JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1995, p. 835–839 Vol. 33, No. 4
0095-1137/95/$04.00+0

Detection and Identification of Aquatic Birnaviruses by PCR Assay†

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Received 2 September 1994/Returned for modification 8 November 1994/Accepted 4 January 1995

A reverse transcriptase polymerase chain reaction (RT-PCR) assay was developed for the detection and identification of aquatic birnaviruses. The four sets of primers (PrA, PrB, PrC, and PrD) that we used are specific for regions of cDNA coded by genome segment A of aquatic birnaviruses. PrA identifies a large fragment (1,180 bp) within the pVP2-coding region, and PrB identifies a 524-bp fragment within the sequence amplified by PrA. Primer set PrC frames a genome fragment (339 bp) within the NS-VP3-coding region, and PrD identifies a 174-bp sequence within the fragment identified by PrC. PrB and PrD amplified cDNAs from all nine recognized serotypes of aquatic birnavirus serogroup A as well as the N1 isolate that may represent a 10th serotype. These results indicate that these three primer sequences are highly conserved and can be used in PCR assays for group identification of these viruses. PrA routinely produced amplification products from eight serotypes but exhibited variable results with one serotype, and primer PrC identified 6 of the 11 virus isolates tested. The qualitative sensitivity of the RT-PCR assay was evaluated by comparison of the results with those of cell culture isolation assays. With the exception of one sample, the RT-PCR assay with primer PrD was as accurate as cell culture isolation for detecting virus in kidney and spleen tissues from naturally infected, asymptomatic carrier fish. These results indicate that the RT-PCR assay can be a rapid and reliable substitute for cell culture methods for the detection of aquatic birnaviruses.

The aquatic birnaviruses are the largest and most diverse group of viruses within the family *Birnaviridae* and include viruses from numerous species of fish and invertebrates (16). Many of these viruses, such as infectious pancreatic necrosis virus (IPNV), have been proven or implicated as the etiological agents of diseases in cultured and wild freshwater and marine species. In addition, aquatic birnaviruses have been isolated from a variety of apparently healthy freshwater and marine species. All of the aquatic birnaviruses are similar in morphology and biochemical and biophysical properties, and most are closely related antigenically.

Aquatic birnaviruses are characterized by a bisegmented double-stranded RNA genome within a nonenveloped, icosahedral capsid of about 60 nm in diameter (4). The smaller genome segment (segment B) encodes a single protein, the virion-associated transcriptase (VP1, 90 to 110 kDa). The larger genome segment (segment A) contains one large open reading frame of about 3,000 bp that encodes a precursor polyprotein (100 kDa). This polyprotein is subsequently cleaved to form three viral proteins (pVP2, 63 kDa; NS, 29 kDa; and VP3, 29 to 31 kDa) by the protease activity associated with the NS protein (10). The gene order of segment A is 5′-pVP2-NS-VP3-3′. The pVP2 protein is further processed to yield the major capsid protein VP2 (50 to 55 kDa). The cDNAs from the A genome segments of two virus isolates (Jasper [Ja] and N1) have been cloned and sequenced (5, 6).

The vast majority of aquatic birnaviruses, regardless of host species or geographic origin, are related antigenically and form a major serogroup (serogroup A) (2, 3, 13). A relatively few antigenically unrelated aquatic birnaviruses represent a second, minor serogroup (serogroup B). On the basis of the results of reciprocal neutralization tests with polyclonal antisera and enzyme immunoassays with monoclonal antibodies, serogroup A contains nine cross-reacting serotypes: West Buxton (WB; originally VR299), Sp, Ab, He, Te, Canada 1 (C1), Canada 2 (C2), Canada 3 (C3), and Ja. Virtually all aquatic birnavirus isolates from freshwater and marine fish in the United States belong to the WB serotype. Four serotypes (C1, C2, C3, and Ja) occur in Canada, and four serotypes (Sp, Ab, He, and Te) as well as the N1 isolate are found in Europe. Isolates representing Ab, Sp, and WB have been found in Asia and South America.

