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Authors: McAllister, P. E., and Schill, W. B.

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IMMUNOBLOT ASSAY: A RAPID AND SENSITIVE METHOD FOR IDENTIFICATION OF SALMONID FISH VIRUSES

P. E. McAllister and W. B. Schill
U.S. Fish and Wildlife Service, National Fish Health Research Laboratory,
Box 700, Kearneysville, West Virginia 25430, USA

ABSTRACT: An immunoblot assay was used to identify the viruses of infectious pancreatic necrosis, infectious hematopoietic necrosis, and viral hemorrhagic septicemia. Viral antigen in infected cell culture supernatant was adsorbed onto nitrocellulose membrane or Whatman 541 filter paper and detected by enzyme-linked immunosorbent assay techniques. The immunoblot assay took less than 4 hr to perform and required no special instrumentation. Assays using cell culture supernatant fluids showed immunoblot sensitivity was 10^5–10^6 PFU/ml. Assay sensitivity, determined using purified virus, is 0.85–4.0 ng of viral antigen. The immunoblot assay was used to detect and identify virus in cell culture fluids.

INTRODUCTION

Infectious pancreatic necrosis (IPN), infectious hematopoietic necrosis (IHN), and viral hemorrhagic septicemia (VHS) are major viral diseases affecting the culture of salmonid fishes (McAllister, 1979; Pilcher and Fryer, 1980). Rapid and accurate diagnosis of these diseases is critical if dissemination of these viruses is to be controlled. Diagnosis now requires isolation of the causative viruses in cell culture and their subsequent identification by reaction with specific immune serum. The serum neutralization assay is the most widely used procedure because of its sensitivity and technical simplicity; however, it requires prolonged incubation of cell cultures before the results can be interpreted. The identification of IPN and VHS viruses can take 2–7 days (Nicholson and Caswell, 1982; Dixon and Hill, 1983) and that of IHN virus 4–10 days (Leong et al., 1983; Dixon and Hill, 1984).

Other serological techniques used for virus identification are immunofluorescent and immunoperoxidase staining of infected cells (Piper et al., 1973; Jorgensen, 1974; McAllister et al., 1974a; Nicholson and Henchal, 1978; Faisal and Ahne, 1980; Ahne, 1981; Swanson and Gillespie, 1981), counterimmunoelectrophoresis (Dea and Elazhary, 1983), coagglutination (Kimura et al., 1984), complement fixation (Finlay and Hill, 1975), radioimmunoassay (Leong et al., 1983), and enzyme-linked immunosorbent assay (ELISA) (Nicholson and Caswell, 1982; Dixon and Hill, 1983; Hattori et al., 1984; Hsu and Leong, 1985). These techniques have seen limited application because of their low sensitivity, technical complexity, difficulty of interpretation, or need for specialized equipment.

We have developed an immunoblot assay—a variation of ELISA—as a rapid, sensitive, and economical technique for virus identification in cell culture fluids. The assay requires no special instruments and takes less than 4 hr to perform.

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MATERIALS AND METHODS

Viruses and cell cultures

Stocks of IHN virus, IPN virus (serotype VR-299), and VHS virus (serotype F1) were prepared in chinook salmon, *Oncorhynchus tshawytscha*, embryo (CHSE-214) cells. Cells were maintained in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS). For virus production, drained monolayer cultures were infected at a multiplicity of infection (MOI) of ≤ 0.01 plaque forming unit (PFU) per cell. Virus was allowed to adsorb for 60–90 min at 15 C, and the cells were then overlaid with EMEM containing 2% FBS. Incubation was continued at 15 C until

cytopathic effect (CPE) was complete—about 48 hr for IPN and VHS viruses and about 96 hr for IHN virus. The viruses were harvested and stored at −70 C, and infectivity was determined by plaque assay as described by Wolf and Quimby (1973). For MOI and growth curve studies, cells were counted with a hemocytometer.

