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NOTE

Diagnosis of infectious hematopoietic necrosis virus in Atlantic salmon *Salmo salar* by enzyme-linked immunosorbent assay

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ABSTRACT. An indirect double-antibody enzyme-linked immunosorbent assay (ELISA) was developed for rapid detection of infectious hematopoietic necrosis virus (IHNV). The detection limit of the assay was 70 plaque forming units (PFU) of virus in fish tissue homogenate derived from 100 µg of tissue. Viral antigen could be detected in both tissue homogenates and fluid from infected cell cultures. Four types of IHNV cross-reacted with antibodies used in the ELISA, enabling detection of any of the tested types. The assay will allow the detection of virus-infected fish within a day.

Several techniques including fluorescent antibody detection in cell culture (LaPatra et al. 1989), immunoblot assay (McAllister & Schill 1986), detection of viral nucleic acids by means of polymerase chain reaction (Arakawa et al. 1990) and use of a biotinylated DNA probe (Deering et al. 1991) have been used for IHNV detection. Although these techniques are sensitive, they are time consuming and technically intensive. An alternate method, the enzyme-linked immunosorbent assay (ELISA), is generally less expensive and more expedient. Previous studies using an ELISA formulated with a single labeled detecting antibody have reported sensitivity of 1.5×10^6 PFU using a 0.2 ml assay volume of cell culture fluid (Way & Dixon 1988). This paper reports a double sandwich ELISA having improved sensitivity.

Materials and methods. ***Virus strains and antisera:*** Four isolates of IHNV received from Dr Joann Leong (Hsu et al. 1986) were propagated in cell culture as described and used as test antigens.

IHNV Type 3 (Dworshak isolate) virus was purified from an alternate line of infected cell culture fluid by the procedure described by Hsu & Leong (1985) and used as previously described to prepare antisera in chickens and rabbits (Sadasiv et al. 1989).

Experimentally infected fish: Four-month-old Atlantic salmon *Salmo salar*, weighing 3 to 5 g and maintained at 10 °C, were infected by waterborne exposure to 10^2 PFU of IHNV ml^{-1} in 10 l of water (Dworshak, Type 3). As the fish became moribund, a 10 % w/v whole fish tissue homogenate was prepared (Bradley et al. 1989) and examined for the presence of IHNV by both plaque formation assay (PFU) (Burke & Mulcahy 1980) and ELISA.

ELISA: A solid phase indirect double-antibody ELISA system was employed (Voller et al. 1976) using equal volumes (50 µl) of each reagent in the following manner: (a) Chicken anti-IHNV serum, diluted 1 : 500 in 0.1 M carbonate-bicarbonate buffer, pH 9.6, was added to wells of Immulon II MicroElisa plates (Dynatech Laboratories, Chantilly, VA, USA). The plates were incubated either at 37 °C for 2 h or 4 °C for 16 h and the wells washed 3 times at 10 min intervals with PBS containing 0.08 % Tween-20 (PBS-T). Blocking buffer (PBS-T plus 2 % bovine serum albumin) was deposited into each well and the plates incubated for 15 min at 37 °C; the blocking buffer was removed. (b) Fluid from IHNV infected cell cultures or homogenates of fish tissue (diluted 1 : 10 or 1 : 50 in PBS-T) was added to replicate wells in alternate rows, and the plates incubated at 37 °C for 2 h. Intervening rows contained similarly diluted negative 'spent' cell culture fluid or fish tissue homogenate from age- and weight-matched virus-negative fish. The plates were washed and blocked as described above. (c) Rabbit anti-IHNV serum, diluted 1 : 250 in PBS-T, was added to each sample well and incubated at 37 °C for 2 h. Washing and blocking procedures were repeated. (d) Peroxidase-conjugated serum (goat anti-rabbit IgG, Organon Teknika-Cappel, West Chester, PA, USA), diluted 1 : 2500 in PBS-T, was added to each well and incubated at 37 °C for 30 min. The plates were washed 3 times at 10 min intervals with PBS-T, followed by a water wash for 5 min. (e) Substrate (40 mg *o*-phenylenediamine dissolved in 100 ml of 0.05 M phosphate-citrate buffer, pH 5, and mixed with 40 µl of 30 % H_2O_2)

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was added to each well. The plates were incubated at room temperature for 30 min and acidified. The optical densities (OD) of the reactions at 490 nm were measured with a MicroElisa Mini Reader (Dynatech Laboratories, Chantilly, VA, USA). In addition to antigen controls, 4 replicate reagent controls (in which PBS-T was substituted for antigen) were run on each plate and served as a blank for all other tests on that plate. The sensitivity of the ELISA was determined by testing 3 stocks of IHNV produced in the same cell line on different days (Dworshak, Type 3) (Table 1). Each was diluted to concentration of 50 000, 5000, 500, 50, and 5 PFU assay^{-1}. Four replicates of each dilution were tested by ELISA.

