Staphylococcal Coagglutination, a Rapid Method of Identifying Infectious Hematopoietic Necrosis Virus†

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A staphylococcal coagglutination test was developed for the rapid detection of infectious hematopoietic necrosis virus (IHNV) in cell cultures and infected fish. The test could be completed in 15 min but required a minimum IHNV titer of \(10^6\) PFU/ml to obtain a positive reaction. All IHNV isolates, representing the five electrophoretotypes taken from a wide variety of species and different geographic ranges, caused coagglutination of Staphylococcus aureus cells sensitized with rabbit polyclonal serum against the Round Butte IHNV isolate. The coagglutination reaction was blocked by preincubation of IHNV with homologous antisera, and IHNV did not cause coagglutination of S. aureus cells sensitized with normal rabbit serum. In specificity tests, cells sensitized with rabbit anti-IHNV serum or normal serum did not coagglutinate in the presence of infectious pancreatic necrosis virus, viral hemorrhagic septicemia virus, cell culture medium components, or media from cell lines of salmonid and nonsalmonid origin. Most importantly, the coagglutination test was able to detect and identify IHNV directly from experimentally infected rainbow trout fry, the organs of naturally infected adult kokanee salmon and winter steelhead trout, and ovarian fluids of the winter steelhead trout. The coagglutination test is very suitable for field use, since it is inexpensive, simple to interpret, sensitive, and rapid and requires no specialized equipment.

Infectious hematopoietic necrosis (IHNV) virus, a rhabdovirus, is the etiological agent of IHN, an economically significant disease that affects the young of several salmonid species. The mortalities of fry and fingerlings can be as high as 90%, and occasional less severe epizootics in smolts and 2-year-old fish have been reported (5, 27).

Currently, there are no effective chemotherapeutic agents or commercially available vaccines against IHNV, so the only control measure is avoidance. Because IHNV is highly contagious in populations of young fish, it is imperative that infected fish be quickly quarantined or destroyed to prevent further spread of the virus. Hence, it is necessary to have rapid, accurate methods of identifying IHNV. Clinical signs, histopathology, and typical cytopathic effects in cell culture provide a presumptive diagnosis of IHNV, but a confirmatory diagnosis is primarily made by a serum neutralization test (1) that requires 1 to 2 weeks. Examples of more rapid methods of confirming IHNV infections include an enzyme-linked immunosorbent assay (6, 28), Western blots (10), immunoblots (19, 26), and a fluorescent antibody test (17). Of these methods, only the enzyme-linked immunosorbent assay and the fluorescent antibody test can detect virus directly from fish tissue without passage of the samples in cell culture; with these two tests IHNV can be identified in 2 h to 2 days, but specialized equipment or technical expertise is required.

For a test to be suitable for field use, it should be rapid, sensitive, and reliable, require no specialized equipment, and be able to detect a pathogen directly from infected fish. A diagnostic test for IHNV that is suitable for field use has not been reported. However, a staphylococcal coagglutination test was previously developed to identify several fish pathogens such as Vibrio anguillarum, Yersinia ruckeri, Aeromonas salmonicida, and Renibacterium salmoninarum (12, 13, 24); infectious pancreatic necrosis virus (IPNV) (4, 14); and Oncorhynchus masou virus (14). This test has all the criteria needed for field use and is being used in Japan as a field diagnostic method to identify IPNV and O. masou virus (11a). This serological test employs Formalin-fixed Staphylococcus aureus cells sensitized with polyclonal rabbit anti sera. Rabbit antibodies, primarily of the immunoglobulin G class, bind to the protein A on the surface of the staphylococcal cells via the Fc portion, leaving the Fab portion of the immunoglobulin free to bind antigen (16). When these antibody-coated cells are mixed with the homologous antigen, the Fab portion of the antibody specifically binds to the antigen, causing the cells to coagglutinate (15).

The purpose of this study was to develop a staphylococcal coagglutination test for the rapid detection and identification of IHNV in cell cultures and in tissue samples from infected fish. The results indicate that the coagglutination test, which takes less than 15 min to complete, is a specific and reliable test for identifying IHNV infections in cell culture and tissues from naturally or experimentally infected fish. This test is also sensitive, does not require any specialized equipment, and would be suitable for field use.