At present, the only truly effective approach for the control of aquatic birnavirus infection in aquaculture is to prevent exposure to virus. In some areas, governmental regulations control the movement of fish and fish products by specifying that artificially propagated fish stocks be free of aquatic birnaviruses and other specific pathogens. Consequently, fish health inspection programs have been developed to identify infected populations. The success of such fish health management programs depends on the rapid detection and identification of specific pathogens. With aquatic birnaviruses, relatively high titers of infectious virus are found in moribund fish, but virus titers in asymptomatic virus carriers can be negligible. The standard method for the detection of aquatic birnaviruses requires inoculation of fish cell cultures with homogenates of fish tissue (1). Once a virus has been isolated, it must be identified by infectivity-neutralization tests with polyclonal antisera or by enzyme immunoassays with monoclonal antibodies or polyclonal antisera (2, 3, 11, 13). Recently, several investigators have described PCR assays for the identification of specific strains of aquatic birnaviruses in infected cell cultures (7, 9, 12, 14). The general objective of the study described here was to

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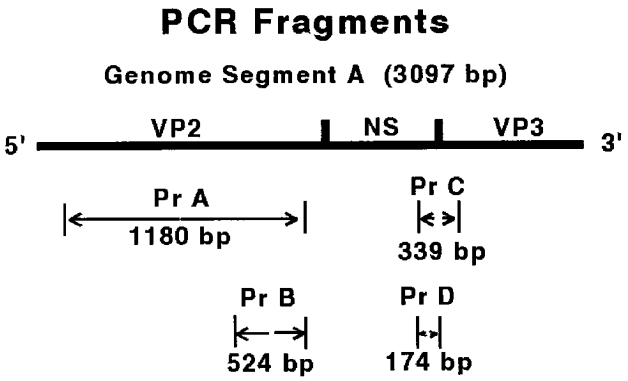
† This is Maine Agricultural and Forest Experiment Station Publication 1895.



Primer Sequences

Pr A (bp 151-1330)
Pr A1 5'-TGAGATCCATTATGCTTCCAGA-3'
Pr A2 5'-GACAGGATCATCTTGGCATAGT-3'

Pr B (bp 807-1330)
Pr B1 5'-GCCGACATCGTCAACTCCAC-3'
Pr B2 5'-GACAGGATCATCTTGGCATA-3'

Pr C (bp 2139-2477)
Pr C1 5'-AAAGCCATAGCCGCCCATGAAC-3'
Pr C2 5'-ATCCTCCTTTGACCACTCATAC-3'

Pr D (bp 2139-2312)
Pr D1 5-'AAAGCCATAGCCGCCCATGAAC-3'
Pr D2 5'-TCTCATCAGCTGGCCCAGGTAC-3'

FIG. 1. Relative locations of amplification target sequences for primer sets PrA, PrB, PrC, and PrD on genome segment A of aquatic birnaviruses and primer pair sequences obtained on the basis of the published sequence of strain Ja (5).

develop a reverse transcriptase PCR (RT-PCR) assay as a rapid and sensitive method for the general identification of aquatic birnaviruses from infected cell cultures but, more importantly, to demonstrate also that the RT-PCR assay has sensitivity comparable to that of cell culture isolation for the direct detection and identification of virus from clinical samples.

MATERIALS AND METHODS

Viruses, cells, and virus plaque assay. The following strains of aquatic birnaviruses, representing the nine established serotypes and the proposed N1 serotype, were used: WB (serotype type strain), Sp (serotype type strain), Ab (serotype type strain), He (serotype type strain), Te (serotype type strain), Atlantic salmon virus (ASV; Canada 1 serotype), Canada 2 (serotype type strain), Canada 3 (serotype type strain), Ja (serotype type strain), and European eel virus (EEV; Ab serotype). All viruses were propagated at 20°C in chinook salmon embryo (CHSE-214) cell cultures (8) in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS) as described previously (2).

Collection, processing, and assay of clinical samples. Clinical specimens of kidneys (posterior half), spleen, mucus, and feces were collected from a hatchery production stock of yearling brook trout (*Salvelinus fontinalis*) that were IPNV carriers and survivors of natural IPNV infections. Samples were held at 4°C until they were processed as pools of samples from five fish. Samples were processed and assayed for viral infectivity within 6 h of collection, and subsets of the samples were shipped by express mail to Orono, Maine, for PCR assays. Kidney and spleen samples were processed by homogenization with mortar and pestle, diluted 1:20 (wt/vol) in 0.1 M phosphate-buffered saline (PBS; pH 7.2), and centrifuged at 1,500 × *g* for 20 min at 4°C. Supernatant fractions were collected and clarified by passage through 0.45-μm-pore-size, low-protein-binding membrane filters.

