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NOTES

Collaborative Evaluation of a Method for the Detection of Norwalk Virus in Shellfish Tissues by PCR

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A multicenter, collaborative trial was performed to evaluate the reliability and reproducibility of a previously described method for the detection of Norwalk virus in shellfish tissues with the PCR (R. L. Atmar, F. H. Neill, J. L. Romalde, F. Le Guyader, C. M. Woodley, T. G. Metcalf, and M. K. Estes, Appl. Environ. Microbiol. 61:3014–3018, 1995). Virus was added to the stomachs and hepatopancreatic tissues of oysters or hard-shell clams in the control laboratory, the samples were shipped to the participating laboratories, and viral nucleic acids were extracted and then detected by reverse transcription-PCR. The sensitivity and specificity of the assay were 85 and 91%, respectively, when results were determined by visual inspection of ethidium bromide-stained agarose gels; the test sensitivity and specificity improved to 87 and 100%, respectively, after confirmation by hybridization with a digoxigenin-labeled, virus-specific probe. We have demonstrated that this method can be implemented successfully by several laboratories to detect Norwalk virus in shellfish tissues.

Norwalk virus (NV) and Norwalk-like viruses cause gastroenteritis in association with the consumption of raw or undercooked shellfish (6, 7, 20, 30). The use of bacterial indicators of fecal pollution, the current method of ensuring the sanitary quality of shellfish, may fail to detect viral contamination (6, 20, 21). To circumvent this problem, several investigators have developed methods to directly detect these viruses in shellfish (3, 4, 10, 12, 14, 23, 25–27, 31, 33). We have previously reported a method for the detection of NV and hepatitis A virus from shellfish tissues with reverse transcription-PCR (RT-PCR) (4). This method was more sensitive and had fewer problems with the presence of inhibitors than an earlier virus detection method in which whole shellfish were utilized (3).

To establish the reliability and reproducibility of this method among potential users, a multicenter, collaborative evaluation of the method was performed. Participating laboratories were ones that could be involved in the evaluation of virus contamination of shellfish. To minimize interlaboratory variability not attributable to assay performance, all reagents and disposable supplies were provided by the control laboratory at Baylor College of Medicine. The protocol did not control for interlaboratory variability of nondisposable equipment used in the study.

Five laboratories participated in the collaborative study. All of the laboratories first demonstrated the ability to detect NV which had been extracted from stool and diluted. NV was obtained from the stool of a human subject (no. 551) who had been challenged with virus (13, 16). A 50% suspension of stool was made in phosphate-buffered saline (PBS; 145 mM sodium chloride, 7.7 mM disodium phosphate, 2.3 mM monosodium phosphate, pH 7.4) and extracted with an equal volume of trichlorotrifluoroethane (Freon 113; E. I. du Pont de Nemours & Co., Wilmington, Del.). NV RNA was extracted from stool as previously described (18), and a solution of extracted viral RNA was sent to each laboratory. The RNA was serially diluted 10-fold, and 20 μl of each dilution was used to synthesize cDNA. RT was performed at 43°C for 1 h in 30 μl of a reaction mixture containing 10 mM Tris hydrochloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 3.3 μM NVp35 (5′-CTTGTTGGTTTGAGGCCATAT-3′), 667 μM deoxynucleoside triphosphates, 20 U of RNasin (Promega, Madison, Wis.), and 5 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.). A 70-μl portion of a PCR mixture was added to each sample to yield a solution with final concentrations of 10 mM Tris hydrochloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 1 μM (each) NV p35, NVp36 (5′-ATAAAAGTTGGC ATGAACA-3′), 200 μM deoxynucleoside triphosphates, and 5 U of *Taq* polymerase (The Perkin-Elmer Corp., Norwalk, Conn.) (3, 18). As appropriate for each laboratory, the samples were overlaid with mineral oil and the cDNA was amplified with a thermal cycler (the model and the need for mineral oil varied among laboratories). Cycling conditions were as follows: initial heat denaturation at 94°C for 4 min; 40 cycles of template denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min 30 s, and primer extension at 72°C for 1 min; and a final extension at 72°C for 15 min. A positive and a negative reagent control were included in each assay. NV-specific am-

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plicons 470 bp in length were detected by agarose gel electrophoresis and ethidium bromide staining. Prior to shellfish trials, an initial test was conducted to ensure that the level of detection of each laboratory's RT-PCR assay was comparable to that of the control laboratory's RT-PCR assay. All laboratories reached the same end point by successfully detecting RNA to the 10^{-4} dilution (equivalent to 42 copies of NV RNA).