MATERIALS AND METHODS

Cell lines. Monolayer cultures of EPC cells from common carp (Cyprinus carpio) were used for the isolation, propagation, and quantitation of IHNV isolates. The chinook salmon embryo (CHSE-214) cell line was used to propagate and quantitate IPNV and viral hemorrhagic septicemia virus (VHSV). Cells were grown in Eagle minimal essential medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS), 100 IU of penicillin per ml, 100 \(\mu\)g of streptomycin per ml, 2 mM L-glutamine, 10 \(\mu\)g of gentamicin sulfate per ml, and 0.25 \(\mu\)g of fungizone per ml and

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buffered with 7.5% sodium bicarbonate to a pH of 7.5. This medium was called complete medium or cMEM. Cells were maintained at 22°C for EPC cells and 18°C for CHSE-214 cells.

Virus isolates. IHNV isolates used included representatives of the five electropherotypes defined by Hsu et al. (9) and were taken from a wide variety of species and different geographic ranges. The IPNV isolates used included Buhl, Sp, and Quebec, and the VHSV isolate was an F1 serotype. For virus growth, cell monolayers were infected at a multiplicity of infection of 0.5 PFU per cell and grown at 18°C, the virus was harvested when complete cytopathic effect was observed, usually in 4 to 5 days. Virus titers in plaque-forming units per milliliter were determined by plaque assays with a 0.75% methylcellulose overlay and without pretreatment of the cell monolayers with polyethylene glycol (PEG).

Quantification of anti-IHNV antibodies in rabbit serum. Rabbit polyclonal antisera to a 1975 IHNV isolate from Round Butte, Oreg., and normal serum were kindly provided by Mark Engelking, Oregon Department of Fish and Wildlife. The anti-IHNV antibody titers of the sera were determined by using immunodot blot methods modified from those of Hsu and Leong (10). Briefly, 100 µl of purified Round Butte IHNV was added to each well of an immunodot blot manifold containing a Nytran filter; the filter was blocked in 5% (wt/vol) skim milk powder in water and then reacted for 1 h with rabbit anti-Round Butte IHNV serum or normal serum. The filter was washed three times with PBS, reacted with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin for 1 h, and washed, and then the blot was developed in 4-chloro-1-naphthol and hydrogen peroxide. The antibody titer was expressed as the highest dilution at which a purple dot was visible.

Growth and sensitization of S. aureus cells. S. aureus (ATCC 12598, Cowan I strain) cells were grown, inactivated, and sensitized with rabbit antibody by using the methods of Bootland (4) as modified from Kimura et al. (14). Briefly, cells were grown in 400 ml of nutrient broth supplemented with 0.5% peptone at 37°C for 18 h, harvested, and washed five times in 100 ml of phosphate-buffered saline (PBS; 0.14 M NaCl, 2.68 mM KCl, 13.3 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.2). Cells were resuspended and fixed in 100 ml of 0.5% Formalin-PBS for 3 h, washed three times in PBS, and heat treated for 1 h at 80°C. After the cells were washed three times in 50 ml of PBS, they were resuspended to a final 10% (vol/vol) concentration in PBS containing 0.1% (wt/vol) sodium azide and stored at 4°C. Formalin-fixed S. aureus cells (10%, wt/vol) were also purchased (BRL, Gaithersburg, Md.).

To sensitize the cells with antibodies, 1 ml of fixed cells was mixed with 0.5 ml of immune or normal rabbit serum. After 1 h at room temperature with occasional shaking, the cells were washed three times in 10 ml of PBS to remove unbound antibody, resuspended to a final 1% (vol/vol) concentration in PBS containing 0.1% sodium azide, and stored at 4°C.

Coagglutination test. Tests were performed on a glass microscope slide by mixing 10 µl of the sensitized S. aureus cells with an equal volume of the sample. The slide was rocked for approximately 5 min and then examined for cell agglutination with a light microscope (×250 magnification).

