The aquatic birnaviruses are the largest and most diverse group of viruses within the family Birnaviridae and include a variety of viruses from numerous species of fish and marine invertebrates (12, 24). Many of these viruses, such as infectious pancreatic necrosis virus (IPNV), have been proven or implicated to be the etiological agents of diseases in a variety of species used in fish farming and aquaculture worldwide.Aquatic birnaviruses are characterized by a bisegmented, double-stranded RNA genome within a nonenveloped, icosahedral capsid approximately 60 nm in diameter (7). The smaller genome segment (B) encodes a single protein (VP1, 90 to 110 kDa), the virion-associated transcriptase. The larger genome segment (A) (approximately 3,000 bp) contains a large open reading frame that encodes a precursor polyprotein (100 kDa) which is subsequently cleaved to form three viral proteins (pVP2, 63 kDa; NS, 29 kDa; and VP3, 29 to 31 kDa) by protease activity associated with the NS protein (16). The proteins are encoded in the following order: pVP2, NS, and VP3. The pVP2 protein is further processed to yield the major capsid protein VP2 (50 to 55 kDa). The vast majority of aquatic birnaviruses, regardless of host species or geographic origin, are closely related antigenically and form a major serogroup (serogroup A) comprising nine serotypes (5, 6, 19).

Infectious hematopoietic necrosis virus (IHNV) is a member of the family Rhabdoviridae. It causes a lethal disease in several salmonid species (23). Epizootics of the disease generally occur in juvenile fish through waterborne horizontal transmission. Mortality levels from IHNV in the most susceptible species can reach 100%. In chronically infected populations, animals are typically debilitated and secondary bacterial infections are common. Viral hemorrhagic septicemia virus (VHSV) is another rhabdovirus which also causes a serious disease in several salmonid species (23). The disease has been found in all age groups of susceptible fish, with mortality rates reaching 80 to 100%, particularly in fry and fingerlings. It has presented serious problems to rainbow trout culture in Europe for over 50 years. Indeed, VHSV is arguably the most important cause of economic loss in the European trout farming industry. Moreover, cases of VHSV have been increasingly reported in other species, including brown trout, grayling, pike, and whitefish.

IHNV is a bullet-shaped virion 150 to 190 nm in length by 65 to 75 nm in diameter (11, 23). The enveloped, helical nucleocapsid contains a negative-sense, single-stranded RNA genome (14). The genome organization for the six separately translated genes is 3'-N, M1, M2, G, NV, L-5' (18). The proteins encoded by these viral genes are the nucleoprotein (N), matrix proteins (M1 and M2), the envelope glycoprotein (G), a nonviral protein (NV), and the RNA polymerase (L). The morphology of VHSV is similar to those of IHNV and other rhabdoviruses. The virion is approximately 120 to 180 nm long by 60 to 90 nm wide. The viral RNA genome organization and gene products are similar to those of IHNV.

The aquatic birnavirus infectious pancreatic necrosis virus (IPNV) and the rhabdoviruses IHNV and VHSV are significant pathogens that cause high levels of mortality among artificially propagated populations of fish. Currently, the most effective method of controlling 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 these three viruses. The current method for detection of fish viruses requires isolation of virus by inoculation of cell cultures with homogenates of tissue samples collected from a statistically significant portion of the population (1). When a virus is isolated, identification of the virus is required. This usually is accomplished by neutralization tests with specific polyclonal antisera, which can take 1 to 4 weeks, or by enzyme immunoassays with monoclonal antibodies or polyclonal antisera (1, 5). However, individual PCR assays for detecting and identifying these fish viruses have been developed (2–4, 6, 8, 9, 15, 21–23). Increasingly, PCR assays are becoming incorporated into fish health management programs. However, the need to perform separate, individual PCR assays for each virus complicates the diagnostic process and increases the costs. McAllister et al. (17) demonstrated the potential of using a single PCR assay for the simultaneous identification of several fish viruses in a single PCR assay. Subsequently, however, it was shown that the IPNV-specific primers used in their investigation failed to identify all aquatic birnavirus serotypes (3).

In this investigation, we developed a multiplex PCR assay for the simultaneous detection of three fish viruses in a single assay: all serotypes of aquatic birnavirus serogroup A, IHNV,
and VHSV. Routine testing for all three viruses is required in many fish health management programs worldwide.

**Viruses and cell cultures.** IPNV (West Buxton strain) and IHNV (unknown electropherotype) were propagated in Chinook salmon embryo 214 (13) cell cultures, and VHSV (Spain strain; provided by Paul Reno, Oregon State University, Newport) was propagated in epitheliuma papulosum cyprini (10) cell cultures at 15 to 16°C in Eagle’s minimum essential medium with l-glutamine and Earle’s balanced salts solution (Sigma Chemical Co., St. Louis, Mo.) supplemented with 0.2% sodium bicarbonate (Sigma Chemical Co.) and 10% fetal bovine serum (Atlanta Biologicals, Norcross, Ga.).

**RNA extraction and reverse transcription.** Total RNA was extracted from 90 to 300 μl of infected cell culture supernatant with TriReagent LS (Sigma Chemical Co.) by the protocol supplied by the manufacturer. Viral cDNA was obtained by reverse transcription by incubating 4 μl of viral RNA preparation with 1 μl of random hexamer primers (1.25 mM random primer; Promega) and 2.2 μl of nuclease-free water (Promega) at 80°C for 5 min, followed by cooling to 37°C for 5 min. The sample was brought to a final volume of 20 μl with a reverse transcription mixture consisting of 4 μl of 5× reverse transcription (RT) buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂, 50 mM dithiothreitol [pH 8.3]; Promega), 200 U of Moloney murine leukemia virus RT (Promega), 20 U of RNasin (Promega), and 200 μM each dNTP. The cDNA was synthesized at 37°C for 1 h, and the reaction was terminated by heating to 95°C for 5 min.