Three laboratories participated in the first two trials, and all five laboratories took part in the final four trials. Shucked oysters (*Crassostrea virginica*) packaged in a glass jar were obtained from a retail market and used for trials 1 to 3; live oysters, obtained from a local wholesale seafood outlet (The Dutchman's Seafoods, Houston, Tex.), were used in trial 4. Live hard-shell clams (*Mercenaria mercenaria*; South Carolina Department of Natural Resources, Charleston) were used in trials 5 and 6. The stomachs and digestive diverticula were removed from the shellfish by dissection. For each trial, three separate pools of shellfish digestive diverticula and stomachs were made; 100 RT-PCR units of NV per 1.5 g of shellfish tissue was added to one of the pools, and 1,000 RT-PCR units was added to a second pool. An RT-PCR unit represented approximately 42 copies of the NV genome and was used because NV cannot be grown in cell culture or quantitated by electron microscopy (4). The third pool, containing no added virus, served as a negative control. The tissues were homogenized in a solution containing 7.3 ml of PBS and 0.2 ml of antifoam B (Sigma, St. Louis, Mo.) per 1.5 g of shellfish for four 60-s intervals at high speed with a semimicrocontainer (Eberbach Corp., Ann Arbor, Mich.) attached to a Waring power unit (Dynamics Corp. of America, New Hartford, Conn.). For trials 1 and 2, five aliquots of each sample were made, while for trials 3 to 6, six aliquots of each sample were made. The extra aliquots were stored at the control laboratory. For trial 5, approximately 0.03 g of oyster stomach and digestive diverticulum was added to each clam tissue sample to reach a final total of 1.5 g per sample, and in trial 6, approximately 0.3 g of oyster tissue per sample also was used to reach a final total of 1.5 g per sample. The samples were prepared on a Monday, placed on wet ice, and shipped overnight (via Federal Express) to the participating laboratories, including the control laboratory.

The samples were processed on Tuesday-Thursday with reagents which had been provided by the control laboratory. Each laboratory followed a detailed protocol supplied by the control laboratory which included a slight modification of a previously described method (4). Upon receipt, the 9-ml samples, prepared in the control laboratory, were transferred into 50-ml polyallomer tubes. The shipping tube was rinsed with an additional 3 ml of PBS, and the resulting material was combined with the original sample. A 6-ml aliquot of chloroform-butanol (1:1 [vol/vol]) was added to each sample, and the mixtures were vortexed for 30 s twice, with a 15-s rest interval. Cat-Floc T (Calgon Corp., Elwood, Pa.) was added to each sample to a final concentration of 2.7 ppt, and the tubes were mixed by inversion. After being rocked gently for 5 min at room temperature, each sample was allowed to settle for 15 min at room temperature. The samples then were centrifuged at 13,500 × *g* for 15 min at 4°C, and the aqueous phases were transferred to clean tubes containing 6.5 ml of a solution of polyethylene glycol 6000 (24% [wt/vol]; BDH Ltd., Poole, England) and sodium chloride (1.2 M). The samples were rocked for 1 h at 4°C and centrifuged for 20 min at 11,000 × *g* and 4°C. The supernatants were removed by decanting, and the pellets were stored overnight at 4°C. The next day, each of the polyethylene glycol pellets was suspended in 3 ml of water. After digestion of the virus with proteinase K (0.2-mg/ml final concentration) (Amresco, Solon, Ohio) for 30 min at 56°C, the samples were extracted twice with an equal volume of phenol-chloroform-water (68:18:14) (Applied Biosystems, Foster City, Calif.) and the aqueous phases were precipitated in a solution containing 3 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2) for 30 min with a dry-ice–ethanol bath. Following centrifugation at 15,000 × *g* for 30 min, the resulting pellets were suspended in water, and cetyltrimethylammonium bromide (Sigma) and sodium chloride were added to final concentrations of 1.4% and 0.11 M, respectively. The samples were incubated for 15 min at room temperature and then centrifuged for 30 min at 15,000 × *g* and 25°C. The pellets were suspended in 1 M sodium chloride and precipitated in a solution containing 3 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2). The precipitated nucleic acids were suspended in 100 μl of water, and 20 μl of each suspension was used for RT-PCR as described above.