Coagglutination test. Tests were performed on a glass microscope slide by mixing 10 µl of the sensitized S. aureus cells with an equal volume of the sample. The slide was rocked for approximately 5 min and then examined for cell agglutination with a light microscope (×250 magnification). To determine the specificity of the coagglutination test for detecting IHNV, cells sensitized with either anti-IHNV or normal serum were mixed with reagents commonly used in viral diagnostic tests, cMEM, media from cell cultures, IPNV, VHSV, or IHNV. The titer of each of these virus isolates was greater than 10^7 PFU/ml. Specificity was also tested by determining whether the coagglutination reaction could be blocked by preincubation of Round Butte IHNV in an equal volume of homologous antiserum or normal serum for 30 min at 22°C. The sensitivity of the test was determined by calculating the minimum titer of each of the IHNV isolates of the five electropherotypes needed to cause cell agglutination.

Detection of IHNV directly from fish tissue. Fish samples tested for IHNV by coagglutination included 100 experimentally infected and 10 control rainbow trout fry, 6 adult kokanee salmon from Lake Billy Chinook, Oreg., and 5 broodstock winter steelhead trout from Clackamas Hatchery, Oreg. The fry were sampled from 8 to 20 days after an initial 6-h immersion in 3 × 10^3 PFU of either a Round Butte (type 1) or an International Aquaculture Research Centre (type 2) IHNV isolate per ml. The kokanee were undergoing a natural epizootic of IHN, and the winter steelhead trout were from a hatchery with a history of IHN. Frozen whole kokanee, individual winter steelhead homogenates of pooled kidney, spleen, and pyloric cecum-pancreas diluted 1:20 (wt/vol), and steelhead ovarian fluids diluted 1:2 were kindly provided by Richard Holt, Mark Engelking, and John Kaufman of the Oregon Department of Fish and Wildlife. To prepare samples, individual whole fry were diluted 1:5 (wt/vol) in Hanks’ balanced salt solution (HBSS), and the liver, spleen, kidney and pyloric cecum-pancreas of thawed kokanee were each diluted 1:2 (wt/vol) in HBSS. The samples were homogenized in a Stomacher and centrifuged. The resulting supernatant fluids were diluted 1:2 (vol/vol) in N-2-hydroxyethylpipеразине-N'-2-этансулфonic acid-buffered HBSS containing 1,000 IU of penicillin, 1 mg of streptomycin, 2.5 µg of fungizone and 50 µg of gentamicin sulfate per ml.

The coagglutination test was performed as described above with 10 µl of the final fish tissue sample used in a concomitant infectious virus titer assay. The dilutions used were as follows: for fry homogenates, 1:10; for individual organs of thawed kokanee salmon, 1:4; for steelhead organs, 1:20; and for ovarian fluids, 1:2. Infectious virus titers in the fish samples tested by coagglutination were determined by plaque assay, and virus titers of the kokanee spleens and kidneys, assayed before freezing, were provided by the Oregon Department of Fish and Wildlife.

RESULTS

Rabbit antisera. Two batches of polyclonal rabbit anti-IHNV (Round Butte) sera were used to sensitize S. aureus cells. By immunodot blots, these sera had anti-IHNV binding antibody titers of 163,840 and 192,000 PFU/ml of serum. S. aureus cells sensitized with 0.5 ml of either of these sera agglutinated when mixed with IHNV. The lowest binding anti-IHNV antibody titer needed to effectively sensitize 1 ml of a 10% suspension of staphylococcal cells was approximately 60,000 PFU/ml of serum. The strength of the coagglutination reaction occurring with IHNV was equivalent when the cells were sensitized with an antibody titer of greater than 60,000 PFU/ml of serum. Cells sensitized with an antibody titer lower than 20,000 PFU/ml of serum did not coagglutinate when mixed with IHNV. Normal rabbit serum did not have any anti-IHNV binding antibodies, as determined by using immunodot blots. It was not necessary to adsorb rabbit serum before using it for sensitizing staphylococcal cells.

