APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2006, p. 1800–1809 Vol. 72, No. 3
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.3.1800–1809.2006

Human and Animal Enteric Caliciviruses in Oysters from Different Coastal Regions of the United States

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Received 15 September 2005/Accepted 14 December 2005

Food-borne diseases are a major cause of morbidity and hospitalization worldwide. Enteric caliciviruses are capable of persisting in the environment and in the tissues of shellfish. Human noroviruses (HuNoVs) have been implicated in outbreaks linked to shellfish consumption. The genetic and antigenic relatedness between human and animal enteric caliciviruses suggests that interspecies transmission may occur. To determine the occurrence of human and animal enteric caliciviruses in United States market oysters, we surveyed regional markets. Oysters were collected from 45 bays along the United States coast during the summer and winter of 2002 and 2003. Samples were analyzed by reverse transcription-PCR, and results were confirmed by hybridization and sequence analysis. Nine samples (20%) were positive for HuNoV genogroup II after hybridization. Animal enteric caliciviruses were detected in 10 samples (22%). Seven of these samples were positive for porcine norovirus genogroup II, and one sample was positive for porcine sapovirus after hybridization and confirmation by sequencing. Bovine noroviruses were detected in two samples, and these results were confirmed by sequencing. Five HuNoV samples sequenced in the polymerase region were similar to the norovirus genogroup II US 95/96 subset (genogroup II-4) previously implicated in diarrhea outbreaks. Different seasonal and state distributions were detected. The presence of animal enteric caliciviruses was associated with states with high livestock production. Although the presence of human caliciviruses in raw oysters represents a potential risk for gastroenteritis, disease confirmation by investigation of outbreaks is required. The simultaneous detection of human and animal enteric caliciviruses raises concerns about human infection or coinfection with human and animal strains that could result in genomic recombination and the emergence of new strains.

Food-borne diseases are a major cause of morbidity and hospitalization worldwide. Each year 76 million food-borne illnesses and more than 300,000 hospitalizations and 5,000 deaths due to these illnesses are reported in the United States alone, indicating that one in four Americans develops food-borne illness each year and at least 1 in 1,000 is hospitalized (40, 41). Despite major advances and improvements in food and water quality (32), diagnostic methods (3, 56), and surveillance systems (8, 40), food-borne diseases remain a global public health problem. Only 20% of food-borne illnesses are attributed to known pathogens. Although attention has been focused on preventing bacterial infections, viral agents are estimated to account for more than two-thirds of the food-borne illnesses caused by known pathogens (41), but no methods (antiviral agents or vaccines) are currently available for prevention or treatment of these illnesses.

The percentage of outbreaks associated with seafood is around 10 to 20% in countries such as the United States and Australia, but this percentage increases to 70% in countries in which seafood consumption is greater, such as Japan, or wherever seafood is eaten raw (7, 33). Seafood includes both fish and shellfish (mollusks and oysters). Mollusks are filter feeders that can concentrate more than fourfold in their tissue particles present in the surrounding water, including fecal coliforms and viruses (6). An etiological agent has been confirmed in only 44% of the outbreaks associated with seafood, and 47% of the outbreaks associated with seafood and with confirmed etiology are caused by viruses (7, 64).

Viruses in the family *Caliciviridae* are divided into four genera, *Norovirus*, *Sapovirus*, *Vesivirus*, and *Lagovirus*. Human and animal caliciviruses associated with gastroenteritis belong to the genera *Norovirus* and *Sapovirus*. Human noroviruses (HuNoVs) cause illness in people of all ages, whereas human sapoviruses (HuSaVs) cause illness primarily in children (19). The genus *Norovirus* is divided into five genogroups, genogroups I, II, III, IV, and V, which can be subdivided into 8, 19, 2, 1, and 1 genotypes, respectively, based on phylogenetic tree topology and distance analysis of the capsid gene (62). Human noroviruses belong to genogroups I, II, and IV. The genus *Sapovirus* is also divided in five genogroups that can be subdivided into three, three, one, one, and one genotypes based on a similar analysis of the capsid sequence (14). Human sapoviruses belong to genogroups I, II, IV, and V.

Animal enteric caliciviruses cause gastroenteritis in calves and pigs and have also been isolated from healthy pigs. Porcine norovirus (PoNoV) has been detected in Japan and Europe and was recently found in the United States (55, 57, 62). The

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PoNoVs detected in Japan and the United States belong to three different genotypes in genogroup II, the most widely detected norovirus genogroup in humans. Bovine noroviruses (BoNoVs) have been detected in Germany, England, and the United States, and they belong exclusively to genogroup III (9, 39, 53). Porcine sapovirus (PoSaV) has emerged as an important pathogen associated with diarrhea and subclinical infections in pigs of all ages since it was discovered in 1980 (15, 21).

Low infectious doses (19), prolonged asymptomatic shedding (17), environmental stability (49), and great strain diversity (1, 14) increase the risk of infection by members of this virus family. Moreover, the identification of closely related animal enteric caliciviruses in cattle and pigs and the existence of recombinants of BoNoVs (23, 46), PoNoVs, HuNoVs, and also HuSaVs (26, 29, 30) raise concerns about possible human infections or coinfection of animals or humans with human and animal enteric caliciviruses.

In the United States, HuNoVs accounted for 93 to 96% of the outbreaks of nonbacterial acute gastroenteritis submitted to the CDC from 1997 to 2000 (13). It is estimated that 50 to 66% of all food-borne illness whose etiology is known is due to HuNoVs, and 52% of the gastroenteritis cases associated with consumption of raw or partially cooked shellfish are attributable to HuNoVs, which makes these viruses the leading cause of seafood-associated (especially oysters) food-borne illness (7). The first oyster- and NoV-associated gastroenteritis outbreak involved about 2,000 persons, and it occurred during the summer of 1978 in Australia (43). Since then, several reports worldwide have indicated that NoV-contaminated oysters were associated with outbreaks of gastroenteritis (4, 11–13, 31, 37, 50, 51).

The objectives of this study were to conduct a survey of regional markets in the United States in order to determine the presence of human and animal enteric caliciviruses in market oysters and to compare the calicivirus strains detected in these market oysters with strains previously detected and associated with human gastroenteritis.