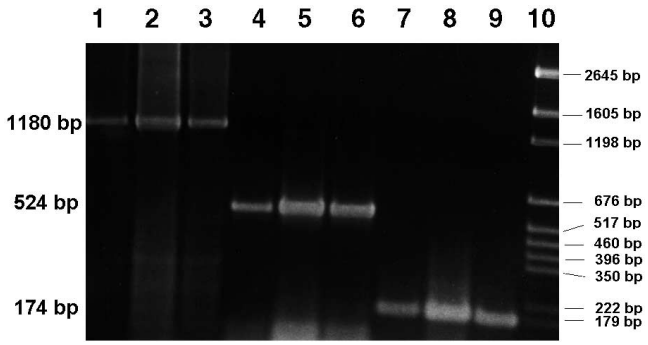


FIG. 2. Typical agarose gel showing the identification of WB virus in infected cell cultures by RT-PCR assay with primer sets PrA (lanes 1 to 3), PrB (lanes 4 to 6), and PrD (lanes 7 to 9). Lane 10, molecular size markers. The figure was prepared with a HP ScanJet II cx and Adobe Photoshop 2.5.

Surface mucus was collected by scraping with a sterile stainless steel spatula. Mucus was diluted 1:2 (wt/vol) in PBS, mixed thoroughly, sonicated at 75 W for 10 s, and centrifuged and filtered as described above. Fecal samples were collected by manually expressing fecal material from the anal vent and were processed as described above for mucus samples.

Sample dilutions were plated in duplicate, and replicate assays were performed at 15°C with CHSE-214 cells or rainbow trout mesothelioma cells grown at 18°C in EMEM containing 10% FBS and 100 μg of gentamicin per ml. The titer of infectious virus was determined by the plaque method of Wolf and Quimby (17), and the titer was expressed as the number of PFU per milliliter. Virus from clinical samples was identified as IPNV by dot blot enzyme-linked immunosorbent assay (11).

Nucleic acid extraction. Virus samples (100 μl of fluids from infected cell cultures or from clinical sample homogenates) were incubated with 400 μl of lysis buffer (1% sodium dodecyl sulfate, 0.15 M NaCl, 1.25 mM EDTA, 0.1 M Tris-HCl [pH 7.5]) and 6.75 μl of proteinase K (10 mg/ml in diethylpyrocarbonate-treated water) for 3 h at 37°C. One volume of phenol-chloroform (1:1) was added, the contents were mixed, and the mixture was centrifuged at 10,000 × *g* for 5 min. The aqueous layer was recovered and mixed with 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol. Samples were held overnight at −20°C and were then centrifuged at 15,000 × *g* for 30 min. Nucleic acid pellets were washed with 70% ethanol, air dried, and resuspended in 10 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]) and were stored at −70°C.

Primer selection and production. Primers were selected on the basis of published sequences of the cDNAs of genome segment A of the Ja and N1 strains of aquatic birnaviruses (5, 6). Primer sequences and their relative positions are shown in Fig. 1. Primer pairs were developed to identify and amplify one moderately sized sequence (524 bp; PrB) within the cDNA region encoding pVP2 and one relatively large sequence including most of the pVP2-coding region (PrA). Primer set PrC was designed by computer analysis to amplify a 339-bp region within the NS-VP3-coding region and was previously described by McAllister et al. (12). Seminested primer set PrD was designed in concert with primer set PrC to frame a smaller sequence (174 bp) within the NS-VP3-coding region identified by PrC (15).

cDNA synthesis. Samples were heated for 5 min at 95°C, cooled on ice, and briefly (5 s) centrifuged at 10,000 × *g*. Three microliters of each RNA sample was added to 17 μl of a reaction mixture containing 5 mM $MgCl_2$, 1 mM (each) the deoxyribonucleotide triphosphates, 40 U of RNasin, 1.25 mM random hexamer primers, and 200 U of Moloney Murine leukemia virus RT in Mg-free PCR buffer (Promega, Inc., Madison, Wis.). The contents of the tube were mixed, and the mixture was incubated for 1 h at 37°C.

PCR. A single PCR protocol was used with all four primer sets. Samples were heated for 5 min at 95°C and cooled on ice. PCR was carried out in a total volume of 100 μl of PCR buffer (Promega, Inc.) containing 2 mM $MgCl_2$, 42 pmol of each primer (Operon Technologies, Inc., Alameda, Calif.), and 2.5 U of *Taq* polymerase (Promega, Inc.). After the addition of 70 μl of mineral oil to prevent evaporation, the samples were subjected to 35 cycles (denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 1 min at 72°C) in an MJ Research (Watertown, Mass.) model PTC-100 thermal cycler.