Results and discussion. In order to standardize absorbance values, results have been expressed as positive/negative (P/N) ratios. The P/N is the mean OD of replicate wells of a putative virus-containing sample divided by the mean OD of the corresponding uninfected control (negative antigen). A test reading was considered positive when the test antigen OD reading exceeded the value of matched negative antigen OD by at least 3 standard deviations (3 SD) of the negative antigen and the P/N ratio equaled or exceeded 2 (P/N $\geq$ 2).

Detection of cell culture produced IHNV by ELISA: Assays containing 50 000 PFU gave positive virus detection (Table 1), as did 5000 and 500 PFU. At 50 PFU, the P/N ratios were in the positive range for 2 samples and only one of those exceeded 3 SD of the negative control. The detection is considered negative. At 5 PFU, again one virus stock was above 3 SD of the diluted negative cell culture medium but the P/N ratio was 1.5, below the value set for positive virus detection.

The 4 IHNV types reacted with Type 3 antiserum (Table 2). They did not react with an unrelated fish virus produced in the same cell line as the IHNV (data not shown).

Detection of IHNV in whole fish tissue homogenates by ELISA: The ELISA was able to detect IHNV in fish tissue homogenates with titers as low as 70 PFU (Table 3). Although OD readings greater than 3 SD above the mean of the negative antigen were found at lower titers (23 PFU), the P/N ratio of 1.6 indicates that this detection is below the level of confidence selected for this test.

The double criteria of both P/N and 3 SD above the mean of the negative provide a level of confidence of valid virus detection of greater than 99 %. When these criteria have been used, no false negatives or false positives have been encountered in comparison to plaque-forming units. Virus titers exceeding 10^4 to 10^5 PFU ml^{-1} of tissue homogenate are common in fish during IHNV epizootics (Wolf 1984), and are well

Table 1 Sensitivity of IHNV detection by ELISA

Virus conc. (PFU 50 µl^{-1})	Optical density (OD)[a] Stock virus 1[b]	Stock virus 2[b]	Stock virus 3[b]	Virus detection[c]
5×10^4	1.18 ± 0.07	1.35 ± 0.06	1.77 ± 0.09	
Neg[d]	0.05 ± 0.01			
P/N[e]	24	27	35	+
5×10^3	0.63 ± 0.13	0.70 ± 0.07	1.24 ± 0.33	
Neg[d]	0.04 ± 0.00			
P/N[e]	16	17	31	+
5×10^2	0.27 ± 0.02	0.26 ± 0.03	0.50 ± 0.18	
Neg[d]	0.04 ± 0.01			
P/N[e]	7	7	12	+
5×10^1	0.05 ± 0.02	0.07 ± 0.02	0.14 ± 0.05	
Neg[d]	0.03 ± 0.01			
P/N[e]	1.7	2.3	4.7	–
5	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	
Neg[d]	0.04 ± 0.01			
P/N[e]	1	1.25	1.50	–

[a] Mean optical density of 4 wells
[b] Stock virus consisted of Type 3 infectious hematopoietic necrosis virus (IHNV) (Dworshak strain) produced from cell culture on different dates
[c] For positive detection on IHNV, the P/N must equal or exceed 2 and a positive reading must exceed the negative antigen plus 3 SD of the negative antigen
[d] The negative antigen is matched dilutions of uninfected 'spent' cell culture fluids. The value is the mean OD ± SD of 4 wells
[e] Positive/negative ratio

Table 2. Comparison of detection of IHNV strains by ELISA

Virus isolate and type[a]	Titer of virus (PFU 50 µl^{-1})	ELISA[b] OD[c]	ELISA[b] P/N[d]	Virus detection[e]
IHNV Type 1	1×10^6	1.85 ± 0.03	10.9	+
IHNV Type 2	2×10^5	1.93 ± 0.03	11.4	+
IHNV Type 3	3×10^6	1.94 ± 0.11	11.4	+
IHNV Type 5	1×10^5	≥ 2.00	≥ 11.8	+
Cell culture fluid[f]		0.18 ± 0.02		

[a] Type 1 – Suttle Lake, 1980 – K
Type 2 – Beaver Creek, 1981 – STW
Type 3 – Trinity, 1980 – CHS
Type 5 – Cedar River, 1980 – CHF
All strains promulgated in the same cell line
[b] ELISA utilized capture and detecting antibodies produced against Type 3 (Dworshak strain) IHNV
[c] Mean optical density reading of 4 wells ± SD
[d] Positive/negative ratio
[e] For positive detection of IHNV, the OD must exceed the mean OD plus 3 SD of the same dilution of negative cell culture fluid (which in this case would be 0.18 + (3 × 0.02) = 0.24). It must also demonstrate a P/N of ≥ 2
[f] Negative antigen – Mean OD reading of 4 wells of uninfected 'spent' cell culture fluid ± SD

Table 3. Detection of IHNV in tissue homogenates by cell culture system and ELISA