To prepare cell lysates, we washed monolayer cultures (about 2×10^7 cells) three times with calcium- and magnesium-free 0.1 M phosphate buffered saline (PBS, pH 7.2) and scraped them from the culture flask. The cells were concentrated by centrifugation at 800 *g* for 10 min at 4 C and washed three times in PBS. The final cell pellet was resuspended in 1.0 ml of PBS and disrupted by ultrasonic treatment (100 W for 60 sec). Cell debris was sedimented by centrifugation at 1,500 *g* for 15 min at 4 C, and the supernatant fluid was used in the immunoblot assay.

Virus purification and antiserum production

Virus stocks were concentrated by polyethylene glycol precipitation—IHN and VHS viruses as described by de Kinkelin (1972) and IPN virus as described by Macdonald and Yamamoto (1977). The viruses were purified by velocity sedimentation at 97,000 *g* for 45 min at 4 C in linear 10–50% sucrose gradients, further concentrated by centrifugation at 97,000 *g* for 60 min at 4 C, and stored at −70 C. Protein concentration was determined by the method of Lowry as described by Shatkin (1969), and purity was ascertained by polyacrylamide gel electrophoresis (PAGE) as described by McAllister and Wagner (1975).

We prepared antisera against whole virus in rabbits, using a modification of the protocol of McAllister et al. (1974b). At 14-day intervals, rabbits were injected intravenously with 500 μg of viral protein and intramuscularly and subcutaneously with 500 μg of viral protein emulsified in complete or incomplete Freund's adjuvant. All rabbits immunized with virus received a primary injection and either one (IPN) or three (IHN or VHS) booster injections. Rabbits were bled, and the serum was collected and assayed for neutralizing activity.

Fractionation and adsorption of IgG from rabbit antiserum

The IgG fraction of the rabbit antiserum was separated and purified by affinity chromatography as described by Goding (1978) and Cold Spring Harbor Laboratory (1980). Whole serum or serum diluted in 0.1 M PBS was loaded onto a column of Protein A–Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, New Jersey 08854, USA), and the column washed with 0.1 M PBS until baseline optical density at 280 nm was recorded. Bound IgG was eluted with 0.1 M acetic acid containing 0.15 M NaCl, dialyzed against two changes of 0.1 M PBS for 24 hr, and stored at 4 C in 0. 1 M PBS containing 0.02% sodium azide until adsorbed. The IgG's used for immunoblot assays were diluted 1/1,000 or 1/2,000 (v/v) in 0.1 M PBS containing 20% FBS and 0.02% sodium azide, and adsorbed overnight at 37 C on monolayer cultures of CHSE-214 cells. The adsorbed IgG's are hereafter referred to as primary antibody.

Immunoblot assay

The immunoblot assay was performed using modifications of the procedures for the Bio-Rad Immun-Blot (GAR-HRP) Assay (Bio-Rad Laboratories, Richmond, California 94804, USA). Two types of solid matrix were used for immunoblots—nitrocellulose membrane (BA85, 0.45 μm; Schleicher and Schwell, Inc., Keene, New Hampshire 03431, USA) and 541 hardened ashless filter paper (Whatman Chemical Separation, Inc., Clifton, New Jersey 07014, USA) (Larsson, 1981). The nitrocellulose membrane was wetted in 0.1 M PBS and air dried on blotting paper for 5 min before use; the 541 filter paper was used directly from the package. Medium from infected cell cultures or medium diluted in 0.1 M PBS was spotted (10 μl) onto the desired matrix and air dried for 5 min. Medium from noninfected cell cultures and 0.1 M PBS were used as negative controls. To block nonspecific reactive sites, the matrix was immersed for 20 min in 3% gelatin (EIA grade; Bio-Rad Laboratories, Richmond, California 94804, USA) and then transferred to the primary antibody solution. To conserve antibody, we placed the matrix in a Seal-N-Save pouch (Sears, Roebuck, and Co., Chicago, Illinois 60607, USA), and the pouch was agitated every 15 min to redistribute the antibody. After incubation for 60 min at 37 C, the matrix was washed in a dish for 30 min in several changes of 0.1 M PBS containing 0.02% Tween 20 and then transferred to the second antibody solution—affinity purified goat anti-rabbit IgG conjugated with horseradish peroxidase (1/3,000 [v/v] dilution in 0.1 M PBS; Bio-Rad Laboratories, Richmond, California 94804, USA). After incubation for 60 min at 37 C, the matrix was washed as described previously and immersed in color-developing solution (15 ml of 0.1 M PBS containing 16 μl of 30% H_2O_2 and 0.25 ml of 16 mM o-dianisidine dihydrochloride). After about 3 min, virus reacting with