Gel electrophoresis. Samples of PCR products (15 to 25 μl) were analyzed on 2.4% NuSieve GTG/Agarose LE (3:1; wt/wt) gels (FMC Bioproducts, Rockland, Maine) in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA) at 60 V for 3 h. The gels were stained with ethidium bromide and photographed.

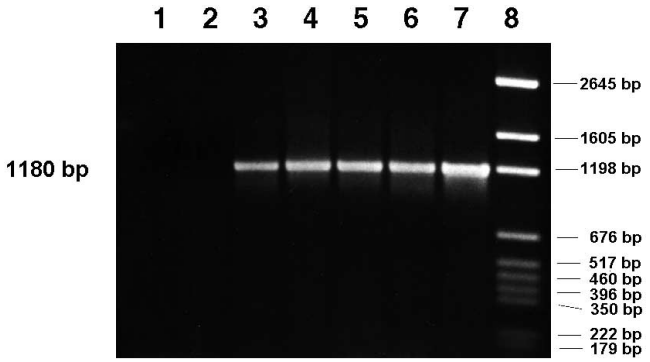


FIG. 3. Typical agarose gel showing the detection and identification of several different serotypes of aquatic birnaviruses in infected cell cultures by RT-PCR assay with primer set PrA. Lane 1, RNA control; lane 2, cell control; lane 3, ASV (serotype C1); lane 4, WB virus serotype WB); lane 5, EEV (serotype Ab); lane 6, N1; lane 7, Ab; lane 8, molecular size markers. The figure was prepared with a HP ScanJet II cx and Adobe Photoshop 2.5.

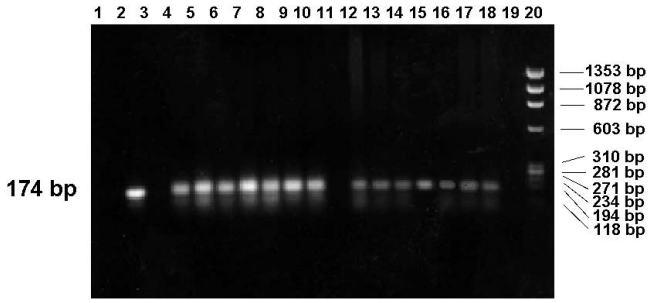


FIG. 4. Typical agarose gel showing the detection and identification of aquatic birnavirus in homogenates of kidney and spleen tissue from naturally infected, asymptomatic brook trout that were IPNV carriers by RT-PCR assay using primer set PrD. Lane 1, negative cell control; lane 2, no sample; lane 3, positive control (WBV); lanes 4 to 10, kidney tissue samples; lane 11, no sample; lanes 12 to 18, spleen tissue samples; lane 19, no sample; lane 20, molecular size markers. The figure was prepared by using HP ScanJet II cx and Adobe Photoshop 2.5.

RESULTS

Identification of aquatic birnaviruses from infected cell cultures. An RT-PCR assay was developed and evaluated for its capability to detect and identify representative strains of all nine recognized serotypes of serogroup A aquatic birnaviruses as well as the N1 strain, a proposed 10th serotype. Several sets of primers were designed and tested. Primer sets PrA and PrB were developed at the University of Maine, and primer sets PrC and PrD were developed at the National Fish Health Research Laboratory. A typical ethidium bromide-stained agarose gel of the amplification products of the WB type strain with primer pairs PrA, PrB, and PrD is shown in Fig. 2. Figure 3 shows typical results of the amplification of cDNAs from several different strains of aquatic birnaviruses with a single set of primers (PrA). The abilities of all four primer sets to identify representative strains of all serotypes of serogroup A and the N1 strain in infected cell cultures are summarized in Table 1. The PCR assay was capable of detecting and identifying all birnaviruses tested when primer sets PrB and PrD were used. PrA also was capable of identifying eight of the nine serotypes and strain N1, but it gave variable results with the He type strain virus. Primer set PrC identified 6 of the 11 virus strains tested; viruses representing Ab, C1, C2, and C3 were not detected by PrC. Negative controls included uninfected cell cultures and infectious hematopoietic necrosis virus, an unrelated fish virus (data not shown). The specificities of the reactions were confirmed by Southern blot hybridization with an Sp strain cDNA probe (data not shown). The sensitivity of the PCR assay was between 1 and 10 (50% tissue culture infective doses) per ml of tissue homogenate (data not shown).