TABLE 1. Detection of NV in shellfish tissues by laboratories participating in the collaborative trial

Laboratory	Result with no. of RT-PCR units[a]								
	Oysters			Clams			Total		
	0	100	1,000	0	100	1,000	0	100	1,000
A	1/4[b]	3/5[c]	3/3	0/2	2/2	2/2	1/6[b]	5/7[c]	5/5
B	1/3[b]	3/3	2/3				1/3[b]	3/3	2/3
C	0/4	5/5	2/3	0/2	0/2	2/2	0/6	5/7	4/5
D	0/2	3/3	1/1	0/2	2/2	2/2	0/4	5/5	3/3
E	0/2	3/3	1/1	0/2	1/2	2/2	0/4	4/5	3/3
Total	2/15	17/19	9/11	0/8	5/8	8/8	2/23	22/27	17/19

[a] Results were obtained by visual interpretation of ethidium bromide-stained agarose gels and are expressed as the number of positive samples per the total number of samples tested.
[b] Positive sample negative after hybridization with NV-specific probe.
[c] One additional positive sample after hybridization with NV-specific probe.

The results of each trial were faxed to the control laboratory on Friday, and photographs of agarose gels depicting electrophoretic analyses of the RT-PCR products were sent to the control laboratory for independent interpretation. An assay was considered interpretable if the negative reagent control was negative and the positive reagent control (containing 10 RT-PCR units, or approximately 420 copies, of NV RNA) was positive.

The results of the six trials for detecting 100 or 1,000 RT-PCR units in oyster and clam tissues are shown in Table 1. All laboratories except laboratory B had interpretable results for all trials. Laboratory B had interpretable results only for the first three trials; thereafter, the positive control in each RT-PCR assay was negative. Attempts to improve the performance of the RT-PCR assay in laboratory B, including the replacement of all reagents, failed to increase the sensitivity of the assay. Laboratories D and E participated only in the final four trials. The overall sensitivities (the ratios of the number of shellfish with virus added that were assay positive to the total number of shellfish with virus added) of the extraction-amplification method were 81 and 89% for 100 and 1,000 RT-PCR units, respectively, while the specificity (the ratio of the number of shellfish with no added virus that were assay negative to the total number of shellfish with no added virus) was 91%. Only one laboratory (D) correctly identified all virus-containing samples.

In several trials, the presence of extra bands after agarose

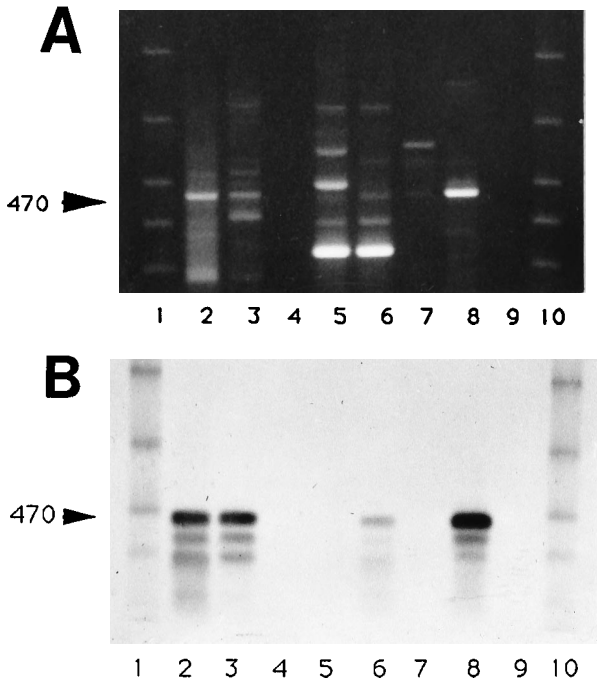

FIG. 1. Detection of NV in shellfish tissues. (A) Detection of RT-PCR products by using an ethidium bromide-stained agarose gel. Lanes 1 and 10, molecular weight marker; lanes 2 to 4, clams spiked with 1,000 and 100 RT-PCR units and no NV, respectively (from laboratory C, trial 5); lanes 5 and 7, oysters with no NV but initially interpreted as positive (from laboratories A and B, respectively); lane 6, oyster spiked with 100 RT-PCR units initially interpreted as negative (from laboratory A); lane 8, NV RNA (positive control); lane 9, negative reagent controls. (B) Southern blot of gel from panel A. A digoxigenin-labeled, NV-specific probe (NVp69-dig) was used. Lanes are the same as in panel A. Numbers at arrowheads are the numbers of base pairs.