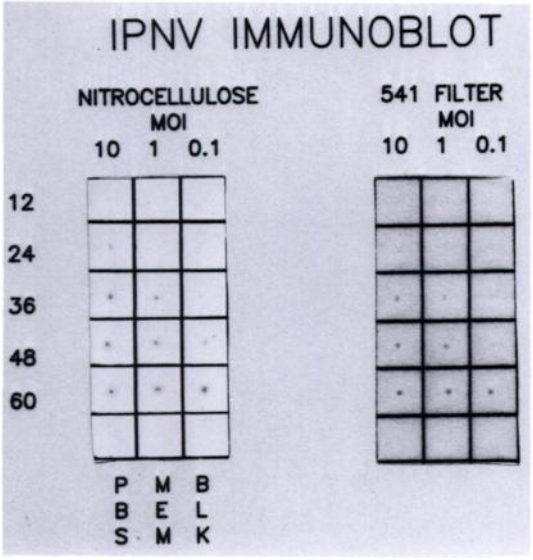

FIGURE 1. Detection of IPN virus in cell culture medium by immunoblot assay. Culture media from IPN virus replication studies were assayed for viral antigen by the immunoblot method using nitrocellulose and 541 filter paper matrices. Medium was spotted (10 μl) with glass microtiter pipettes, and the immunoblot assay was performed as described in the text. For negative controls, the matrix was spotted with PBS and MEM or left unspotted (BLK).

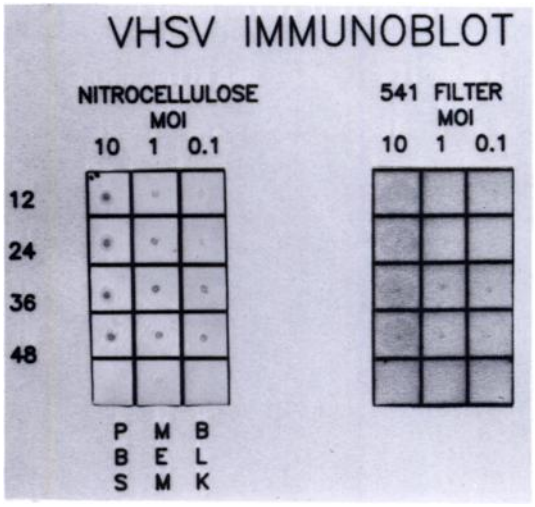

FIGURE 2. Detection of VHS virus in cell culture medium by immunoblot assay. Culture media from VHS virus replication studies were assayed for viral antigen by the immunoblot method; nitrocellulose and 541 filter paper matrices were used, as described in the caption for Figure 1.

specific immune serum appeared as an orange-brown dot on the white matrix. The matrix was removed from the color-developing solution and washed in several changes of water.

Protein binding capacity of immunoblot solid phase matrix

Nitrocellulose membrane and 541 filter paper disks (8.5 mm in diameter) were soaked for 2 hr in 10 μg/ml bovine serum albumin (BSA) in 0.1 M PBS. To remove unbound BSA, we rinsed disks in three 1-hr washes in 0.1 M PBS and then soaked them overnight in 0.1 M PBS. We then blotted the filters dry and placed them in cuvettes, and determined bound protein by the method of Lowry as described above, using BSA as the standard. Ten replicates of each filter type were assayed, and the mean ± one standard deviation was determined.