Detection of aquatic birnaviruses from tissue specimens. In order to evaluate the potential of the RT-PCR assay to directly detect aquatic birnavirus in infected fish, several types of clinical specimens were collected from a population of naturally infected brook trout that were IPNV carriers. Homogenates of kidney and spleen tissues as well as samples of surface mucus and feces were tested by the RT-PCR assay with primer sets PrB and PrD and by virus isolation and quantification in cell culture. Although the PCR assay with PrB detected virus in infected cell cultures, amplification products from the PCR assay with PrB either were not detected in the clinical specimens or appeared as large, diffuse bands (data not shown). However, the PCR assay with primer set PrD detected virus in kidney and spleen tissues but not in mucus or feces. A typical agarose gel of the amplification products of kidney and spleen tissue samples tested with primer set PrD is shown in Fig. 4, and the ability of the PCR assay to detect virus in comparison with virus isolation is summarized in Table 2. With the exception of one pool of spleen tissue, the PCR assay with primer set PrD was as qualitatively accurate as cell culture for the detection of virus in kidney and spleen samples that contained relatively low titers of virus ($10^{3.2}$ to $10^{4.6}$ PFU/ml). Neither PCR

TABLE 1. Abilities of primers to identify all 10 serotypes of aquatic birnaviruses in infected cell cultures[a]

Virus	Amplification with primers[b]:			
	PrA	PrB	PrC	PrD
WB	+	+	+	+
Sp	+	+	+	+
Ab	+	+	−	+
He	±	+	+	+
Te	+	+	+	+
ASV	+	+	−	+
C2	+	+	−	+
C3	+	+	−	+
Ja	+	+	+	+
N1	+	+	+	+
EEV	+	+	−	+

[a] Amplifications were carried out as described in the text. +, amplification and a positive result; −, a negative result or failure to detect the indicated virus through amplification; ±, inconsistent amplification and detection.
[b] Primer sequences and locations are shown in Fig. 1 and 2, respectively.

TABLE 2. Comparison of RT-PCR with virus isolation in cell culture for detection of aquatic birnaviruses in samples from brook trout[a]

Sample	No. of positive samples/total no. of samples[b]	
	PCR with primer set PrD	Virus isolation (titer [PFU/ml])
Kidney	12/12	12/12 ($10^{3.3}$–$10^{5.5}$)
Spleen	11/12	12/12 ($10^{3.2}$–$10^{4.6}$)
Mucus	1/12	0/12 (ND)[c]
Feces	0/12	1/12 (ND–$10^{3.0}$)

[a] Amplifications and titer determinations were carried out as described in the text.
[b] Each sample consisted of a pool of tissues from five individual fish.
[c] ND, none detected. The sensitivity of the isolation assay was $10^{2.0}$ PFU/ml.

nor the cell culture isolation method detected a substantial prevalence of virus in samples of mucus or feces. The specificities of the reactions were confirmed by Southern blot hybridization with an Sp strain cDNA probe (data not shown).

DISCUSSION

Aquatic birnaviruses are the etiological agents of several diseases in wild and artificially propagated fish (16). Infectious pancreatic necrosis is a particularly significant disease in salmonid species worldwide. Currently, the only effective method of controlling the diseases caused by these viruses is by prevention of exposure of fish to the virus. Consequently, a number of countries, including the United States, have mandated fish health management programs that include inspections of artificially propagated fish for the presence of various fish pathogens including aquatic birnaviruses. The current method for the detection of aquatic birnaviruses requires isolation of virus by inoculation of cell cultures with homogenates of tissue samples collected from a statistically significant portion of the population (1). If virus is isolated, identification of the virus is required, usually by infectivity neutralization with specific polyclonal antisera or by enzyme immunoassay with monoclonal antibodies or polyclonal antisera (2, 3, 11, 13). Currently, no assay is available for the direct detection of aquatic birnaviruses in fish tissue samples.