MATERIALS AND METHODS

Shellfish sampling. Oysters were collected from bays on the East, West, and Gulf coasts from which licensed shippers (Interstate Shellfish Sanitation Conference Shippers List) harvest oysters for consumer markets. When identified, each bay was assigned a number and then placed in an Excel spreadsheet and randomized, and 12 bays were chosen for each coast. Samples were obtained from 12 bays on the West Coast, 12 bays on the East Coast, and nine bays on the Gulf Coast in four, six, and three states, respectively, during the summer of 2002 (May 2002 to September 2002). During the winter (November 2002 to March 2003), samples were obtained from four bays on the West Coast, four bays on the East Coast, and four bays on the Gulf Coast. A total of 610 oysters (at least 12 oysters from each bay in each season) were obtained from 45 bays (Table 1). A lower harvesting rate during winter was the reason for the reduced sampling during this season. Oysters were purchased from farmers, wholesalers, and retailers, and at least 12 oysters were obtained from each bay for this study. A specific code was assigned to each bay; the codes for the West Coast were 1W to 12W, the codes for the East Coast were 1E to 12E, and the codes for the Gulf Coast were 1G to 12G. Each bay was considered one sample, and the oysters from each collection from a bay were pooled. The oysters were kept at −20°C and shipped overnight to our lab.

Shellfish processing. For each bay, oyster samples were rinsed in water prior to opening and counted. Oysters were shucked with a sterile knife, and the oyster tissue was removed. The digestive diverticula was removed and dissected with sterile scissors and forceps. Digestive tissues from oysters obtained on the same collection date from the same bay were pooled and homogenized, and the homogenates were then subdivided into 1.5-g aliquots. The homogenates were frozen (−20°C) until analysis. The number of oysters from each bay, the harvest and arrival dates, the tissue weight (entire oyster tissue without shell), and the digestive tissue weight were recorded.

TABLE 1. States and number of bays sampled in each state between the summer of 2002 and the winter of 2002/2003

State	No. of bays	
	Summer	Winter
West Coast		
Alaska	4	0
Washington	4	2
California	1	0
Oregon	3	2
Total	12	4
East Coast		
South Carolina	1	0
Delaware	2	0
Maine	3	2
New Jersey	1	0
New York	4	1
Virginia	1	1
Total	12	4
Gulf Coast		
Florida	3	4
Louisiana	5	0
Mississippi	1	0
Total	9	4

Virus elution and concentration. A 1.5-g aliquot of homogenate was thawed on ice and processed as previously described by Atmar et al. (3), with minor modifications. The homogenate was homogenized in phosphate-buffered saline (pH 7.4) with a grinder and transferred to a centrifuge tube. Chloroform-butanol was added to remove tissues, and Cat-Floc T (20% polydiallyldimethyl ammonium chloride) (Calgon Corp.) was added to coagulate proteins. After centrifugation the supernatant was recovered, and polyethylene glycol 6000 was added to concentrate the virus. After centrifugation, the pellet was resuspended in 3 ml of diethyl pyrocarbonate (DEPC)-treated water for nucleic acid extraction.

Nucleic acid extraction. Nucleic acids were purified from concentrated virus as previously described by Atmar et al. (3), with minor modifications. Briefly, after digestion of a pellet with proteinase K (20 mg/ml) (AMRESCO), double extraction was performed with 70% phenol–chloroform–water (Applied Biosystems) and chloroform-isoamyl alcohol (24:1). The aqueous phase was precipitated with ethanol, the pellet was resuspended in DEPC-treated water, and 5% cetyltrimethylammonium bromide (Sigma) was added to remove PCR inhibitors. After 15 min of incubation at room temperature, samples were centrifuged for 30 min at 14,000 × *g*, and the supernatant was discarded. The pellet was resuspended in 1 M NaCl and reprecipitated with ethanol and 3 M sodium acetate (pH 5.2) for 30 min at −80°C. The precipitated nucleic acids were resuspended in 50 μl of DEPC-treated water with 0.8 U/μl RNasin (Promega, Madison, WI). The presence of natural PCR inhibitors was tested by using an internal control (IC) and performing reverse transcription (RT)-PCR with primers NVp35 and NVp36, which were specific for this IC, as described below.

Detection of PCR and RT-PCR inhibitors in oysters. An IC was used in the RT-PCR to assess the presence of RT-PCR inhibitors. The IC (kindly provided by M. K. Estes and R. L. Atmar) was generated from a DNA clone containing the region that was amplified by primers NVp35 and NVp36 (4,487 to 4,956 bp) with a 123-bp deletion that yielded, after RNA synthesis by SP6 polymerase, a 347-bp RNA amplicon (50). One microliter of IC was added to each sample during the RT-PCR, and primers NVp35 and NVp36, which were specific for this IC, were used (Table 2). Briefly, for RT, 3 μl of RNA was added to 22 μl of an RT mixture containing 2.5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 9.0 at 25°C], 500 mM KCl, 1% Triton X-100), 2.5 μl of a 25 mM $MgCl_2$ solution, 1 μl of a solution containing each deoxynucleoside triphosphate (dNTP) at a concentration of 10

TABLE 2. Primers and probes used to detect human and animal noroviruses and sapoviruses in oysters