RESULTS

Detection of viral antigen

Preliminary experiments were performed to define the conditions, procedures, and sensitivity of the immunoblot assay. The protein binding capacity of each of the two types of solid matrix was determined. Nitrocellulose membrane bound 59.5 ± 10.2 μg of protein/cm^2, which was about four times the binding capacity of the Whatman 541 filter paper (15.3 ± 8.8 μg of protein/cm^2). Three blocking reagents—10% FBS, 4% BSA, and 3% gelatin—were tested; 3% gelatin in 0.1 M PBS proved to be the most effective in suppressing nonspecific antibody binding (data not shown). Purified virus and primary antibody were allowed to react in a two dimensional assay to determine appropriate concentrations of primary antibody for the immunoblot reaction and to determine assay sensitivity. For all three viruses, strong dot reactions occurred up to 1/2,000 (v/v) dilution of homologous primary antibody (the greatest dilution tested). The assay was sensitive to 0.85 ng of IPN virus and 4.0 ng of IHN and VHS viruses, which represented about 10^3 PFU by infectivity assay using CHSE-214 cells. By visual inspection, nitrocellulose mem-

brane was a more sensitive matrix than 541 paper. Immunoreactive dots on nitrocellulose were discrete and intensely colored, whereas those on 541 paper were rather diffuse and weakly colored, and consequently more difficult to interpret (Figs. 1, 2).

Because varied amounts of virus occur in clinical samples, we conducted a series of experiments comparing virus incubation time in cell culture at different MOI's and appearance of immunoblot reactivity. Strong immunoblot reactions were evident for IPN virus at 24 hr post-cell culture inoculation for MOI = 10, 36 hr for MOI = 1, and 48 hr for MOI = 0.1 (Fig. 1). Similarly, VHS virus immunoblot reactions were readily apparent after 12 hr for MOI = 10, 24 hr for MOI = 1, and 36 hr for MOI = 0.1 (Fig. 2). Strong correspondence was evident between the accumulation of viral antigen and its detection by immunoblot assay. Immunoblot reactivity coincided with the earliest signs of CPE in cell culture. The level of infectivity detected in cell culture was 10^5–10^6 PFU/ml.

Cross-immunoblot virus identification

In our first cross-immunoblot assays, strong reactions occurred between unadsorbed homologous primary antibody and virus, but multiple heterologous reactions were evident also, particularly in assays in which primary antibody to IHN and VHS viruses was used. The adsorption of the antibody with 20% FBS in 0.1 M PBS eliminated the heterologous reaction with cell culture medium, but reactivity with concentrated CHSE-214 cell lysate remained (data not shown). After adsorption of the primary antibody with both FBS and CHSE-214 cells, no heterologous reactions were observed with any of the virus reactions (Fig. 3). Reactivity with the CHSE-214 cell lysate was not evident on the nitrocellulose matrix, but was evident on the 541 paper matrix (Fig. 3).

In cross-immunoblot virus identification assays, intense color reaction occurred only with homologous preparations, and the reaction was strong even at the 1/100 (v/v) dilution of the virus (Fig. 4).

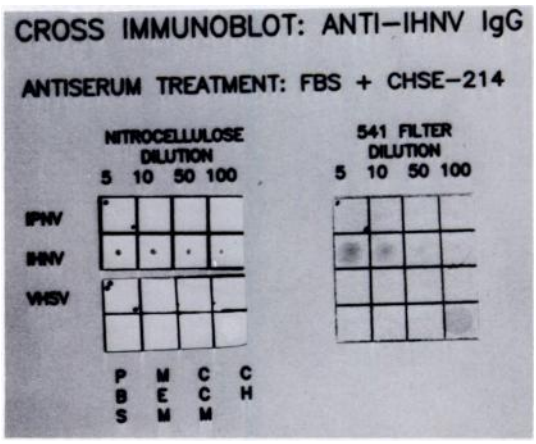

FIGURE 3. Cross-immunoblot assay using anti-IHNV IgG was adsorbed with FBS and CHSE-214 cells. Cell culture harvests of IPN, IHN, and VHS viruses were diluted in PBS and 10 μl spotted onto nitrocellulose and 541 filter paper matrices. We performed the immunoblot assay as described previously, using a 1:1,000 dilution of anti-IHNV IgG that was adsorbed for 18 hr at 37 C in PBS containing 20% FBS, on a monolayer culture of CHSE-214 cells. As negative controls, the matrices were spotted with PBS, MEM, medium from noninfected CHSE-214 cells (CCM), and CHSE-214 cell lysate (CH).

DISCUSSION

In the immunoblot assay antigen is bound by simple adsorptive processes to a solid phase matrix and detected by immuno-enzymatic reactivity.