Visualization of the coagglutination reaction. Macrospecti-
Round Butte IHNV preincubated with, coagglutination cause with any three Cells sensitized virus for serum anti-IHNV from CHSE-214 medium occurred when tion coagglutination reaction, cell lines, negative (Fig. 2C) diagnostic normal by mixing staphylococcal cells sensitized nates the coagglutination reaction, indicating the cells still coagglutinate in in cell cultures from an infection with IPNV or VHSV.

One nonspecific coagglutination reaction was observed. This occurred when cells sensitized with antiserum or normal serum were resuspended in PBS and then mixed with PEG at concentrations of >0.9% (wt/vol) (Table 1). This reaction was indistinguishable from a true positive reaction. When the sensitized cells were washed and resuspended in water instead of PBS immediately before use, the nonspecific reaction was eliminated in the presence of up to 14% PEG. The sensitized cells resuspended in water still coagglutinated in the presence of IHNV, but the limit of sensitivity decreased by approximately 1 log unit to 10⁷ PFU/ml. To identify IHNV in cell cultures pretreated with PEG, the sensitized staphylococcal cells should be resuspended in PBS.

Detection of IHNV isolates from the five electropherotypes. All cell culture-grown IHNV isolates tested in this study caused a positive coagglutination reaction when mixed with staphylococcal cells sensitized with anti-Round Butte IHNV serum (Table 2). These IHNV isolates were representative of the five electropherotypes and were taken from a wide

![FIG. 1. Macroscopic coagglutination reactions of antibody-sensitized staphylococcal cells. Cells had an agranular appearance in a negative reaction (A), whereas a positive coagglutination reaction, indicating the presence of IHNV, often had a granular appearance (Fig. 1B). When fish homogenates or ovarian fluids were assayed by coagglutination, macroscopic observation of the slides often gave misleading results. This was due mainly to the presence of lipid globules in the samples which, when mixed with sensitized staphylococcal cells, resulted in a granular appearance (Fig. 1C). Although results could sometimes be read macroscopically, the coagglutination reaction was much more accurately interpreted when the slides were observed microscopically (×200 to ×400 magnification). A positive reaction was characterized by large clumps of cells under the microscope (Fig. 2A), whereas in a negative reaction the cells were evenly distributed and not clumped (Fig. 2B). Fish homogenates or ovarian fluids could easily be interpreted as IHNV negative (Fig. 2C) or positive (Fig. 2D), and it was easy to distinguish lipid globules from staphylococcal cells. Slides should be examined for coagglutination within 10 min, since staphylococcal cells tend to autoagglutinate after this time.

A negative control should be run for each set of samples to ensure that autoagglutination has not occurred by the time slides are read, and a positive control should be included to ensure that sensitized cells still coagglutinate in the presence of IHNV. Generally, sensitized cells remain stable for 3 months at 4°C.

Specificity of the coagglutination reaction. The specificity of the coagglutination test for identifying IHNV was evaluated by mixing staphylococcal cells sensitized with anti-IHNV or normal serum with reagents and media commonly used in viral diagnostic tests, cell culture media from salmonid and non-salmonid cell lines, or five fish viruses. No coagglutination occurred when staphylococcal cells sensitized with antiserum were mixed with PBS, HBSS, FBS, cMEM, or medium from CHSE-214 or EPC cells (Table 1). A strong coagglutination reaction occurred when cells sensitized with anti-IHNV serum were mixed with IHNV, but no coagglutination occurred when these cells were mixed with any of the three isolates of IPNV or VHSV, another fish rhabdovirus (Table 1). The titers of these viruses were >10⁷ PFU/ml. Cells sensitized with normal serum did not coagglutinate with any of the above samples (Table 1). Round Butte IHNV preincubated with an equal volume of anti-Round Butte serum for 30 min at 22°C and then used in the assay did not cause coagglutination of sensitized staphylococcal cells. Round Butte IHNV preincubated with normal serum, however, was still capable of coagglutinating antibody-sensitized cells. These results indicated that the coagglutination test was specific for IHNV and could detect and differentiate an IHNV infection in cell cultures from an infection with IPNV or VHSV.