Primer or probe	Sense	Genus	Sequence (5′–3′)[f]	Location[a]	Reference
Primers					
Broadly reactive primers					
NVp110	−	NoV, SaV	ACDATYTCATCATCACCATA	4865–4884	35
p290	+	NoV, SaV	GATTACTCCAAGTGGGACTCCAC	4568–4590	27
JV12Y	+	NoV	ATACCACTATGATGCAGAYTA	4552–4572	58
JV13I	−	NoV	TCATCATCACCATAGAANGAG	4858–4878	58
NVp35	−	NoV	CTTGTTGGTTTGAGGCCATAT	4936–4956	3
Human calicivirus primers					
NoV genogroup I					
NVp36	+	NoV	ATAAAAGTTGGCATGAACA	4487–4505	2
NoV genogroup II					
NI	+	NoV	GAATTCCATCGCCCACTGGCT	4495–4515[b]	18
NV-4611	+	NoV	CWGCAGCMCTDGAAATCATGG	4611–4631	65
Animal enteric calicivirus primers					
Porcine					
PEC66-Bio[e]	+	SaV	Bio-GACTACAGCAAGTGGGATTCC	4327–4347[c]	22
PEC65	−	SaV	ATACACACAATCATCCCCGTA	4636–4656[c]	22
PNV7-Bio[e]	+	NoV	Bio-AGGTGGTGGCCGAGGAYC	4422–4443	62
PNV8	−	NoV	TCGCCATAGAAGTARAAG	4613–4632	62
Bovine					
CBEC-UF	+	NoV	AGTTAYTTTTCCTTYTAYGGGA	868–889[d]	53
CBEC-UR	−	NoV	AGTGTCTCTGTCAGTCATCTTCAT	1376–1399[d]	53
Probes					
GGIa		NoV	ATGGATGTAGGTGAYTAYGT	4685–4704	59
GGIb		NoV	ATGGAYGTTGGYGAYTATGT	4685–4704	59
GGII		NoV	GGAAYTCCATYRCMCAYTG	4494–4512[b]	59
PoNoro 1A		PoNoV	AGCCAGTGGGCGAAGGAGTTCCACTGTGATGTGCA		61
PoNoro 1B		PoNoV	AGCCAATGCGCTATGGAGTTCCACTGTGATGTGCA		61
PoNoro 1C		PoNoV	AGCCAATGGGCAAAGGAATTCCATTGTGATGTGCA		61
PoSapo 1A		PoSaV	ATRACACTGGTGAAGGGCATGCCAGAGGGGAG		61
PoSapo 1B		PoSaV	ATCACACTGGTGAAGGGCATGCCTGATGGTAA		61
PoSapo 1C		PoSaV	ATGACGCTGGTGAATGGCATGCCAGAGGGAAG		61

[a] Nucleotide positions are the positions in Hu/NLV/Norwalk/68/US (accession no. M87661), unless indicated otherwise.
[b] Nucleotide positions are the positions in Hu/NLV/Lordsdale/93/UK (accession no. X86557).
[c] Nucleotide positions are the positions in Po/SLV/Cowden/US (accession no. AF182760).
[d] Nucleotide positions are the positions in Bo/NLV/CV95-OH/02/US (accession no. AF542083).
[e] Oligonucleotides are 5′ biotin (Bio) labeled for detection by the microwell probe capture hybridization assay.
[f] Y = C + T; R = A + G; M = A + C; D = A + T + G; W = A + T.

mM, 1 μl of a 50 μM reverse primer NVp35 solution, 2 U of avian myeloblastosis virus (AMV) reverse transcriptase (Promega), and 8 U of RNasin (Promega), and the mixture was incubated at 42°C for 1 h; this was followed by heat inactivation at 94°C for 3 min. For PCR, another 25-μl PCR mixture containing 2.5 μl of 10× PCR buffer, 2.5 μl of a 25 mM $MgCl_2$ solution, 1 U of *Taq* DNA polymerase, and 1 μl of a 50 μM forward primer NVp36 solution was added to the RT reaction mixture. After initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s were performed, followed by a final extension at 72°C for 10 min. Amplified products were analyzed by 9% acrylamide gel electrophoresis, stained with ethidium bromide, and visualized under UV light. If inhibition was detected, the RNA template was diluted 1:10 to overcome the inhibition, and the RT-PCR was repeated.

RT-PCR for calicivirus detection. Several primer sets combined into nine different RT-PCR protocols and one seminested PCR were used to detect human and animal enteric caliciviruses. Based on the conserved RdRp region, broadly reactive primers for both NoVs and SaVs and specific primers for human or animal NoVs or SaVs were used, as described in Table 2. RT-PCRs with broadly reactive primers NVp110 and p290 or HuNoV genogroup-specific primers NI (genogroup II), NV-4611 (genogroup II), and NVp36 (genogroup I) were performed in two steps. For RT, 4 μl of reverse primer NVp110 (50 μM) was added to 12 μl of RNA, incubated at 94°C for 2 min, and transferred to ice. Twenty-four microliters of an RT mixture containing 11 μl of DEPC-treated water, 4 μl of 10× PCR buffer (Promega), 4 μl of a 25 mM $MgCl_2$ solution (Promega), 4 μl of a solution containing each dNTP at a concentration of 10 mM, 5 U of AMV reverse transcriptase (Promega), and 20 U of RNasin was added and incubated at 42°C for 1 h, and this was followed by heat inactivation at 94°C for 3 min. For PCR, 10-μl aliquots of the RT product were transferred to four PCR tubes, and 40 μl of a PCR mixture containing 30.8 μl of DEPC-treated water, 4 μl of 10× PCR buffer, 4 μl of a 25 mM $MgCl_2$ solution, 5 U of *Taq* DNA polymerase, and 1 μl of a 50 μM solution of one forward primer (p290, NI, NV-4611, or NVp36) was added to each tube. After initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s were performed, followed by a final extension at 72°C for 10 min.

RT-PCR with primers JV12Y and JV13I was also performed in two steps, as previously described by Vennema et al., with minor modifications (58). For RT, 4 μl of reverse primer JV13I (50 μM) was added to 5 μl of RNA, incubated at 94°C for 2 min, and transferred to ice. Six microliters of an RT mixture containing 2.3 μl of DEPC-treated water, 1.5 μl of 10× PCR buffer, 1.5 μl of a 25 mM $MgCl_2$ solution, 0.3 μl of a solution containing each dNTP at a concentration of 10 mM, 2 U of AMV reverse transcriptase, and 8 U of RNasin was added and incubated at 42°C for 1 h, and this was followed by heat inactivation at 94°C for 3 min. For PCR, a 5-μl aliquot of the RT product was added to 45 μl of a PCR mixture, and the PCR was performed as previously described (58).