Because reactive antigen appears as a vivid colored dot on the matrix, immunoblot results can be interpreted by visual inspection. Reflectance scanning densitometry can be used to quantify assay results, but it is not necessary for our assay. As in any system related to ELISA, background color can occur if the matrix is left too long in the color-developing solution, but the reactive dots remain in contrast to the background. The dried matrix can be sealed between sheets of adhesive plastic

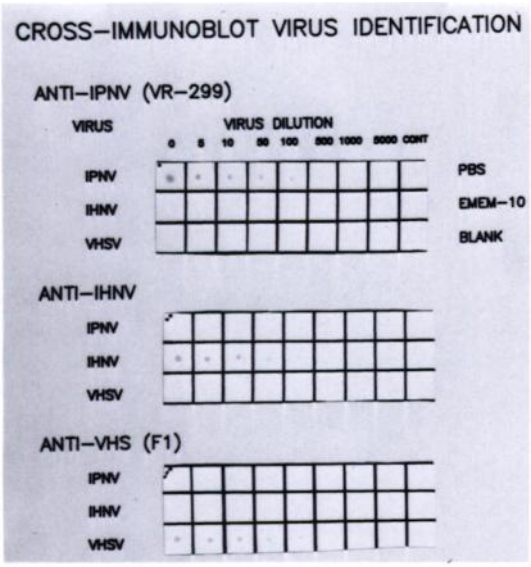

FIGURE 4. Cross-immunoblot assay for the identification of IPN, IHN, and VHS viruses. Cell culture virus harvests were diluted in PBS, spotted (10 μl) onto nitrocellulose matrix, and reacted with 1/2,000 dilution of adsorbed homologous or heterologous antibody in the immunoblot assay as described in the text. As negative controls, the matrices were spotted with PBS and medium from noninfected CHSE-214 cells (EMEM-10), or were left unspotted (blank).

film for hard-copy records and the color of the reactive dots remains for months.

Only small volumes of antibody reagents are needed for the immunoblot assay; furthermore, the primary antibody can be recovered and reused several times. We routinely used a 1:2,000 dilution of primary antibody. The 50% plaque-neutralization titers of our original antisera were 1:3,000,000 for IPN virus, 1:553 for IHN virus, and 1:506 for VHS virus. Preparation of the IgG fractions caused no loss of reactivity other than through dilution. In the undiluted IgG preparations the IgG protein concentration was about 1.5 mg/ml. A high degree of immunoblot reactivity occurred with all three primary antibody preparations. Hsu and Leong (1985) titered IHN virus antibody using an assay format very similar to ours and found that binding antibody titer was much higher than virus neutralizing titer. In contrast, Dixon and Hill (1984) reported that only antisera with 50% plaque-neutralization titers of 1:50,000 or greater were suitable for ELISA.

Our immunoblot assay requires less than 4 hr total time to perform. The ELISA procedures of Dixon and Hill (1983, 1984), Hattori et al. (1984), and Nicholson and Caswell (1982) require 8–24 hr including 6–16 hr to prepare ELISA plates, unless the plates have been prepared in advance. The PAGE protein transfer assays of Hsu and Leong (1985) require 51–66 hr including 9 hr for virus replication in cell culture. The time required to perform the immunoblot assay could be decreased to about 2.5 hr by direct assay if enzyme–primary antibody conjugates were used, as described by Dixon and Hill (1983, 1984). In the indirect assay we describe here, commercially available conjugates were used to detect primary antibody and provided increased versatility, specificity, and sensitivity.

Initially we observed heterologous immunoblot reactions. We assayed CHSE-214 cells for endogenous peroxidase activity but detected none. The heterologous activity was due to antibody reacting with medium and host cell components and was eliminated by adsorbing diluted primary antibody with FBS and CHSE-214 cells. We found that antibody to IPN virus did not require adsorption with CHSE-214 cells, but antibody to IHN and VHS viruses did. Dixon and Hill (1984) reported that their attempts to adsorb out antibody against host cell antigens were successful with antisera to IHN virus, but were not entirely successful with antisera to VHS virus and spring viremia of carp virus, causing serious background color to develop in ELISA. Hsu and Leong (1985) reported that background staining in PAGE protein transfer assays could be virtually eliminated when antibody was adsorbed adequately with noninfected cells.