gel electrophoresis made interpretation of the results difficult, including the results for the two samples which were incorrectly identified as containing virus. To evaluate whether the false positives were due to (i) contamination of the sample during processing or (ii) visual misinterpretation of the gel due to the presence of nonspecifically amplified nucleic acids, the amplified products were further evaluated by Southern blot hybridization, using a digoxigenin-labeled oligomer (NVp69) (5′-GGCCTGCCATCTGGATTGCC-3′) homologous to a region of the NV genome amplified by NVp35 and NVp36. This assay was performed by the control laboratory on all samples from each trial, as described previously (4). Neither of the two false positives was detected by Southern blot hybridization (Fig. 1), and one of the samples originally interpreted as negative (an oyster sample spiked with 100 RT-PCR units and processed by laboratory A) was found to be positive. Thus, the addition of a hybridization assay increased the overall specificity of the assay to 100% and increased the sensitivity of detection of 100 RT-PCR units to 85%. Similar findings have been noted previously (4, 22). A confirmatory probe hybridization assay should be used when shellfish tissues are examined by RT-PCR for the presence of NV.

False-negative and a few true-negative samples were evaluated in the control laboratory for the presence of inhibitors of RT-PCR. An internal RNA standard for the detection of PCR inhibitors was prepared as previously described (4, 15). A total of 420 copies of RNA standard were added to the test sample and amplified with NVp35 and NVp36 as described above. In the absence of significant inhibition, amplicons that were 445 bp in length, 25 bp shorter than the NV amplicons, could be detected by agarose gel electrophoresis. Of the seven false negatives, four failed to yield specific RT-PCR products after amplification with the transcripts serving as an internal RNA standard (i.e., inhibitors were present [Fig. 2]). Of the three remaining samples, two samples previously identified as negative yielded RT-PCR products of the correct size for both NV and the transcripts and one yielded only amplicons of the size expected from amplification of the internal RNA transcripts. One of the new positives was the oyster sample processed by laboratory A which had previously been positive by hybridization, and the other sample was a clam sample spiked with 100 RT-PCR units and processed by laboratory E. All six true-negative samples tested yielded appropriately sized RT-PCR products. These results were confirmed by Southern hybridization.

Three of the five laboratories had at least one false negative due to the persistence of inhibitors, and at least one laboratory detected viral RNA from each shellfish-virus mixture, suggesting that the persistence of inhibitors in the final samples was not due to the presence of excessive amounts of inhibitors in the original samples distributed for testing. Inhibitors of RT-PCR have been shown to interfere with the detection of nucleic acids in a wide variety of samples (4, 5, 22). The reason(s) for the persistence of inhibitors in some extracted samples is not known. Although this was not specifically tested by the