For animal enteric caliciviruses, RT-PCR was performed with primers CBEC-UF and CBEC-UR for BoNoVs as previously described by Smiley et al. (53) and with primers PEC66-Bio and PEC65 for PoSaVs as previously described by Guo et al. (21), and for PoNoVs RT-PCR was performed with primers PNV7-Bio and PNV8 as described by Wang et al. (62).

Seminested PCR was performed by using the product of RT-PCR obtained with primers NVp110 and NV-4611 as the template for a reaction with primers NVp110 and NI. Briefly, 3 μl of the RT-PCR product was added to 47 μl of a PCR mixture containing 5 μl of 10× PCR buffer, 5 μl of a 25 mM $MgCl_2$ solution, 1 μl of a solution containing each dNTP at a concentration of 10 mM, 1 μl of a 50 μM forward primer NI solution, 1 μl of a 50 μM reverse primer NVp110 solution, and 1 U of *Taq* DNA polymerase. After initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s were performed, followed by a final extension at 72°C for 10 min. All amplified products were analyzed by electrophoresis using 9% acrylamide gels with ethidium bromide staining and were visualized under UV light.

Hybridization assay for HuNoVs. Dot blot analysis was used to confirm RT-PCR products, as previously described by Le Guyader et al. (36). Probes GGIa, GGIb, and GGII (59) (Table 2) were labeled with digoxigenin using a 3′DIG oligonucleotide tailing kit (Boehringer Mannheim). The RT-PCR products (obtained with NVp110 and p290 or with JV12Y and JV13I) were diluted in buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and denatured at 95°C. The RT-PCR products were blotted onto a positively charged nylon membrane

TABLE 3. Number of bays positive for human caliciviruses as determined by RT-PCR, nested PCR, and hybridization during the summer of 2002 and the winter of 2002/2003

Coast	No. of bays tested	No. of bays positive						
		Human calicivirus					Total	
		Human norovirus						
		Genogroup I		Genogroup II		RT-PCR[c]	RT-PCR	Hybridization
		RT-PCR	Hybridization	RT-PCR[a]	Hybridization[b]			
West	16	0	0	8	5	0	8	5
East	16	0	0	8	2	1	9	2
Gulf	13	0	0	4	2	2	6	2
Total	45	0	0	20	9	3	23	9

[a] Samples were positive with broadly reactive primers and/or genogroup II-specific primers.
[b] Samples were positive as determined by hybridization with probe GGII.
[c] Samples were positive only with broadly reactive primers NVp110 and p290 or primers JV12Y and JV13I.

(Sigma) under a vacuum and fixed by UV light. Positive controls were introduced onto each membrane. After prehybridization at 50°C for 30 min, hybridization was performed for 2 h at 50°C. Hybridized probes were detected by chemiluminescence using a CDP-Star ready-to-use kit (Roche) according to the manufacturer's protocol.

Microwell probe capture hybridization assay for PoNoV and PoSaV. For PoNoV and PoSaV a microplate probe capture hybridization assay with specific probes was used to confirm RT-PCR products, as previously described by Wang et al. (61). Briefly, EIA/RIA eight-well strips (Corning Inc., Corning, NY) were coated with 100 ng/well of probes PoNoro 1A, PoNoro 1B, and PoNoro 1C to detect PoNoVs or with 100 ng/well of probes PoSapo1A, PoSapo 1B, and PoSapo 1C to detect PoSaVs. The RT-PCR products (obtained with PNV7-Bio and PNV8 or with PEC66-Bio and PEC65) were diluted 1:1 in denaturation buffer and transferred to the strips. Positive and negative RT-PCR controls were included. After incubation, hybridized products were detected with Neutravidin-horseradish peroxidase conjugate (Pierce Biotechnology, Rockford, IL) and tetramethylbenzidine as the substrate (KPL, Gaithersburg, MD). The absorbance at 450 nm was determined with a spectrophotometer.

Sequence analysis. RT-PCR products were purified from polyacrylamide gels with a Gebaflex kit used as described by the manufacturer (Bioworld, Ohio). The DNA was sequenced directly (when the amount of DNA was enough) or after cloning into the pCR2.1-TOPO (T/A) vector (Invitrogen), using BigDye terminator cycle chemistry and a 3700 DNA analyzer (Applied Biosystems, Foster City, CA). Sequence data were aligned using the Lasergene software package (DNASTAR Inc., Madison, WI) and were compared with the previously published sequences using the Basic Local Alignment Search Tool (BLAST) and Clustal methods.

RESULTS

In this study, 610 oysters that represented 45 bays (or 45 samples) were purchased from different markets on coasts around the United States (Table 1). All of the oysters were supplied by licensed shippers that harvest oysters approved for human consumption from bays on the East, West, and Gulf coasts. To assess the presence of human or animal enteric caliciviruses, 33 bays (12 bays on the East Coast, 12 bays on the West Coast, and 9 bays on the Gulf Coast) during the summer and 12 bays (4 on each coast) during the winter (a total of 45 bays) were randomly selected. Oysters that were harvested from one bay at one time were considered one sample. Each sample was composed of at least 12 oysters. Tags were checked to determine the harvest location.

Crude shellfish extracts often contain low virus concentrations compared with the concentrations in feces, in addition to natural RT-PCR inhibitors. To overcome these problems, virus was eluted from the oyster tissue and concentrated. In addition, cetyltrimethylammonium bromide was included in the solution used for extraction of RNA to remove inhibitors, and the presence of inhibitors after RNA extraction was assessed by using an IC. The use of the IC showed that 32 of 45 samples (71%) displayed inhibition (data not shown). To overcome this problem, RNAs were diluted 1:10 in DEPC-treated water before analysis.

Primarily genogroup II HuNoVs were detected in oysters, as determined by RT-PCR and hybridization. The HuNoVs were detected with one or two genogroup-specific primer sets in 44% (20/45) of the samples (Table 3). In addition, human caliciviruses were detected with broadly reactive primers but not with NoV-specific primers in 3/45 samples (7%). No positive samples were detected with primers NVp110 and NVp36 for genogroup I HuNoVs.