Our adsorption may have been more complete because we adsorbed diluted rather than whole serum.

We observed some differences between 541 paper and nitrocellulose matrices in the binding of cellular and viral antigens. The affinity for cellular antigens seemed to be greater for 541 paper than for nitrocellulose, but this increased affinity did not interfere with interpreting assay results when adsorbed antibody was used. The 541 paper gave a diffuse dot profile that was more difficult to interpret, whereas the discrete dot profile on nitrocellulose was easy to interpret. Although nitrocellulose costs about 12 times more than 541 paper and is more difficult to handle, it is the preferred assay matrix.

We measured the sensitivity of our immunoblot assay using purified virus and found that the assay was sensitive to 0.85–4.0 ng of viral antigen, which represented about 10^3 PFU by infectivity assay using CHSE-214 cells. Because only 10 μl was applied to the matrix, the measured infectivity was about 10^5 PFU/mL. Thus a sample having equivalent antigenic mass would produce a visible immunoblot reaction. Assays using cell culture virus preparations showed immunoblot reactivity at titers of 10^5 to 10^6 PFU/ml. Dixon and Hill (1983, 1984), Hattori et al. (1984), Hsu and Leong (1985), and Nicholson and Caswell (1982) reported similar levels of sensitivity with their assay systems. Immunoblot sensitivity could be enhanced by increasing sample volume. A greater sample volume could be applied to the matrix using a Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Richmond, California 94804, USA) or similar equipment.

Initially we had hoped that the immunoblot assay would be suitable for detecting virus directly in clinical samples, but unfortunately the matrix was clogged by the high protein content of the sample. Therefore, we used stock virus or medium from infected cell cultures as assay specimens. Passage in cell culture amplifies available antigen and moderates saturation of matrix binding sites caused by the high protein content of sex products and tissue homogenates and fluids. Monitoring cell cultures infected at different MOI's showed that viral antigens could be detected by immunoblot when the earliest signs of CPE appeared. Infectivity assay showed that the cell culture supernatant fluids had virus titers between 10^5 and 10^6 PFU/ml. The immunoblot assay might be suitable for assaying clinical specimens if specimens are extracted and diluted as described by Dixon and Hill (1983, 1984).

Although we have focused on IPN, IHN, and VHS viruses, the immunoblot technique could easily be adapted for identifying any of the fish viruses. The immunoblot assay has the advantages of technical simplicity, ease of interpretation, and economy of time and reagents.

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LITERATURE CITED

AHNE, W. 1981. Serological techniques currently used in fish virology. *In* Developments in Biological Standardization, Vol. 49, D. P. Anderson and W. Hennessen (eds.). S. Karger, New York, New York, pp. 3–27.

COLD SPRING HARBOR LABORATORY. 1980. Hybridoma Techniques. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 65 pp.

DEA, S., AND M. A. S. Y. ELAZHARY. 1983. Counterimmunoelectrophoresis for identification of infectious pancreatic necrosis virus after isolation in cell culture. Can. J. Fish. Aquat. Sci. 40: 2200–2203.

DE KINKELIN, P. 1972. Le virus d'Egtved. II.—Purification. Ann. Rech. Vet. 3: 199–208.

DIXON, P. F., AND B. J. HILL. 1983. Rapid detection of infectious pancreatic necrosis virus (IPNV) by the enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. 64: 321–330.

———, AND ———. 1984. Rapid detection of fish rhabdoviruses by the enzyme-linked immunosorbent assay (ELISA). Aquaculture 42: 1–12.

FAISAL, M., AND W. AHNE. 1980. Use of immunoperoxidase technique for detection of fish virus antigens. *In* Fish Diseases, Third COPRAQ-Session, W. Ahne (ed.). Springer-Verlag, New York, New York, pp. 186–192.

FINLAY, J., AND B. J. HILL. 1975. The use of the complement fixation test for rapid tying of infectious pancreatic necrosis virus. Aquaculture 5: 305–310.

GODING, J. W. 1978. Use of staphylococcal protein A as an immunological reagent. J. Immunol. Methods 20: 241–253.