**Primers.** The aquatic birnavirus-specific primers WB1 and WB2 (WB1, GCGCAACTTACTTGGGATCTGCTATTGTC; WB2, CGTCTGTTCTAGATTCCACCTATGTG) and the IHNNSpecific primers IHN3 and IHN4 (IHN3, GTTCACACTCAACCCGAACAGG; IHN4, TGAAGTACCCACCCAGCCAGATCATT) had been developed previously in our laboratory (22). Primers WB1 and WB2 (which recognize a 206-bp cDNA fragment within the VP2 gene of aquatic birnaviruses) previously had been shown to identify representative isolates of all nine serotypes of aquatic birnavirus serogroup A (23). Primers IHN3 and IHN4 recognize a 371-bp cDNA fragment within the N gene of IHNV.

Several sets of VHSV-specific primers were designed and evaluated with the aim of developing primers to specifically identify VHSV and not produce any amplification products from the other rhabdovirus, IHNV. Bruchhof et al. (4) determined that VHSV and IHNV could not be differentiated based on genomic sequences of the N gene. Considerable variation in the matrix protein genes (M1 and M2) has been demonstrated among the VHSV serotypes, and the NV genes of the North American and European strains of VHSV are markedly dissimilar. Little genomic sequence information is available for the L gene. However, considerable sequence information is available for the G gene of VHSV. Therefore, the G gene of VHSV was chosen as the target for RT-PCR amplification in this investigation. G gene sequences, including conserved regions, were available for the four serotypes from the following strains: Makah (representing the North American isolates, serotype 1), He-70 (Denmark, serotype 2), 23/75 (France, serotype 3), and 02-84 (France, serotype 4). These sequences were aligned and used to design primers that would identify common, conserved sequence fragments with the program Oligo (National Biosciences, Inc., Plymouth, Minn.). Ultimately, primers VHS3 and VHS4 (VHS3, CGGCCAGCTCAACTCAAGTCGCTCC; VHS4, CCAGGTCGCTCCATGATCCATTCTGTC), which amplify a 625-bp cDNA fragment within the G gene, were selected for use in the multiplex RT-PCR assay.

**Multiplex PCR.** For multiplex RT-PCR, IPNV, IHNV, and VHSV viral RNA templates were reverse transcribed simultaneously, and PCR was performed with different combinations of multiple primer pairs for each virus. Various thermal cycler programs were evaluated to optimize the reaction conditions. In the optimal procedure, PCR was performed with 10 μl of reverse transcribed cDNA in a 50-μl reaction mixture consisting of 4 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 1.0% Triton X-100; Promega), 1.5 mM MgCl₂, 0.5 to 0.6 μM each primer, and 5 μl of AmpliTaq DNA polymerase (Perkin-Elmer Co.). Amplification (40 cycles) was performed in an MJ Research (PTC-100) programmable thermal cycler by the following protocol: initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min 30 s, and final extension at 72°C for 10 min. Agarose gel electrophoresis was used to separate the PCR products. Agarose gels (2% agarose; SeaKem LE; FMC, Rockland, Maine) containing 0.5 μg of ethidium bromide per ml in 1× TAE electrophoresis buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA) were loaded with 12 μl of PCR sample and electrophoresed at 65 V for 2 h or 100 V for 1 h. Either PCR Markers (50 to 1,000 bp), 100-bp DNA ladder (100 to 2,072 bp), or an HaeIII-digested λX174 DNA marker ladder (118 to 1,353 bp) were loaded on either end of the gel. A UV transilluminator was used to visualize the bands, and results were recorded by photography.

Typical ethidium bromide-stained agarose gels of the amplification products of the multiplex RT-PCR assay are shown in Fig. 1. Figure 1A shows amplification by the multiplex RT-PCR assay from infected cell culture supernatants of each viral template separately and of all three target templates from a mixture of the three viruses. Also, the multiplex RT-PCR assay was tested for ability to amplify the three viral templates from uninfected kidney and spleen tissue homogenates from Atlantic salmon which had been mock infected with all three viruses. Figure 1B shows amplification of all three viral templates from both infected cell culture supernatants and mock-infected tissue homogenates.
Sensitivity of multiplex PCR assay. The sensitivity of the PCR assay was determined with 10-fold serial dilutions of each virus. RNA was extracted from 90 μl of each sample. RT-PCR was performed as previously described. The sensitivity was determined from the highest dilution of the sample exhibiting a positive PCR result. The sensitivity levels of the multiplex RT-PCR assay were 100, 1, and 32 % tissue culture infective doses [TCID50/ml by the method of Reed and Muench (20)] for IPNV, IHNV, and VHSV, respectively (Fig. 2). These levels of sensitivity are comparable to those of direct isolation of virus in cell culture with dilutions of fish tissue homogenates.

In summary, the multiplex RT-PCR assay, which can be completed in 8 h or less, provides significant savings in time, cost, and materials in comparison to the antibody neutralization test or the separate, individual PCR assays presently used to identify viruses isolated in cell culture from clinical materials. Furthermore, the data presented here and from other preliminary investigations (data not shown) in our laboratory indicate that the multiplex RT-PCR assay compares in sensitivity to isolation of virus in cell cultures for the direct detection of these viruses in clinical specimens. Confirmation of the sensitivity of the multiplex RT-PCR assay in field trials using tissue samples from naturally infected fish will provide a diagnostic assay with significant advantages over the current method of virus isolation in cell culture, which requires 3 to 4 weeks.

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