Several recent reports have indicated that PCR assays can be used for the detection and identification of specific strains of aquatic birnaviruses in infected cell cultures (7, 9, 12, 14). In the investigation described here, we evaluated four different sets of primers (PrA, PrB, PrC, and PrD) in a PCR assay for the identification of all serotypes of serogroup A aquatic birnaviruses, including the N1 strain that may represent a 10th serotype. The primers were selected on the basis of the conserved regions in the published sequences of the Ja and N1 strains of aquatic birnaviruses (5, 6). Primer sets PrA and PrB frame sequences within the pVP2-coding region, whereas primer sets PrC and PrD define sequences within the NS-VP3-coding region. Primer set PrC was previously used in a PCR assay to identify the VR299 strain of virus in infected cell cultures (12), as was PrB for several isolates of aquatic birnaviruses from Asia (9). In our study, primer sets PrA, PrB, and PrD were capable of identifying, in infected cell cultures, representative viruses from all nine serotypes of serogroup A including the N1 strain of virus, except that variable results were sometimes obtained with type strain He by using PrA (Table 1). In contrast, primer set PrC identified 6 of the 11 virus strains tested, failing to identify serotypes Ab, C1, C2, and C3. We have previously shown that this PCR assay with primer set PrB identified a variety of recent serogroup A aquatic birnavirus isolates from Asia that apparently do not belong to any of the currently recognized monoclonal antibody serotypes (9). Thus, our results indicate that the sequences identified by primer sets PrA, PrB, and PrD are highly conserved among serogroup A aquatic birnaviruses and that the PCR assay described here is a simple and rapid method for the general identification of this group of viruses.

A diagnostic assay capable of detecting aquatic birnaviruses directly in fish samples would be of significant benefit for fish health management programs worldwide. Therefore, the PCR assay was evaluated for its efficacy in detecting the presence of virus directly in samples from a population of naturally infected, asymptomatic carrier fish. Homogenates of kidney and spleen and samples of mucus and feces were prepared and assayed in cell culture for the presence of aquatic birnaviruses at the National Fish Health Research Laboratory. Subsets of the samples were sent by express mail to the University of Maine and were tested for virus by RT-PCR. Unfortunately, during shipment, the samples were delayed and experienced a period of several days without refrigeration, which provided an opportunity for possible degradation of the viral RNA by the nucleases in the tissue homogenates. Nevertheless, by using primer set PrD, the PCR assay proved to be an accurate and sensitive method of directly detecting virus in samples of kidney and spleen in comparison with virus isolation (Table 2). Neither the PCR assay nor virus isolation in cell culture reproducibly detected substantive virus in samples of mucus or feces.

Although the PCR assay with primer set PrB was effective in identifying all of the viruses tested in infected cell cultures (Table 1), this primer set was not effective in detecting virus in the clinical fish samples used in the study (data not shown). Use of primer set PrB failed to detect virus in samples shown to be positive by cell culture tests, or amplification products appeared as broad, diffuse bands in agarose gels. A possible cause of these problems with PrB may have to do with the fact that this primer set defines a relatively large amplification target (524 bp), and substantial degradation of the viral RNA could have occurred in these samples during the prolonged shipping time without refrigeration. Since primer set PrA frames an even larger target (1,180 bp), no attempts were made to use this primer set in the PCR assay of the clinical samples. Primer set PrC was not tested because the studies with infected cell cultures showed that it failed to detect several of the nine serotypes (Table 1). Additional investigations of the efficacy of the PCR assay with primer sets PrA and PrB for the direct detection of virus in clinical samples should be performed with materials that are maintained frozen to minimize the degradation of viral RNA. Presumably, the use of primer set PrD was successful in detecting virus even under conditions of possible viral RNA degradation because the amplification target of this primer is smaller (174 bp). Thus, the PCR assay with primer set PrD has significant potential for use as a general detection assay in fish health management programs, even under less than ideal conditions for storing and processing samples.

The PCR assay with primer set PrD described here is the first diagnostic test that has been shown to be capable of routinely detecting aquatic birnaviruses directly in fish tissue samples at a level of accuracy and sensitivity comparable to those of virus isolation in cell culture. Detection and the confirmed identification of virus can be obtained in 24 to 48 h. Therefore, the results of the present investigation indicate that the PCR assay described here is a rapid and reliable substitute for virus isolation in cell culture.

ACKNOWLEDGMENTS

This work was supported by grants from the USDA Northeastern Regional Aquaculture Center (90-38500-5211), NOAA Sea Grant (R/FMD-217 and R/FMD-235), NSF (INT-8504094), the NSF EPSCoR Program (EHR 91-08766), the Maine Agricultural and Forest Experiment Station, the University of Maine Center for Marine Studies, and the U.S. Fish and Wildlife Service.

We thank the Pennsylvania Fish and Boat Commission and Ken Stark, fish pathologist, Benner Springs Fish Research Station, for cooperation and assistance.

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