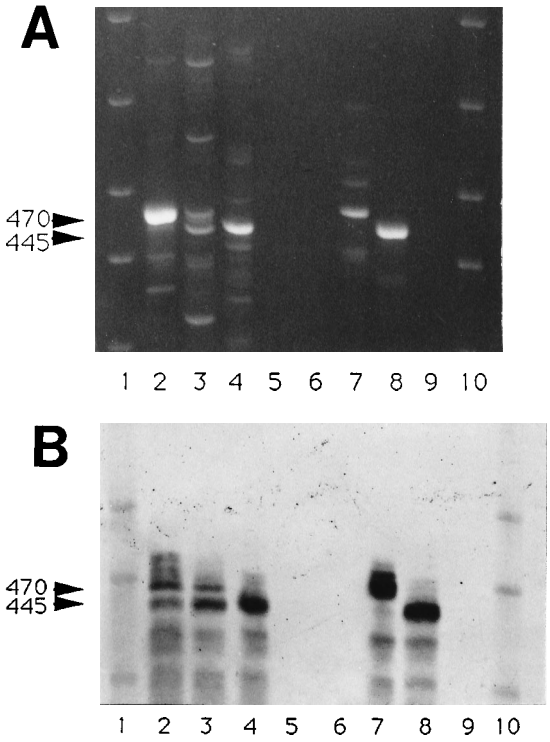

FIG. 2. Detection of NV and inhibitors in shellfish tissues by use of RNA transcripts as an internal standard. (A) Detection of RT-PCR products by using an ethidium bromide-stained agarose gel. Transcripts (420 copies) were added to RT-PCR mixtures for samples in lanes 2 to 6 and lane 8. Lanes 1 and 10, molecular weight marker; lanes 2 to 4, oysters spiked with 1,000 and 100 RT-PCR units and no NV; lanes 5 and 6, oysters spiked with 1,000 and 100 RT-PCR units, respectively, but with negative results due to the persistence of inhibitors (laboratory C); lane 7, NV RNA (positive control); lane 8, transcripts only; lane 9, negative reagent control. (B) Southern blot of gel from panel A. A digoxigenin-labeled, NV-specific probe (NVp69-dig) was used. Lanes are the same as in panel A. Numbers at arrowheads are the numbers of base pairs.

collaborating laboratories, the results in the control laboratory suggest that the use of an internal control would allow the detection of NV at the concentrations tested and the detection of persistent RT-PCR inhibitors.

Several questions concerning the use of this method for the evaluation of viruses in shellfish remain. Four are addressed herein. First, can the method be improved further? The sensitivity (detection of samples with virus in them) is comparable to that seen with commercially available diagnostic assays (e.g., enzyme immunoassay, immunofluorescent-antibody staining) for other viruses in clinical samples (2). However, although the extraction method effectively removes most inhibitors, in some assays inhibitors to RT-PCR persist. The development of an antigen-capture method for Norwalk-like viruses similar to that developed for hepatitis A virus might overcome this problem, but to date no hyperimmune serum which is broadly cross-reactive with different human caliciviruses has been developed (9, 10, 12, 17, 19).

Second, can this method detect virus in shellfish epidemiologically associated with outbreaks of illness? Preliminary studies of oysters associated with a point source outbreak of viral gastroenteritis suggest that the answer to this question is yes (24). However, further studies of this and other outbreaks are needed to determine the ability of the method to detect the epidemic virus and to determine the percentage of shellfish which can be demonstrated to harbor virus. Furthermore, the use of primer sets besides NVp35 and NVp36 will be needed in order to successfully detect the greatest number of human caliciviruses (1, 8, 11, 28, 29, 32).

Third, what types of laboratories will be able to use these methods? This study demonstrated that the method could be performed in three federal laboratories. Scientists at each of these laboratories felt that the method could be performed in a state laboratory, providing the personnel received sufficient training.

Finally, what role does this method have in the evaluation of the pathogen-free status of shellfish harvested from natural waters? At this time there are not enough data to answer this question. The optimal primer sets to be used for virus detection, the number of shellfish to be tested, the need for testing water or sediment in the area from which shellfish are harvested, the comparative utility of the detection of caliciviruses versus other viruses (e.g., enteroviruses and astroviruses), and the economic feasibility of using this method are just some of the issues which should be resolved prior to the use of this method in a regulatory fashion. However, further RT-PCR studies can be undertaken to address these issues.

In summary, this collaborative study demonstrated that the method previously developed by one of the laboratories for the detection of NV in shellfish tissues could be performed successfully by other laboratories with a high degree of sensitivity and specificity. Variables which theoretically could alter test performance, such as the source of reagents and disposable supplies, were not examined. Several problems which emphasize the need for multiple controls in the assay were recognized during the course of the study. In order to obtain optimal sensitivity and specificity, a hybridization assay had to be performed. This was not recognized when the collaborative study was originally designed; thus, a hybridization assay was not incorporated into the collaborative-study protocol. However, when such an assay was used, the sensitivities of the NV detection method ranged from 75 to 100% for the different laboratories and the specificity was 100%. After hybridization, the sensitivities for detecting 100 and 1,000 RT-PCR units of virus were comparable (85 and 89%, respectively), as were those for detecting virus in oysters and clams (90 and 81%, respectively). Further studies using this method will determine its utility in the detection of NV and related viruses in naturally contaminated shellfish.

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