After hybridization, 9 of 45 RT-PCR products obtained with primers NVp110 and p290 or primers JV13I and JV12Y were positive with probe GGII, and the viruses were confirmed to be genogroup II HuNoVs. The same nine samples were also positive with the genogroup II-specific primer pair (Table 3). These samples were obtained from the West Coast (three samples from Oregon, one sample from Alaska, and one sample from Washington), the East Coast (two samples from New York), and the Gulf Coast (one sample from Mississippi and one sample from Louisiana). No positive results were obtained with probes GGIa and GGIb, which agreed with the RT-PCR results which showed that no products were detected with a genogroup I NoV-specific primer set (primers NVp110 and NVp36).

Animal enteric caliciviruses were detected in oysters mainly from the West Coast, as determined by RT-PCR or hybridization. Fifteen of 45 samples (33%) were positive with specific primer sets for animal enteric caliciviruses (Table 2); 2 of these samples were positive with the BoNoV-specific primer set, 1 was positive with the PoSaV-specific primer set, and 12 were positive with the PoNoV primer set. The microwell probe capture hybridization assay was used to confirm the RT-PCR results for PoNoV and PoSaV. Seven of 12 samples that were positive for PoNoV as determined by RT-PCR were positive as determined by hybridization, and the viruses were confirmed to be genogroup II PoNoVs. Six of these seven samples (86%) were from the West Coast (three samples from Oregon, one sample from California, and two samples from Washington),

TABLE 4. Number of bays positive for animal enteric caliciviruses as determined by RT-PCR and microplate hybridization during the summer of 2002 and the winter of 2002/2003

Coast	No. of bays tested	No. of bays positive						
		Bovine norovirus (RT-PCR)[a]	Porcine sapovirus		Porcine norovirus		Total	
			RT-PCR[b]	Hybridization[c]	RT-PCR[d]	Hybridization[e]	RT-PCR	Hybridization
West	16	2	1	1	11	6	14	7
East	16	0	0	0	1	1	1	1
Gulf	13	0	0	0	0	0	0	0
Total	45	2	1	1	12	7	15	8

[a] Samples were positive with primers CBEC-UF and CBEC-UR.
[b] Samples were positive with primers PEC66-Bio and PEC65.
[c] Samples were positive as determined by hybridization with probes PoSapo 1A, PoSapo 1B, and PoSapo 1C.
[d] Samples were positive with primers PNV7-Bio and PNV8.
[e] Samples were positive as determined by hybridization with probes PoNoro 1A, PoNoro 1B, and PoNoro 1C.

and one sample was from the East Coast (Delaware). In the case of PoSaV, the RT-PCR product amplified with primers PEC66-Bio and PEC65 was also confirmed by hybridization to be genogroup III PoSaV. The only sample positive for PoSaV was from Oregon. Two samples were positive with primers for BoNoV; one of these samples was from Washington, and the other was from Oregon (Table 4).

HuNoVs detected in oysters are genogroup II-4 NoVs as determined by sequence analysis. From the nine samples that were positive for HuNoVs as determined by RT-PCR and hybridization, five sequences were obtained from amplicons amplified with primers NVp110 and NI (Table 2). These samples were from Washington (bay 3W), Oregon (bay 1W), Louisiana (bay 12G), Mississippi (bay 10G), and New York (bay 5E). Norovirus strains detected in oysters from these states belonged to genogroup II-4, and they were closely related to the genogroup II 95/96-U.S. subset detected in diarrhea outbreaks in the United States and seven other countries during the same period. The seven amplicons amplified with primers specific for PoNoVs (PNV7-Bio and PNV8) were also sequenced. All of them were confirmed to be amplicons of genogroup II NoVs and originated from bays in Washington (bays 3W and 6W), California (bay 7W), Oregon (bays 1W, 2W, and 10W), and Delaware (bay 8E) (Table 5). The two amplicons amplified with primers specific for BoNoVs were also sequenced and were confirmed to be amplicons of genogroup III NoVs.

Human noroviruses were detected on the three coasts, whereas animal enteric caliciviruses were detected mainly on the West Coast. Samples were collected from 13 states. The results were organized by season, coast, state, and bay (Table 5). In the summer, 33 bays in 13 states were sampled, whereas in the winter 12 of these bays in seven states were sampled. Human and/or animal enteric caliciviruses were detected at least once in one bay in 11 of 13 states (23/45 bays) by RT-PCR or RT-PCR and hybridization; negative results were obtained for samples from New Jersey and South Carolina (Table 5).

Human noroviruses were detected in 10 states (Washington, Oregon, Alaska, Maine, Virginia, New York, Delaware, Florida, Louisiana, and Mississippi) and were not detected in New Jersey, California, and South Carolina. Although it was clear that when HuNoV was detected in a state, it was present in both seasons (Washington, New York, and Maine), only in the summer (Oregon), or only in the winter (Virginia and Florida), it was also clear that within each state, some bays were positive and other bays were negative at different times (Table 5). In Louisiana, samples from three bays (bays 6G, 7G, and 12G) were positive for human caliciviruses, whereas the other two bays (bays 5G and 11G) were negative. Four of six bays in Florida were negative. In New York, three of four bays were positive (bays 4E, 5E, and 11E). In the case of Alaska and Washington, four bays in each state were sampled. In both states, two bays were negative, but HuNoVs were detected in the other two bays (bays 3W, 4W, 8W, and 11W). All samples from Oregon were positive for HuNoVs (bays 1W, 2W, and 10W).

Animal enteric caliciviruses were detected in three of the four West Coast states (Oregon, Washington, and California) and in only one East Coast state (Delaware) (Table 5). In Oregon, PoNoV was detected in the three bays studied, PoSaV was detected in one bay (bay 1W), and BoNoV was detected in one bay (bay 2W). Two of four bays in Washington were positive for PoNoVs (bays 3W and 6W), whereas BoNoV was detected in a third bay (bay 4W) and one bay was negative for all of the viruses. One bay was sampled in California, and it was positive for PoNoV, as was one of two bays sampled in Delaware.

