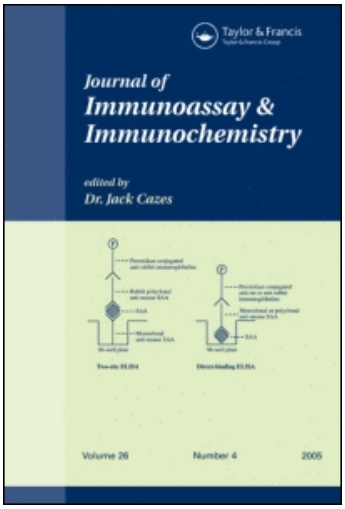

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Detection of Infectious Pancreatic Necrosis Virus Using an Inhibition Enzyme-Linked Immunosorbent Assay

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DETECTION OF INFECTIOUS PANCREATIC NECROSIS VIRUS USING AN INHIBITION ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

An inhibition enzyme-linked immunosorbent assay was used to detect infectious pancreatic necrosis (IPN) virus. In this assay the presence of virus was determined by measuring the decrease in titer of a known antiserum after incubation with a sample suspected to contain virus. The titer of the antiserum was measured with an indirect enzyme-linked assay. Compared to the double antibody sandwich method this assay required fewer reagents (only one anti-IPN serum was required). This assay was also sensitive enough to detect virus at levels of $1x10^2$ $TCID_{50}$/ml. of purified virus and was able to detect virus in samples obtained in the field.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) infects the fry and fingerlings of most salmonid species and can cause high mortality in these fish when grown under hatchery conditions(1). IPN is a highly contagious disease which must be diagnosed during the early stages of an epizootic to protect the uninfected populations in the hatchery facility(2). Several methods of IPNV detection are currently employed in fish disease laboratories but the combi-

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nation of the tissue culture assay with serum neutralization (3) is the only recommended system. However, this approach requires approximately two weeks for a diagnosis. Consequently, the development of rapid, yet sensitive IPNV detection systems has become a top priority in fish immunodiagostic research. A number of assays have been considered. One of these is the enzyme-linked immunosorbent assay (ELISA). This assay has been used for the detection of viruses found in fish (4,5,6). All of the assays previously reported for IPN are of the double-antibody sandwich type. In this work we have used a simpler version of the ELISA called an inhibition ELISA. In this assay the presence of virus was demonstrated by its ability to neutralize the activity of a known anti-IPN serum. The amount of serum remaining after neutralization was measured by a simple indirect ELISA. Using this assay we were able to detect virus at levels as low as $1x10^2$ $TCID_{50}$/ml. We were also able to detect the presence of virus in infected fish. This assay required fewer steps and fewer reagents than the double antibody sandwich method.

MATERIALS AND METHODS

Virus and Cells:

The ATCC-VR 299 strain of IPNV (originally obtained from the National Fish Health Research Laboratory, Kearneysville, W. Va.) was propagated in RTG-2 cells (7). Once the cells were visibly lifting off of the plastic substrate, the virus was extracted and purified from the RTG-2 cell lysate by fluorcarbon extraction and CsCl gradient ultracentrifugation (8). Virus titers were deter-

mined using a microtiter assay (9). Serum neutralization assays were done as described by Aja and Busch (9).

Antiserum Preparation:

Antigen was prepared by diluting stock virus with phosphate buffered saline (PBS; 0.01 M phosphate, 0.15 M NaCl, pH 7.1) to a titer of $1x10^6 TCID_{50}$/ml. New Zealand rabbits were injected with antigen at one week intervals for three weeks. Each week each rabbit received 0.5 ml of antigen intravenously. Each rabbit also received 1 ml subcutaneously with a mixture consisting of 0.5 ml of diluted virus and 0.5 ml of Freund's complete adjuvant. Seven days after the third injection, blood was collected from the marginal ear vein and allowed to clot. Serum was collected and stored at -20 C. IgG was purified from the serum by using a DEAE Affi-gel Blue column (Bio-Rad Laboratories, Richmond, CA.). The purified IgG fractions were pooled, heat inactivated at 56 C for 30 minutes, and then placed onto a confluent monolayer of RTG-2 cells to remove cross reacting antibodies. Preadsorption was allowed to continue for 2 hours at 17 C in a CO_2 incubator at a partial pressure of 3%. After the incubation, the serum was stored at 4 C for later use.

