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A Fluorescent Antibody Test for Detection of the Rickettsia Causing Disease in Chilean Salmonids

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Abstract.—An indirect fluorescent antibody test (IFAT) was developed for detection of the rickettsia that was causing epizootics among salmonids cultured in seawater net-pens in southern Chile. Antiserum against the rickettsial agent was produced in New Zealand white rabbits with a preparation grown in antibiotic-free chinook salmon embryo (CHSE-214) cell cultures and partially purified by a combination of filtration and centrifugation steps. The IFAT was effectively used on blood films, tissue sections, and smears. Two gram-negative and two gram-positive bacterial pathogens of salmonids did not react in this test. Detection of the rickettsial agent has previously been restricted to examination by light microscopy or isolation in salmonid cells. The IFAT provides a simple, rapid, sensitive method for detection of the agent and diagnosis of the disease. The rickettsia is thought to be a member of the tribe Ehrlichieae and was tested by IFAT with sera from animals infected with other rickettsial agents.

An epizootic caused by a newly described rickettsial pathogen (Fryer et al. 1990) is ongoing among salmonids cultured in seawater net-pens near Puerto Montt in southern Chile. Although rickettsial diseases have not previously been reported in fish, mortality attributed to this agent reached 90% in 1989 among coho salmon *Onco-*

rhynchus kisutch held at some locations in the area (Bravo and Campos 1989).

It was originally thought that the rickettsial infection was confined to coho salmon, because early losses were reported only in that species. However, infectivity studies have subsequently shown the rickettsia to be pathogenic for coho salmon and Atlantic salmon *Salmo salar* (Garcés et al. 1991), and in 1990, extensive mortality occurred in Atlantic salmon cultured in the area.

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Losses were also sustained among chinook salmon *O. tshawytscha* and rainbow trout *O. mykiss* held in salt water. The disease does not occur at freshwater rearing sites, but 6–12 weeks after healthy fish are introduced into seawater, mortality begins. Diseased fish are lethargic, dark in color, and anemic. Hematocrits fall to 25% or less. The kidney is swollen, the spleen is enlarged, and gray mottled lesions are occasionally present on the liver. There is extensive necrosis of hematopoietic tissues. Also, the rickettsiae can be observed by light microscopy within membrane-bound cytoplasmic vacuoles or inclusions in Giemsa-stained preparations of kidney and other internal organs.

The organism causing this disease is an obligate, intracellular parasite that does not replicate on bacteriological media. However, it can be grown in salmonid cells cultured in antibiotic-free medium and incubated at 15–18°C, where it produces a cytopathic effect. The organism is pleomorphic, predominantly coccoid, and has a diameter of about 0.5–1.5 μm; pairs and ring forms are commonly seen (Fryer et al. 1990). Fryer et al. (1990) tentatively placed it in the order Rickettsiales, family Rickettsiaceae, and tribe Ehrlichieae—a group of intracellular bacteria that are morphologically similar to this agent and produce lesions in other animals similar to those observed in the disease affecting coho salmon.

With the extensive movement of fish and fish products, the rickettsia warrants the attention of the entire salmonid-culture industry. Its transfer from the west coast of South America to other continents is of particular concern. A clear need exists for diagnostic methods that are sensitive, rapid, and simple to perform to prevent introduction of this agent into areas where it does not presently occur. Sensitive methods are also required if the source of the infection in the marine environment is to be identified.

Detection of the rickettsia has been limited to microscopic examination of Giemsa-stained tissues or isolation of the organism in fish cell lines. However, microscopic observation of the agent in Giemsa-stained preparations is difficult when low numbers are present, and isolation in cell culture can be challenging because assays must be conducted in antibiotic-free medium.

Fluorescent antibody tests are widely used for detection and diagnosis of disease in both human and animal medicine (Bullock and Stuckey 1975; Jones et al. 1978; Ristic et al. 1986). These tests are sensitive, rapid, and easily accomplished in any laboratory equipped with a fluorescence mi-

croscope. The methods have become standardized, and the results are specific. For this reason, we have developed an indirect fluorescent antibody test (IFAT) for detection of the coho salmon rickettsia. The method and results of the test are described.

Methods

Preparation of antigen. —Rickettsiae (strain LF-89) for antigen production were grown in a chinook salmon embryo cell line (CHSE-214; Lannan et al. 1984) cultured in antibiotic-free Eagle's minimum essential medium (MEM) with Earle's salts (Sigma Chemical Co., St. Louis, Missouri) supplemented with 10% fetal bovine serum (MEM-10) (Hyclone Laboratories, Inc., Logan, Utah). The cultures were incubated at 15°C and harvested when the cytopathic effect reached completion (about 14 d).