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<table>
<thead>
<tr>
<th>Sample</th>
<th>Agglutination reaction of cells* sensitized with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>-</td>
</tr>
<tr>
<td>HBSS</td>
<td>-</td>
</tr>
<tr>
<td>FBS</td>
<td>-</td>
</tr>
<tr>
<td>cMEM</td>
<td>-</td>
</tr>
<tr>
<td>Used cMEM*</td>
<td>-</td>
</tr>
<tr>
<td>IPNV</td>
<td>-</td>
</tr>
<tr>
<td>Buhl</td>
<td>-</td>
</tr>
<tr>
<td>Quebec</td>
<td>-</td>
</tr>
<tr>
<td>Sp</td>
<td>-</td>
</tr>
<tr>
<td>VHSV F1</td>
<td>++</td>
</tr>
<tr>
<td>IHNV Round Butte</td>
<td>+++</td>
</tr>
<tr>
<td>PEG (&gt;0.9%, wt/vol)</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Media removed from cultures of EPC and CHSE-214 cells.
* Formalin-inactivated S. aureus cells were sensitized with rabbit polyclonal serum against the Round Butte IHNV isolate or normal serum and resuspended in PBS. Ten microliters of cells was mixed with 10 µl of each sample. The cells were observed macroscopically for coagglutination.
FIG. 2. Microscopic coagglutination reactions (magnification, ×250). A positive reaction (A), indicating the presence of IHNV, was simple to distinguish from a negative reaction (B). Reactions of fish samples were more accurately read microscopically since lipid globules (arrow) could be easily differentiated from staphylococcal cells. A negative reaction with fish homogenates is shown in C, and a positive reaction is shown in D.
variety of species and different geographic ranges (Table 2). In addition to the isolates shown in Table 2, the following IHNV isolates caused a coagglutination reaction: Tamgas Creek (TA2, from sockeye salmon in Alaska; type 1), Cultus Lake (from steelhead trout in Canada; type 1), Metolius River (from kokanee salmon in Oregon; type 1), and Sacramento River Chinook Virus (from California; type 3).

For each virus isolate in Table 2, the minimum virus titer required to cause coagglutination of anti-Round Butte sensitized cells suspended in PBS was determined. The minimum virus titer was not related to the electropherotype but was dependent upon the virus isolate. All minimum virus titers were within 1 log unit of each other; they varied from a low of 6.00 × 10⁵ PFU/ml for Elk River IHNV to a high of 5.52 × 10⁶ PFU/ml for strain 193-110 IHNV (Table 2). The mean minimum IHNV titer needed to cause coagglutination was 2.38 × 10⁶ PFU/ml (Table 2). For reliable results with this assay, the virus titer should be >10⁶ PFU/ml.

Commercially purchased S. aureus cells and laboratory-grown staphylococcal cells were equivalent in specificity and sensitivity when used in coagglutination tests.

**Detection of IHNV in experimentally infected fry.** Staphylococcal coagglutination was an effective method of identifying IHNV in experimentally infected rainbow trout fry. The fry began dying 3 days after an immersion in either RB or IARC IHNV, and daily mortalities remained high until 14 days postimmersion (dpi). Daily mortalities did not increase after 17 dpi. The cumulative percent mortalities were 44% for the RB-exposed fry and 89% for the IRAC-exposed fry at 17 dpi. Coagglutination testing of 10 fry per group at 3-day intervals during the time of high daily mortalities (8, 11, and 14 dpi) indicated that 70 to 100% of the fry in the two groups were infected with IHNV (Table 3). At 17 and 20 dpi, 50 to 80% of the fry were infected (Table 3). The assay also identified IHNV in moribund fry from 17 to 20 dpi; samples from 8 of 14 moribund fry caused coagglutination. Over the 8 to 20 day sampling time, coagglutination identified 78% of the RB-exposed fry and 72% of the IARC-exposed fry as being infected with IHNV (Table 3). Samples from the 10 control fish tested did not cause coagglutination, indicating that false-positive results did not occur.