Human and animal enteric caliciviruses were detected in both seasons in the same or different bays. Human caliciviruses were detected in both seasons by RT-PCR with different primer sets, but only samples obtained in the summer were also positive as determined by hybridization with probe GGII (HuNoV genogroup II) (3). Human noroviruses were detected only in the summer in Oregon (bays 1W, 2W, and 10W) and only in the winter in Virginia and Florida (bays 3E and 1G). However, in some states, such as Washington, New York, and Maine, HuNoVs were detected in both seasons, in the same or different bays. During the summer in Florida, samples from one bay (bay 8G) were positive for human caliciviruses, whereas oysters from the other two bays were negative, including oysters from bay 1G, from which oysters that were later positive for HuNoV in the winter were obtained. Three other samples collected during the winter in Florida were negative. In New York, three of four bays were positive during the summer (bays 4E, 5E, and 11E), and the bay that was sampled in both seasons was also positive in the winter (bay 4E). In Washington, four bays were sampled during the summer; two of these bays were positive for HuNoVs and two were negative. However, the two

TABLE 5. Geographic and seasonal distribution of oysters positive for human and/or animal enteric calicivirus from the East, West, and Gulf coasts during the summer of 2002 and the winter of 2002/2003

Bay	State	HuCV[a]/AECV[b] present	
		Summer	Winter
West Coast			
1W	Oregon	HuNoV[c,d]/PoNoV[d,e]	−/PoSaV[h]
2W	Oregon	HuNoV[c]/PoNoV[d,e]	−/BoNoV[g]
3W	Washington	HuNoV[c,d]/PoNoV[d,e]	HuNoV/−
4W	Washington	HuNoV/−	−/BoNoV[g]
5W	Washington	−/−	NA[i]
6W	Washington	−/PoNoV[d,e]	NA
7W	California	−/PoNoV[d,e]	NA
8W	Alaska	HuNoV/−	NA
9W	Alaska	−/−	NA
10W	Oregon	HuNoV[c]/PoNoV[d,e]	NA
11W	Alaska	HuNoV[c]/−	NA
12W	Alaska	−/−	NA
East Coast			
1E	Maine	−/−	HuNoV/−
2E	Maine	HuNoV/−	−/−
3E	Virginia	−/−	HuNoV/−
4E	New York	HuNoV/−	HuNoV/−
5E	New York	HuNoV[c,d]/−	NA
6E	Maine	−/−	NA
7E	Delaware	HuCV[f]/−	NA
8E	Delaware	HuNoV/PoNoV[d,e]	NA
9E	New York	−/−	NA
10E	South Carolina	−/−	NA
11E	New York	HuNoV[c]/−	NA
12E	New Jersey	−/−	NA
Gulf Coast			
1G	Florida	−/−	HuNoV/−
2G	Florida	NA	−/−
3G	Florida	NA	−/−
4G	Florida	NA	−/−
5G	Louisiana	−/−	NA
6G	Louisiana	HuNoV/−	NA
7G	Louisiana	HuCV[f]/−	NA
8G	Florida	HuCV[f]/−	NA
9G	Florida	−/−	NA
10G	Mississippi	HuNoV[c,d]/−	NA
11G	Louisiana	−/−	NA
12G	Louisiana	HuNoV[c,d]/−	NA

[a] HuCV, human calicivirus.
[b] AECV, animal enteric calicivirus.
[c] Samples were positive as determined by hybridization with probe GGII.
[d] Samples were sequenced and viruses were confirmed to be norovirus genogroup II viruses.
[e] Samples were positive as determined by hybridization with probes PoNoro 1A, PoNoro 1B, and PoNoro 1C.
[f] Samples were positive only as determined by RT-PCR with broadly reactive primers NVp110 and p290.
[g] Samples were sequenced and viruses were confirmed to be norovirus genogroup III viruses.
[h] Samples were positive as determined by hybridization with probes PoSapo 1A, PoSapo 1B, and PoSapo 1C.
[i] NA, not available.

positive bays were also sampled in the winter, but only oysters from bay 3W were positive for HuNoVs. In Oregon, HuNoV genogroup II was detected in all bays sampled during the summer. However, in the winter samples collected from two of these bays (bays 1W and 2W) were negative with all of the human calicivirus primers used (including genogroup II-specific primers that showed positive reactions with summer samples from these bays).

Animal enteric caliciviruses were detected in both seasons. However, whereas the seven samples that were positive for PoNoVs were from Oregon, Washington, California, and Delaware bays sampled during the summer, the two samples that were positive for BoNoVs and the one sample that was positive for PoSaVs were obtained from two bays (bays 2W and 4W) and one bay (bay 1W), respectively, in the winter in Oregon and Washington.

Human and animal enteric calicivirus were simultaneously detected in oysters collected from the same bay during the same season. In the states surveyed with high cattle or swine livestock production, such as Oregon and Washington, HuNoVs and different animal enteric caliciviruses were detected in the same bay in the same or different seasons (Table 5). In Oregon, HuNoVs and PoNoVs were simultaneously detected in oysters collected from all bays sampled during the summer (bays 1W, 2W, and 11W), whereas in the winter PoSaVs and BoNoVs were detected in different bays (bays 1W and 2W) and no PoNoVs or HuNoVs were detected. In Washington, HuNoVs were detected in one bay (bay 4W), PoNoVs were detected in another bay (bay 6W), and both were detected in bay 3W during the summer. In the winter only HuNoVs were detected in bay 3W, and BoNoV, but not HuNoV, was detected in bay 4W.

DISCUSSION

Human health problems associated with bivalve shellfish are well documented (32). Viruses are strict intracellular pathogens that cannot replicate in food or water. Therefore, food-borne viral infectious diseases depend on the initial concentration of virus in the food, host susceptibility, virus stability, and the dose required for infection. Although SaVs preferentially cause disease in children, NoVs do not distinguish between children, the young, and adults, and host susceptibility is based on ABH-histo blood group antigens, with different patterns of attachment for each strain (25). In addition, NoVs and SaVs are nonenveloped viruses that are resistant to disinfection, heat, and pH changes, and the infectious dose is as low as 10 to 100 particles (42). Therefore, enteric caliciviruses (particularly NoVs) are ideally suited to their role as food-borne disease-associated pathogens, which explains why an estimated 50 to 66% of all food-borne illnesses with known etiology are caused by HuNoVs (64).

