C+) obtained from American Type Culture Collection (see Materials and Methods) were subjected to phylogenetic analysis. As shown in Fig. 2, the neighbor-joining tree constructed from the sequence alignments in Fig. 1 indicates that BEV isolates found in cattle can be grouped into three clusters. In addition, all of the BEV isolates found in the

environmental samples and in the feces from deer, except D4-2 and WF1, -3, and -5, belong to one of these groups.

DISCUSSION

This study reports the results of the first survey of an animal enterovirus linked to both biological and environmental specimens from the same location. Numerous reports have identified animal waste as a source of environmental contamination with nutrients such as phosphorus and nitrogen (40, 41). There are also numerous reports of waterborne pathogens, such as *Escherichia coli* O157 H7 and *Cryptosporidium parvum*, circumstantially linked to cow manure (22–24). Many other reports concern the stability and resistance of enteroviruses and the detection of these viruses affecting humans in groundwater and surface waters (5, 8, 10, 32). Based on these reports, the aim of the present study was first to detect BEV in cattle and then to determine if this virus (BEV), thought to be specific to cattle, could be detected in the environment and thereby serve as an indicator of bovine fecal contamination.

The high percentage of cattle that excreted BEV indicated that this virus was endemic in the area studied, as reported for other countries (3, 21, 45). Because BEV was shed in large numbers in the feces of these animals, the study was extended to look for BEV in environments potentially affected by feces from animal agriculture. Results of in vitro infectivity and molecular studies with viral RNA extracted from environmental samples indicated that all were contaminated with BEV. In water samples collected from standing water, runoff, and animal watering tanks, the concentration of BEV was high enough to be detected without any concentration: that is, at least 1.4×10^4 PFU/liter of water. Water and oysters collected from the adjacent Wye River were also contaminated with BEV. The survey also revealed that approximately half of 50 fecal specimens from deer that utilized the same pastures as the cattle were positive for BEV by PCR. Deer also appeared to be carriers of other pathogens affecting MDBK cells. Because deer are abundant in this area and excrete viral particles in their feces, they also must be considered as potential sources of detectable enterovirus contamination in environmental specimens.

To further analyze the BEV detected in the biological and environmental samples, the fragments of DNA amplified by RT-PCR with the BEV primers were sequenced. The region sequenced was the 5′ NTR, a highly conserved region in many picornaviruses often used for detection of enterovirus (15, 26, 32, 35). Although all sequences studied were more homologous to the BEV PS87 strain than to other BEV sequences, significant differences were found among the cattle isolates. In the cattle population, sequences could be clustered into three groups. Each group had similar specific nucleotide changes compared to the PS87 sequence, but all had at least 84% homology with this strain. Although the sequence divergence was relatively low, the region of the viral genome that was analyzed was very conserved; thus, a sequence identity score of less than 85% in this region could indicate that these isolates were variants belonging to different serotypes. Since all samples were taken at the same time from a closed herd and a relatively small area, these results indicate that there are different coexisting BEV populations. The sequence variability within the BEV population was remarkably high, suggesting interesting implications for viral evolution. Sequencing and analysis of the VP1 region of the viral genome should be useful to further characterize these isolates and to confirm their serotype, as has been reported in other enterovirus typing studies (26–28).

Analysis of BEV sequences from cattle and deer feces and from water and oyster samples showed that most nucleotide changes were found in four regions. In addition, compared to the PS87 GenBank sequence, there were six identical changes in all of the isolates, in the BEV isolates RM2 and VG527, and in the BEV isolate used in this study as a positive control (C+). Sequence alignments and phylogenetic analyses indicated that BEV isolates found in water samples taken from the island and from most of the oysters contained the same variants as those in one of the cattle groups. Analysis of the oyster samples indicated they were contaminated with at least two BEV variants (groups I and II), both found in the cattle population. Analysis of the deer specimens showed that these animals shed BEV particles in their feces. However, it is not clear whether the deer are mechanical vectors or are susceptible to infection with BEV. The sequences of the 5′ NTR region of the BEV isolates from deer were similar to those of group I of cattle, except for one deer. To more fully characterize the BEV isolates from deer feces, an in-depth series of experiments are needed, including infectivity of other cell lines and molecular analysis of the capsid region. Finally, even Canada geese appear to be carriers of BEV and may play a role in its dissemination from pasture to pasture or from pasture to water, a role the geese also appear to play in the dissemination of *C. parvum* (13).

In summary, this study has found BEV throughout the environment associated with animal agriculture, including a closed herd of Angus cattle, resident white-tailed deer, a Canada goose, animal watering tanks, surface waters, and oysters in an adjacent river. The BEV population consists of several variants within that limited geographic area that can serve as sensitive markers for fecal contamination. The possibility that BEV can serve as a specific marker for bovine fecal contamination depends on additional analyses of BEV from other animals and farms and whether the virus found in deer feces can be differentiated by capsid or other gene sequences.

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