The plaque assay was more sensitive than the coagglutination test. With the plaque assays 100% of the fish in both groups were identified as being IHNV infected on all sample days except on 14 dpi, when 90% of the fish in the RB group were identified as IHNV positive (Table 3). Plaque assays indicated that 98% of the RB group and 100% of the IARC group were infected with IHNV over the 8- to 20-dpi sampling period (Table 3); the virus titers in individual fish ranged from 250 to 3 × 10⁷ PFU/g. The mean virus titers ± standard errors obtained from 8 to 20 dpi were 7.25 ± 10⁶ ± 1.6 × 10⁶ and 1.86 ± 10⁶ ± 2.23 × 10⁵ PFU/g for the groups exposed to RB and IRAC IHNV, respectively.

In spite of the higher sensitivity of plaque assays, a positive coagglutination test produced much quicker results regarding fish infection (15 min versus 7 to 14 days). When a coagglutination assay did not coincide with the plaque assay, the virus titer was generally below 10⁶ PFU/g, the detection limit of the coagglutination assay. For only 3 of the 100 fish examined did the coagglutination test result negative when the virus titer was greater than 10⁷ PFU/g.

**Detection of IHNV in adult kokanee salmon.** Coagglutination was used successfully to diagnose IHNV in kokanee adults undergoing a natural epizootic of IHN. Testing of individual organs by coagglutination indicated that five of the six fish were infected with IHNV, with stronger coagglutination reactions observed with the kidney and liver homogenates (Table 4). Plaque assays of kidneys and spleens before freezing indicated that all six fish were infected. The virus titers of the kidneys and spleens of fish 1 to 5 ranged from 9.3 × 10⁵ to 1.4 × 10⁶ PFU/g, and fish six had virus titers ofless than 10⁵ PFU/g (Table 4). After freezing, the virus titers of organs dropped by more than 100-fold to less than 10⁶ PFU/g (Table 4). Coagglutination testing was performed on homogenates prepared from thawed organs and detection of IHNV correlated with the virus titers originally measured in the fresh fish tissues (Table 4). Only fish 6, which had a virus titer of less than 10⁶ PFU/g, was not identified as being infected by coagglutination. These results demonstrated that IHNV did not need to be infectious to be detected by the coagglutination test.

**Detection of IHNV in ovarian fluid and organs of adult steelhead trout.** The coagglutination test detected IHNV directly from the ovarian fluids and organs of asymptomatic winter steelhead trout from Clackamas Hatchery. Two of the organ and ovarian fluid samples from the three females were

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**TABLE 2. Sensitivity of staphylococcal coagglutination in detecting the five electropherotypes of IHNV**

<table>
<thead>
<tr>
<th>IHNV isolate</th>
<th>Typea</th>
<th>Minimum detectable IHNV titer (PFU/ml, 10⁶)</th>
<th>Speciesb</th>
<th>Location</th>
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<tbody>
<tr>
<td>Round Butte 1</td>
<td>1</td>
<td>3.12</td>
<td>Steelhead</td>
<td>Oregon</td>
</tr>
<tr>
<td>Little White salmon</td>
<td>2</td>
<td>2.55</td>
<td>Steelhead</td>
<td>Washington</td>
</tr>
<tr>
<td>Hagerman (193-110)</td>
<td>2</td>
<td>5.52</td>
<td>Rainbow</td>
<td>Idaho</td>
</tr>
<tr>
<td>Hagerman (03982)</td>
<td>2</td>
<td>3.10</td>
<td>Rainbow</td>
<td>Idaho</td>
</tr>
<tr>
<td>IARCc</td>
<td>2</td>
<td>2.05</td>
<td>Rainbow</td>
<td>Idaho</td>
</tr>
<tr>
<td>Elk River</td>
<td>3</td>
<td>6.00</td>
<td>Chinook</td>
<td>Oregon</td>
</tr>
<tr>
<td>Coleman River (CO₂)</td>
<td>4</td>
<td>1.00</td>
<td>Chinook</td>
<td>California</td>
</tr>
<tr>
<td>Cedar River (CDS)</td>
<td>5</td>
<td>1.10</td>
<td>Chinook</td>
<td>Washington</td>
</tr>
</tbody>
</table>