Inhibition ELISA Procedure:

The inhibition ELISA was designed to take advantage of the fact that when virus and antiserum are mixed, the interaction results in inactivation of the antibody. The amount of inactivation was measured using a modification of the indirect ELISA (10,11). In this assay, stock IPNV was first diluted to 10^5

$TCID_{50}$/ml in coating buffer (carbonate-bicarbonate; 1.59g Na_2CO_3, 2.93g $NaHCO_3$/liter H_2O), and a 200 ul amount of diluted virus was placed into each of the desired number of wells of a Gilford cuvette pack (Gilford Instrument Laboratories Inc., Oberlin, Ohio). The cuvettes were then incubated at 37 C for 2 hours. The contents of the cuvettes were then aspirated and the wells washed 3 times with PBrij [8.0 g NaCl, 0.2 g KH_2PO_4 2.9 g $Na_2HPO_4 \cdot 7H_2O$, 0.2g KCl, and 30 ml 5% (w/v) Brij 35 (polyoxethylene 23-lauryl ether, Sigma Chemical Co. St. Louis MO.) per liter water]. The wells were washed as above, 200 ul of neutralized antiserum (see below for neutralization procedure) was added to each of the IPNV-coated wells and the cuvettes were incubated at 37 C for 1 hour. The wells were again washed with PBrij as above. Goat anti-rabbit IgG, conjugated with alkaline phosphatase (Sigma) diluted 1:1000 in PBrij was added in 200 ul amounts to each well and incubation continued at 37 C for 1 hour. The wells were washed as described above, and substrate [p-nitrophenyl phosphate substrate (Sigma), 1 tablet/5 ml of coating buffer] was immediatedly pipetted into each well (280 ul/well) and incubation continued for 30 minutes. The absorbance was read at 405 nm on a Gilford EIA Manual Reader (Gilford Instrument Laboratories Inc., Oberlin, Ohio). Neutralized antibody was prepared by mixing equal portions of material suspected to contain virus and specific antiserum (rabbit anti-IPNV 1:600 final dilution). The mixture was incubated in a separate tube at 37 C during the last hour of the coating incubation. As a control, material shown to be free of virus (using a tissue culture assay) was incubated with anti-IPNV serum. All

inhibition mixtures were incubated in disposable, glass culture tubes (VWR Scientific Inc., San Francisco, CA., catalog #60825-913) sealed with parafilm.

Determination of ELISA Sensitivity:

Standard curves were performed to determine the sensitivity of the ELISA. ELISAs were performed as described above. The inhibition mixtures were prepared by inoculating normal steelhead tissue homogenate (negative for virus by serum neutralization) with IPNV titers from 10^6 $TCID_{50}$/ml to 10^1 $TCID_{50}$/ml. An equal portion of anti-IPNV was added to each sample (1:600 final dilution of antiserum). Uninoculated normal homogenate was incubated with anti-IPNV as a control. Two standard deviations from the mean control value were subtracted from the mean control value, and this value was designated as the baseline. The lowest virus concentration which produced an absorbance less than the baseline value was considered the limit of sensitivity of each assay.

Fish Sampling Procedures:

Tissue samples (kidney, spleen, and pyoloric caeca) were taken from returning steelhead trout being spawned at the Pahsimeroi Steelhead Collection Facilty, Ellis, Idaho (Idaho Department of Fish and Game). Twelve 5-fish pools of tissue were collected. The tissue, about 1 g per sample, was placed in 7 ml of transport medium (9) Samples were transported on ice and stored at -20 C for later processing.

Tissue Sample Preparation for ELISA:

Tissue samples were partially thawed and homogenized using a glass tissue grinder. The homogenate was then replaced in 15-ml conical centrifuge tubes (Falcon Labware) and centrifuged at 800 x g (2000 rpm) for 10 minutes in an International IEC refrigerated centrifuge. The supernatant was decanted into sterile tubes and stored at 4 C until assayed. The tissue grinder was washed and sterilized (immersed in Clorox bleach for 5 minutes) between each sample to insure that no carryover of viable virus occurred from one sample to the next.

RESULTS

Determination of ELISA Sensitivity:

When the inhibition ELISA was tested using fish tissues seeded with virus samples of known titers, an inverse relationship was observed between virus titer and the absorbance value obtained (see figure 1.) The mean control value (obtained from samples of uninfected tissue incubated with the reference serum) was 0.604. Subtacting two standard deviations from this value yielded a baseline value of 0.534. Values less than the baseline were considered positive for IPN virus. Using this baseline value, we could detect virus in samples which contained greater than 10^2 $TCID_{50}$/ml. (See fig.1).