Most food-borne disease outbreaks are due to direct contamination of food or water by a food handler during food distribution at the end of the chain, but contamination may also occur at any point from harvest to table. In the case of seafood, the association of shellfish-transmitted infectious diseases with sewage pollution, symptomatic or asymptomatic cases, or illegal overboard sewage discharges into harvest areas has been well established worldwide, and human enteric viruses are the most common etiological agents transmitted by bivalve shellfish (48). In United States, the first seafood-associated NoV outbreaks occurred in 1980, and they were due to oysters that were harvested in Florida and were contaminated with NoVs (20). Since then, several outbreaks have been traced back to the harvest location in the same state or different states (4, 11). Oyster-related outbreaks will continue unless

frequent monitoring and more stringent control measures are established.

In this study, oysters from oyster beds were purchased from different markets in the coastal United States; these oysters were approved for human consumption and were supplied by licensed shippers that harvest oysters from bays on the East, West, and Gulf coasts. To assess the presence of human or animal enteric caliciviruses, 33 bays (12 bays on the East Coast, 12 bays on the West Coast, and 9 bays on the Gulf Coast) during the summer and 12 bays (4 bays on each coast) during the winter (a total of 45 bays) were randomly selected. Different methodologies have been used to assess viral contamination in oysters. Here we used a method based on analysis of dissected tissue (3), which allowed us to obtain a more representative sample of the overall contamination, because oysters from the same bay and harvested at the same time were processed together and pooled.

Enteric caliciviruses (human and/or animal) were detected by RT-PCR with broadly reactive and genogroup-specific primers in oysters from 28 of the 45 bays (62%) during the summer or winter (Tables 3 and 4). Human and animal enteric caliciviruses were detected simultaneously in five samples (11%) (three samples from Oregon, one sample from Washington, and one sample from Delaware); only human enteric caliciviruses were detected in 18 samples (40%), and only animal enteric caliciviruses were detected in five samples (11%). Other reports have shown lower levels of detection in areas that were authorized or not authorized for harvesting. Human noroviruses were detected in 0 to 16% of samples collected from 20 different areas authorized for harvesting (European Community category A, less than 230 *Escherichia coli* cells per 100 g of shellfish flesh in 90% of the samples; European Community category B, less than 4,600 *E. coli* cells per 100 g of shellfish flesh in 90% of the samples) in northern and southern Europe (16). In France HuNoVs were detected in 23% of samples collected from authorized harvesting areas (European Community category B) during a 3-year study, and in England NoVs were detected in 27% of samples collected from a highly polluted area (34, 36). In a study performed in the United States in 2003, 52% of samples that were previously depurated and relocated to a prohibited shellfish-growing area were positive for HuNoVs (52). Our results showed a higher percentage of positive samples than the percentages detected in other studies, although the samples came from approved shellfish-growing areas based on the criteria used for bacterial contamination (fecal coliform level). The difference could be a consequence of the sampling protocol, because in our case samples were collected at only two times and in the other studies samples were collected during consecutive months and included many negative samples that decreased the percentage of positive samples. Our results confirm that fecal coliforms are not an appropriate marker for viral contamination and that contamination by virus should be directly assessed until a good marker is found. Other studies have suggested that F-RNA bacteriophage could be used as a viral indicator. However, this proposal is questionable because in some reports these phages correlate well with the presence of human enteric viruses, whereas in other reports the correlation is poor (10, 44).

Hybridization assays with HuNoV genogroup I or II probes were used to confirm RT-PCR products and to group the strains into genogroup I or II. The HuNoV strains detected belonged to genogroup II. However, hybridization did not occur with 11 of 20 RT-PCR samples that were positive for HuNoVs, presumably because of differences in the genome segment used for the probes, as previously reported (60). The samples positive for genogroup II HuNoVs after hybridization included two samples from the Gulf Coast (bays 10G and 12G), two samples from the East Coast (bays 5E and 11E), and five samples from the West Coast (bays 1W, 2W, 3W, 10W, and 11W). Sequence analysis of the 116-bp segment obtained with primers NVp110 and NI for samples from Washington (bay 3W), Oregon (bay 1W), Louisiana (bay 12G), New York (bay 5E), and Mississippi (bay 10G) revealed the highest levels of identity with the norovirus GII/4 strain, particularly with the NoV 95/96-US subset and other Lordsdale strains previously detected in oysters, implicated in diarrhea outbreaks in the United States, and distributed worldwide (13, 45, 51). However, because the sequence analyzed was short, further studies are required to assess strain identity.

Interestingly, three samples (from bays 7E, 7G, and 8G) were positive as determined by RT-PCR with the broadly reactive primers NVp110 and p290 but were negative with all NoV genogroup-specific primers (specific for genogroups I and II). These three samples were also negative with primers designed for animal enteric caliciviruses. These results could be explained by the fact that NVp110 and p290 can also detect SaVs (27). Although SaVs are not frequently associated with food-borne outbreaks (19), the possibility of water contaminated with enteric caliciviruses other than genogroup II NoVs cannot be dismissed. Most of the surveillance systems used so far have tested only for NoVs because of their close association with food-borne outbreaks, but other members of the *Caliciviridae* family (i.e., SaVs) could also be present.