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**TABLE 3. Comparison of staphylococcal coagglutination and plaque assays for detecting IHNV in experimentally-infected rainbow trout fry**

<table>
<thead>
<tr>
<th>Days</th>
<th>IHNV isolate</th>
<th>% of fish shown as IHNV positive by:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Coagglutination*</td>
</tr>
<tr>
<td>8</td>
<td>RB</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>IARC</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>RB</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>IARC</td>
<td>70</td>
</tr>
<tr>
<td>14</td>
<td>RB</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>IARC</td>
<td>70</td>
</tr>
<tr>
<td>17</td>
<td>RB</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>IARC</td>
<td>70</td>
</tr>
<tr>
<td>20</td>
<td>RB</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>IARC</td>
<td>50</td>
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</table>

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* Fry were sampled from 8 to 20 days after a 6 h of immersion in water containing the Round Butte (RB) or International Aquaculture Research Centre (IARC) IHNV isolates. Ten fish per virus isolate were tested on each of sample days.

A 1:5 (wt/vol) dilution of each individual fry homogenate was tested for IHNV by coagglutination.
identified as IHNV infected with the coagglutination test, but neither of the two male organ samples was positive (Table 5). IHNV was detected with plaque assays in all of the samples (Table 5). The coagglutination test was positive when the virus titer was above 10⁶ PFU/ml but was not as sensitive as the plaque assay.

**DISCUSSION**

Staphylococcal coagglutination was shown to be a very rapid, effective method of detecting and identifying IHNV in cell cultures and directly from naturally or experimentally infected salmonids. The test employed Formalin-fixed, heat-treated S. aureus cells coated with rabbit anti-IHNV antibodies and suspended in PBS.

This study is the first to report that coagglutination could be used to detect and identify infectious hematopoietic necrosis virus. Kimura et al. (14) found that staphylococcal cells sensitized with rabbit antiserum against the Yurappu isolate of IHNV did not coagglutinate when mixed with the homologous virus. This difference in results may be due to differences in the antibody titer of rabbit anti-IHNV serum and the titer of cell culture-grown IHNV. In agreement with the data of Hsu and Leong (10), the rabbit anti-IHNV serum used in this present study contained high binding antibody titers. The binding antibody titer of at least 60,000 PFU/ml of serum required to effectively sensitize staphylococcal cells would be equivalent to a neutralization titer of approximately 1:458 (10). The antiserum used by Kimura et al. (14) had a neutralization titer of 1:100, and this may not have been high enough to effectively sensitize the staphylococcal cells. Second, the mean IHNV titer required to consistently cause coagglutination in this study was 10⁶ PFU/ml. The Yurappu IHNV titer in the study by Kimura et al. (14) may not have been this high.

The coagglutination test was developed as a specific method for the universal identification of IHNV. Isolates from five IHNV electropherotypes caused coagglutination of staphylococcal cells sensitized with antiserum to the Round Butte (type 1) IHNV isolate. Polyclonal rabbit anti-IHNV serum clearly recognizes the glycoprotein (G), nucleocapsid protein (N), and a matrix protein (M1) in Western blots (9), but in serum neutralization tests (7, 20) or indirect fluorescent antibody tests (17), it has not been able to differentiate IHNV isolates into serotypes. A positive coagglutination test with all IHNV isolates was likely due to rabbit antiserum binding to epitopes that were common to all the virus isolates. Engeling et al. (7) have shown through cross-neutralization tests with rabbit antiserum to the IHNV G protein that all virus isolates tested from the five electropherotypes were neutralized to some extent. This result indicated that there was at least one conserved neutralizing antigenic site. It has only been with the development of monoclonal antibodies to the G and N proteins of IHNV that identification of common and unique epitopes on these two proteins has become possible (2, 8, 25, 29). Sensitization of staphylococcal cells with monoclonal antibodies that recognize unique epitopes may result in a coagglutination test that will identify different antigenic variants of IHNV.