Comparison of the ELISA to Virus Neutralization Assay:

When the inhibition ELISA was compared to the virus neutralization assay for detection of IPN virus in spawning steelhead, the results shown in table 1 were obtained. (This table repre-

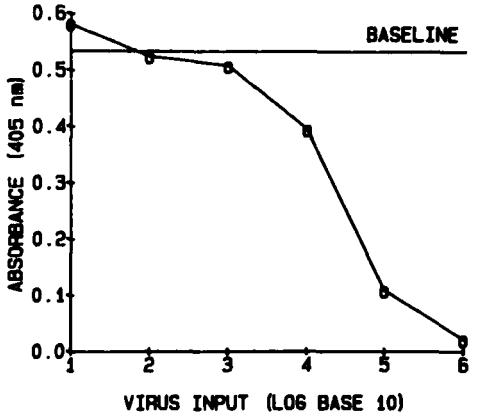

Figure 1. Representative standard curve; Inhibition ELISA. o———o Values obtained from tissue samples seeded with known amounts of virus. ——— Baseline value obtaines by subtracting two standard deviations from the mean of eight control determinations.

sents the data from three different assays. Note that the values in each are relative to the baseline for that group). Nine out of the 12 samples assayed with the ELISA were positive for IPNV while seven of these samples were positive employing the tissue culture/serum neutralization assays. In all cases but one (Group III, sample 2) when the two types of assays produced divergent results, the ELISA result was positive and the serum neutralization result was negative.

DISCUSSION

In this work we investigated the use of an inhibition ELISA to detect IPN virus. We found that this assay had a number of advantages over the more conventional double antibody sandwich method. First, the inhibition assay was simpler, requiring fewer

Table 1. Detection of IPNV in Spawning Steelhead

Group I Sample	A_{405}	Virus Assay: ELISA	Serum neut.
Control	0.350±0.036[a]	-	-
1	0.171[b]	+	+
2	0.268	+	+
3	0.139	+	+
4	0.147	+	+
5	0.220	+	+

[a]Mean of 8 determinations. Baseline (control-2std.dev.)=0.287
[b]Mean of 4 determinations.

Group II. Sample	A_{405}	Virus Assay: ELISA	Serum neut.
Control	0.323±0.024[c]	-	-
1	0.067[d]	+	-
2	0.373	-	-
3	0.185	+	-
4	0.301	-	-
5	0.093	+	-

[c]Mean of 8 determinations. Baseline (control-2std.dev.)=0.275
[d]Mean of 4 determinations.

Group III. Sample	A_{405}	Virus Assay: ELISA	Serum neut.
Control	0.501±0.032[e]	-	-
1	0.423[f]	+	+
2	0.495	-	+

[e]Mean of 8 determinations. Baseline (control-2std.dev.)=0.437
[f]Mean of 4 determinations.

steps and fewer reagents than the double antibody sandwich method. Secondly, the limit of virus detection we observed was lower than that reported by others for the double antibody sandwich method (4,5,6). We could detect 10^2 $TCID_{50}$/ml by using the inhibition assay (fig. 1). The best sensitivity reported by those using the double antibody sandwich method to detect IPN virus has been 10^4-10^5 $TCID_{50}$/ml (4,6). The inhibition assay was also more sensitive than the microtiter cell culture assay that we use. In the cell culture assay, the sensitivity has a practical limit of 10^3 $TCID_{50}$/ml because of the need to dilute samples beyond the point of cytotoxicity. Thus, the two cases where the ELISA indicated the presence of virus where none was detected by cell culture assay (table 1) might be because the ELISA was more sensitive. More work must be done, however, to prove that these cases were not the result of false positive indications.

The results that we have observed may reflect the ability of this assay to detect virus which is not viable in the tissue culture assay but which still remains antigenic. Others have suggested that the ELISA can detect non-viable virus (5). This fact may be useful in detecting carrier states where the virus might be present in large numbers in the form of defective interfering particles, with very few infectious particles present. Previous investigation into the carrier state has utilized the cell culture assay for virus detection (12.13). If non-viable virus was present, it might not have been detected by the cell culture assay. Use of the ELISA might help establish if such a state exists in IPN infections.

This assay was also more rapid than the tissue culture assay. It could be completed in six hours including the time required to initially coat the wells with antigen.

Further studies are under way in our laboratory. We hope to utilize the sensitivity and speed of the assay to help determine the prevalence of virus in wild populations of fish.

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