To our knowledge, animal enteric caliciviruses have not previously been found in shellfish. In our survey, animal enteric caliciviruses were detected by RT-PCR, and the results were confirmed by hybridization or sequence analysis with oysters obtained from 10 of 45 bays distributed on the three coasts. Seven of 45 samples (16%) were confirmed to be PoNoV positive after hybridization. The positive samples included six samples from the West Coast (California, Washington, and Oregon) and one sample from the East Coast (Delaware). Sequence analysis of the 210-bp segment of these samples obtained with primers PNV7-Bio and PNV8 revealed the presence of genogroup II NoVs. In our study PoSaV was also detected in Oregon and BoNoV was detected in samples from Oregon and Washington. BoNoVs, PoNoVs, and PoSaVs have been detected and characterized directly from cattle and swine samples in the United States, as well as in Europe and Japan (55, 57, 62). Phylogenetic analysis has indicated that the BoNoVs and PoNoVs differ from HuNoVs, but they cluster in the NoV genus with BoNoVs in genogroup III and with PoNoVs in genogroup II; the latter is the most frequently detected genogroup in humans. There is little data regarding interspecies transmission of enteric caliciviruses. Animal caliciviruses in the genus *Vesivirus* have wide host ranges, and interspecies transmission has been documented (54). Although animal enteric caliciviruses have not been isolated from humans, human infection with NoVs related to genogroup III BoNoV was suggested by the presence of antibodies against

BoNoV GIII/2 in veterinarians in The Netherlands (63). On the other hand, Oliver et al. (47) and Han et al. (23) have reported that bovine strains are unlikely to be a risk for humans, because they form a third genogroup distinct from the HuNoVs. However in the case of PoNoVs, phylogenetic analysis has indicated that PoNoV strains belong to two distinct PoNoV clusters, clusters 11 and 19, and also to cluster 18, which includes human genogroup II strains (62), the most widely detected genogroup in humans, suggesting that under appropriate conditions interspecies transmission could occur (24).

Human and animal enteric caliciviruses were simultaneously detected in samples collected during the summer in Oregon, Washington, and Delaware. The fact that two or more human viruses can be detected in the same sample has been observed previously. In France simultaneous detection of at least four different human viruses (NoVs, rotavirus, enterovirus, and astrovirus) was described for 10% of samples collected during 3 years (36). The coexistence of several genogroup I and II NoVs and different genotypes of each genogroup was reported for oysters associated with food-borne outbreaks in Japan between December 1998 and January 2001 (28). In this study we focused not only on enteric caliciviruses but also on both human and animal strains. The confirmed presence of two genogroup-related strains (human and/or animal) in the same sample indicates that there is a potential risk of recombination among similar genogroup members in the human host. The detection of HuNoVs and HuSaVs whose genomes were derived from naturally occurring recombinants between members of the same (26, 29, 30) or different (5, 24) genogroups (intragenogroup and intergenogroup recombination, respectively), the existence of chimeric BoNoVs within NoV genogroup III (23, 46), and the recent detection of PoNoVs that belong to genogroup II of the NoV genus suggest that coinfection with genetically closely related human and animal enteric caliciviruses could result in genomic recombination between the viruses (as part of the natural evolution of NoVs) and emergence of new strains relevant to the control of NoV outbreaks in humans.

The seasonal and geographic distribution of the positive samples showed that there was a dynamic pattern. Human caliciviruses were not detected in three states (California, South Carolina, and New Jersey), whereas samples from the other 10 states were positive in the summer (54%, 18/33 samples) and/or the winter (42%, 5/12 samples). Two studies performed in Europe suggested that there was seasonal variation in the number of NoV-positive samples detected and that a larger number of positive samples was obtained during the winter. Formiga-Cruz et al. (16) indicated that there was increased detection of NoVs in samples collected during January and February, whereas Le Guyader et al. (36) showed that in France there was increased detection of HuNoVs in samples collected from November to January. We found a higher prevalence during the summer, but there was not a significant correlation with season, perhaps because a large geographic area was covered by our sampling protocol. Similar to our results, in studies of shellfish from the Norwegian Coast Myrmel et al. (44) did not find a correlation between the presence of HuNoVs in shellfish and season.

Within each state, when calicivirus was detected, it was present in both seasons (Washington, New York, and Maine), only in the summer (Oregon), or only in the winter (Virginia). In addition, some bays were positive and others were negative in the same state at different times. As reported for France, HuNoVs were not always detected at the same site in every season. This observation may be partially explained by the presence of fecal waste from human-associated recreation in the water, illegal dumping, accidental contamination of water with human waste with or without treatment (floods, etc.), or contamination during harvesting by infected but asymptomatic workers.

In contrast to the observations for HuNoVs, animal enteric caliciviruses were present in only four states (Washington, Oregon, California, and Delaware). Also, the seasonal distribution was less variable. Whereas PoNoVs were detected only in the summer, samples positive for BoNoVs and PoSaVs were obtained in the winter. It is not surprising that most of these samples were from Washington and Oregon, because these states have the highest livestock production among the states included in this study. What is noteworthy is the distribution of positive samples in these states. In Washington, HuNoVs were detected in samples from bays 6W and 3W, in which PoNoV was also detected. However, in the winter HuNoVs were detected only in bay 3W and BoNoVs were detected in bay 6W. Also notable was the detection of enteric caliciviruses in Oregon, where HuNoVs and PoNoVs were detected by RT-PCR in the three bays studied, as confirmed by hybridization and sequencing; however, in the winter no HuNoVs or PoNoVs were detected, but PoSaVs and BoNoVs were present.

To our knowledge, no data have been published previously confirming viral contamination in commercial shellfish areas that are representative of a range of geographic locations along the U.S. coasts and during different seasons. Our results support previous reports that confirmed that there was direct enteric calicivirus contamination of harvested seafood (38). Also, the presence of enteric caliciviruses of animal origin in shellfish with potential risk for humans has not been reported previously. To our knowledge, this study is the first study to demonstrate that both human and animal enteric caliciviruses are present in oysters from approved harvesting areas. Because these areas have been approved based on fecal coliform levels, a different marker needs to be used to determine contamination by enteric viruses. The fact that the oysters were purchased from farmers, wholesalers, and retailers suggests that there is a potential disease risk that requires confirmation by outbreak investigation. The simultaneous detection of human and animal enteric caliciviruses raises concerns about possible human infection or coinfection of humans with human and animal enteric caliciviruses, resulting in recombination and the emergence of new strains relevant for the control of disease.

ACKNOWLEDGMENTS

We are grateful to M. G. Han and Q. Wang for their helpful advice and comments. We thank M. K. Estes and R. L. Atmar (Baylor College of Medicine, Houston, TX) for the IC. We thank Ana Azevedo for technical assistance. Sequencing was performed at the Plant-Microbe Genomics Facility of the Ohio State University.

Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University, and by NRICGP/USDA Epidemiologic Approaches to Food Safety award 2001-35212-10876.

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