It was not surprising that IPNV or VHSV did not cause coagglutination of staphylococcal cells sensitized with anti-IHNV serum. Polyclonal anti-IHNV serum has previously been shown not to cross-react with either IPNV or VHSV in plaque reduction assays (18) or immunoblots (19). The specificity of the coagglutination test was supported by
experiments that showed that the reaction could be blocked by preincubation of IHNV with antiserum but not by preincubation of IHNV with normal serum. In addition, no coagglutination reaction was observed with medium components or media removed from continuous cultures of cell lines. It should be noted that it was not necessary to preadsorb the rabbit antibody before sensitization of cells. In other reports of using immune serum to identify IHNV, preadsorption with FBS and established cell lines to prevent high backgrounds or nonspecific reactions were required (6, 10, 17, 19).

It has been recommended that cell monolayers be pre-treated with 7% polyethylene glycol 20000 to enhance the detection of IHNV in cell cultures and decrease incubation time (3). PEG is used for the fractional precipitation of proteins, and precipitation is enhanced in the presence of salts (11). In the coagglutination test, it is likely that the PEG was precipitating the staphylococcal cells when the cells were suspended in PBS. Resuspending the cells in water eliminated the nonspecific reaction with PEG but decreased the sensitivity of the assay. The lower sensitivity should not affect the use of coagglutination for identifying IHNV in cell culture media from PEG-treated monolayers showing complete cytopathic effect where IHNV titers are typically greater than 10⁷ PFU/ml (3, 6).

The three advantages of the coagglutination test over other diagnostic tests for IHNV are its speed (15 min), simplicity, and specificity. Passage of samples in cell culture is required for polycrylamide gel electrophoresis, Western blots (10), and immunoblotting (19, 26); identification of IHNV takes 4 to 52 h. Tissue homogenates and raw ovarian fluids clogged nitrocellulose membranes or produced false-positive results in the immunoblot assay (19, 26). In the enzyme-linked immunosorbent assay, which takes 2 h, whole fry homogenates produced high background readings (6, 28). In contrast to the above methods, whole fry homogenates and raw ovarian fluids were suitable samples for the coagglutination test and no false-positives were obtained. The sensitivity limit of the coagglutination test, 10⁶ PFU/ml, was similar to those of other reported methods. Immunofluorescent antibody testing can detect IHNV directly in blood smears and kidney imprints within 1 h, but ovarian and seminal fluids and tissue homogenates had to be grown in cell culture for 48 h (17). All of the above methods are not well suited to field use, are slower than the coagglutination test, require more than one reagent, and may also require specialized equipment or technical expertise to interpret the results. For the coagglutination test, the single reagent is simple to prepare, requires low amounts of anti-IHNV antibody, and is stable at 4°C for months. Stabilized staphylococcal cells are available commercially as a 10% suspension, and when 1 ml of this suspension is sensitized with 0.5 ml of anti-IHNV antibody there is sufficient reagent to carry out 1,000 coagglutination tests. The only equipment required is a light microscope. A benchtop centrifuge can be used if available, but samples can also be filtered through a 0.45-μm-pore-size filter.

Use of the coagglutination test at a hatchery may eliminate sending samples to a laboratory and waiting for days to obtain a diagnosis. In this study, the coagglutination test identified IHNV in more than 70% of the rainbow trout fry undergoing an experimentally induced IHN epizootic. The test also identified IHNV in the organs of wild kokanee salmon and the organs and ovarian fluids of naturally infected adult steelhead trout. The test could not identify all IHNV-infected samples, since not all samples had a virus titer greater than 10⁶ PFU/ml or 10⁷ PFU/g. However, it is not necessary to identify IHNV from every fish tested to make a diagnosis of IHNV. More sensitive methods of identifying IHNV may have to be used on fish samples that typically have virus titers of less than 10⁶ PFU/ml, such as milk (21–23), or samples that are negative by the coagglutination test. The rapidity of this test is important for management decisions regarding the destruction of infected fish to prevent further spread of the virus.

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