Fish[a]	Titer of virus (PFU 50 µl^{-1})	ELISA OD[b]	ELISA P/N[c]	Virus detection[d]
D1	70	0.46 ± 0.10	2.7	+
D3	200	0.77 ± 0.11	4.5	+
D4	23	0.27 ± 0.03	1.6	–
D5	15000	•	•	+
D6	9000	•	•	+
D7	100	0.56 ± 0.04	3.3	+
Neg[e]		0.17 ± 0.02		

[a] Fish tissue homogenate obtained from whole moribund Atlantic salmon fry infected with IHNV D followed by number indicates the day post-infection upon which the fish died
[b] Mean optical density of 4 wells ± SD
[c] Positive/negative ratio
[d] For positive detection of IHNV at a level of confidence > 99 %, the P/N must equal or exceed 2 and the positive OD must exceed the negative OD + 3 SD of the negative, which in this case would be 0.17 + (3 × 0.02) = 0.23
[e] Negative antigen – Mean OD + SD of 4 wells of tissue homogenate of same age Atlantic salmon fry not exposed to IHNV
• These 2 fish were clearly positive but their values did not fall within the linear portion of the ELISA. The samples were exhausted in the testing

within the detection level of this ELISA. Concentrations of 50 and 5 PFU approached the detection limits of the ELISA for cell culture fluids. These concentrations may be detected when less stringent criteria are used.

Although monoclonal antibodies (Mab) provide a high degree of specificity, the use of polyclonal antisera in ELISA can allow detection of a greater range of virus types and antigenic variants, which would be an advantage for field testing (Xu et al. 1991). Using Mab in a similar ELISA, Mourton et al. (1990) could detect another piscine rhabdovirus, viral hemorrhagic septicemia virus (VHSV), at levels as low as 5×10^4 in a 0.1 ml assay. However, their assay was strain specific, which might be considered a limitation of its value.

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LITERATURE CITED

Arakawa, C. K., Deering, R. E., Higman, K. H., Oshima, K. H., O'Hara, P. J., Winton, J. R. (1990). Polymerase chain reaction (PCR) amplification of a nucleoprotein gene sequence of infectious hematopoietic necrosis virus. Dis. aquat. Org. 8: 165–170

Bradley, T M., Medina, D. J., Chang, P. W., McClain, J. (1989). Epizootic epitheliotropic disease of lake trout *(Salvelinus namaycush):* history and viral etiology. Dis. aquat. Org. 7: 195–201

Burke, J. A., Mulcahy, D. (1980). Plaquing procedure for infectious hematopoietic necrosis virus. Appl. environ. Microbiol. 39: 872–876

Deering, R. E., Arakawa, C. K., Oshima, K. H., O'Hara, P. J., Landolt, M. L., Winton, J. R. (1991). Development of a biotinylated DNA probe for detection and identification of infectious hematopoietic necrosis virus. Dis. aquat. Org. 11: 57–65

Hsu, Y L., Engelking, H. M., Leong, J. C. (1986). Occurrence of different types of infectious hematopoietic necrosis virus in fish. Appl. environ. Microbiol. 52: 1353–1361

Hsu, Y L., Leong, J. C. (1985). A comparison of detection methods for infectious hematopoietic necrosis virus. J. Fish Dis. 8: 1–12

LaPatra, S. E., Roberti, K. A., Rohovec, J. S., Fryer, J. L. (1989). Fluorescent antibody test for the rapid diagnosis of infectious hematopoietic necrosis. J. aquat. Anim. Health 1: 29–36

McAllister, P. E., Schill, W. B. (1986). Immunoblot assay: a rapid and sensitive method for the identification of salmonid fish viruses. J. Wildl. Dis. 22: 468–474

Mourton, C., Bearzotti, M., Piechaczyk, M., Paolucci, F., Pau, B., Bastide, J.-M., deKinkelin, P. (1990). Antigen-capture ELISA for viral haemorrhagic septicaemia virus serotype I. J. virol. Meth. 29: 325–334

Sadasiv, E. C., Piela, T. H., Chang, P. W. (1989). Detection of latent avian adenovirus associated virus proteins in chicken cells. Avian Dis. 33: 125–133

Voller, A., Bartlett, A., Bedwell, D. E., Clark, M. F., Adams, A. N. (1976). The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. gen. Virol. 33: 165–167

Way, K., Dixon, P. F. (1988). Rapid detection of VHS and IHN viruses by the enzyme-linked immunosorbent assay (ELISA). J. appl. Ichthyol. 4: 182–189

Wolf, K. (1984). Diseases caused by microorganisms. In: Kinne, O. (ed.) Diseases of marine animals, Vol. IV, Part 1, Introduction, Pisces. Biologische Anstalt Helgoland, Hamburg, Germany, p. 17–46

Xu, L., Mourich, D. V., Engelking, H. M., Ristow, S., Arnezen, J., Leong, J. C. (1991). Epitope mapping and characterization of the infectious hematopoietic necrosis virus glycoprotein, using fusion proteins synthesised in *Escherichia coli.* J Virol. 65: 1611–1615

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