HATTORI, M., H. KODAMA, S. ISHIGURO, A. HONDA, T. MIKAMI, AND H. IZAWA. 1984. In vitro and in vivo detection of infectious pancreatic necrosis virus in fish by enzyme-linked immunosorbent assay. Am. J. Vet. Res. 45: 1876–1879.

HSU, Y. L., AND J. C. LEONG. 1985. A comparison of detection methods for infectious haematopoietic necrosis virus. J. Fish Dis. 8: 1–12.

JORGENSEN, P. E. V. 1974. Indirect fluorescent antibody techniques for demonstration of trout viruses and corresponding antibody. Acta Vet. Scand. 15: 198–205.

KIMURA, T., M. YOSHIMIZU, AND H. YASUDA. 1984. Rapid, simple serological diagnosis of infectious pancreatic necrosis by coagglutination test using antibody-sensitized staphylococci. Fish Pathol. 19: 25–33.

LARSSON, L. 1981. A novel immunocytochemical model system for specificity and sensitivity screening of antisera against multiple antigens. J. Histochem. Cytochem. 29: 408–410.

LEONG, J. C., Y. L. HSU, H. M. ENGELKING, J. L. FENDRICK, L. K. DURRIN, AND G. KURATH. 1983. Methods for diagnosing IHNV infection in fish. *In* Proceedings: Workshop on Viral Diseases of Salmonid Fishes in the Columbia River Basin, J. C. Leong and T. Y. Barila (eds.). Bonneville Power Administration, Portland, Oregon, pp. 23–47.

MACDONALD, R. D., AND T. YAMAMOTO. 1977. The structure of infectious pancreatic necrosis virus RNA. J. Gen. Virol. 34: 235–247.

MCALLISTER, P. E. 1979. Fish viruses and viral infections. *In* Comprehensive Virology, Vol. 14, H. Frankel-Conrat and R. R. Wagner (eds.). Plenum Press, New York, New York, pp. 401–470.

———, J. L. FRYER, AND K. S. PILCHER. 1974a. Further characterization of infectious hematopoietic necrosis virus of salmonid fish (Oregon strain). Arch. Gesamte Virusforsch. 44: 270–279.

———, ———, AND ———. 1974b. An antigenic comparison between infectious hematopoietic necrosis virus (OSV strain) and the virus of haemorrhagic septicaemia of rainbow trout (*Salmo gairdneri*) (Denmark strain) by cross neutralization. J. Wildl. Dis. 10: 101–103.

———, AND R. R. WAGNER. 1975. Structural proteins of two salmonid rhabdoviruses. J. Virol. 15: 733–738.

NICHOLSON, B. L., AND P. CASWELL. 1982. Enzyme-linked immunosorbent assay for identification of infectious pancreatic necrosis virus. J. Clin. Microbiol. 16: 469–472.

———, AND E. A. HENCHAL. 1978. Rapid identification of infectious pancreatic necrosis virus in infected cell cultures by immunoperoxidase techniques. J. Wildl. Dis. 14: 465–469.

PILCHER, K. S., AND J. L. FRYER. 1980. The viral diseases of fish: A review through 1978. Part I: Diseases of proven viral etiology. *In* CRC Critical Reviews in Microbiology, Vol. 7, H. D. Isenberg (ed.). CRC Press, Boca Raton, Florida, pp. 287–363.

PIPER, D., B. L. NICHOLSON, AND J. DUNN. 1973. Immunofluorescent study of the replication of infectious pancreatic necrosis virus in trout and Atlantic salmon cell cultures. Infect. Immun. 8: 249–254.

SHATKIN, A. J. 1969. Estimation of RNA, DNA, and protein by the use of isotopic precursors followed by chemical fractionation. *In* Fundamental Techniques in Virology, K. Habel and N. P. Salzman (eds.). Academic Press, New York, New York, pp. 238–241.

SWANSON, R. N., AND J. H. GILLESPIE. 1981. An indirect fluorescent antibody test for the rapid detection of infectious pancreatic necrosis virus in tissues. J. Fish Dis. 4: 309–315.

WOLF, K., AND M. C. QUIMBY. 1973. Fish viruses: Buffers and methods for plaquing eight agents under normal atmosphere. Appl. Microbiol. 25: 659–664.