The antigen preparation was partially purified with a modification of the method of Weiss et al. (1975). Briefly, spent medium was collected from five 150-cm^2 flasks of fully lysed CHSE-214 cells, and rickettsiae with accompanying host cells and debris were concentrated by centrifugation at 17,300 × gravity for 15 min. The supernatant was decanted; the cell pellet was resuspended in 10 mL MEM-10, homogenized with 20 strokes in a Ten Broeck® tissue grinder, and centrifuged for 10 min at low speed (200 × gravity) to remove host-cell debris. Further debris was removed by passing the supernatant fluid through an AP20 microfiber glass filter (Millipore Corp., Bedford, Massachusetts). The filter was rinsed with an additional 10 mL of MEM-10, and the total volume filtered through a second AP20 filter. The filtrate was centrifuged for 15 min at 17,300 × gravity, the supernatant was decanted, and the rickettsial pellet was resuspended in 10 mL of 10% formalin in MEM (pH about 7.5). The preparation was stored overnight at 4°C and centrifuged as before, and the pellet was washed twice with 10 mL of 0.01 M phosphate-buffered saline (PBS) at pH 7.0. Following the final centrifugation, the rickettsiae were resuspended in 1 mL of PBS and delivered to a commercial laboratory (Immugenics, Corvallis, Oregon) for injection into two New Zealand white rabbits.

Preparation of test slides. —Spent medium containing rickettsiae, free or attached to host-cell debris, was harvested from fully lysed cultures of infected CHSE-214 cells and spotted onto wells (about 0.05 mL/well) of 10-well microscope slides (Erie Scientific Co., Portsmouth, New Hampshire). Uninfected control cells were harvested with

a cell scraper and also spotted onto multiple-well slides. The preparations were air-dried, heat-fixed, and treated for 5 min with 100% methanol at ambient temperature, then stored at −20°C until used.

Blood films from infected and control fish were air-dried and fixed for 5 min in 100% methanol. Tissue smears were air-dried and either heat-fixed or fixed for 5 min in 100% methanol. Whole fish (fork length, about 8 cm) were fixed in 10% buffered formalin, embedded in paraffin, and cut in 7-μm-thick sections.

Testing of antiserum.—All IFATs were conducted in the following manner. The primary antibody was applied, and the slides were incubated for 30 min at ambient temperature in a darkened, humidified chamber. They were then rinsed thoroughly with PBS and dried in a stream of air before application of the second antibody, which was a commercially prepared antiglobulin labeled with fluorescein isothiocyanate (FITC; Sigma) that was diluted in PBS as specified by the manufacturer. This antibody was applied, and following 30 min of incubation, the slides were rinsed and dried as before. They were counterstained for 2 min with 1% methyl green (Allied Chemical Corp., New York) to reduce nonspecific background fluorescence, then rinsed and dried again, and a coverslip was applied with buffered glycerol mounting medium (pH 8.0). Slides were viewed at 630× magnification with a Zeiss standard microscope equipped with a quartz halogen lamp for incident-light fluorescence and the following combination of filters: KP 490, FT 510, and LP 520.

For titration, hyperimmune serum from each rabbit was diluted in Hanks' balanced salt solution and used as the primary antibody. A series of two-fold dilutions from 2^{-1} to 2^{-15} was applied to test and control slides at one drop per well, one well per dilution. As a negative control, a parallel test was conducted with preimmune serum from the same rabbit. The second antibody used in each of these tests was an FITC-labeled goat anti-rabbit immunoglobulin G (IgG; Sigma).

Evaluation of fluorescence.—Fluorescence was rated with a system adapted from Jones et al. (1978). Maximal fluorescence was rated as 4+, bright fluorescence as 3+, definite but moderate fluorescence as 2+, very subdued fluorescence as 1+, and an absence of specific fluorescence as 0. The highest dilution of the antiserum producing at least 3+ fluorescence on positive control slides and producing 0 fluorescence on negative control slides was defined as optimal for use. After determination of that dilution, it was used for testing

blood films, tissue sections, and smears, which were stained and incubated as described.

Tests with other bacteria.—Two gram-negative and two gram-positive bacterial fish pathogens were tested for reaction with the anti-rickettsial serum. Individual colonies were picked from tryptic soy agar plates containing actively growing pure cultures of the gram-negative bacteria *Vibrio anguillarum* and *Aeromonas salmonicida,* or the gram-positive bacterium *Carnobacterium piscicola.* The bacteria were suspended in PBS, dropped onto multiple-well slides, air-dried, and heat-fixed. These preparations were tested with two-fold dilutions of the antiserum as previously described. In addition, a kidney smear from a fish infected with the gram-positive bacterium *Renibacterium salmoninarum,* the causative agent of bacterial kidney disease, was tested as follows. The smear was heat-fixed and treated with absolute methanol for 5 min. Two circles were drawn on the smear with a wax pencil, and material within one circle was tested in the anti-rickettsial IFAT as described. As a positive control, material within the other circle was tested in a direct fluorescent antibody test with an FITC-conjugated antibody made against *R. salmoninarum.*

Tests with other sera.—To assess the presence of antigens common to the coho salmon rickettsia and members of the tribe Ehrlichieae, sera from animals infected with other ehrlichial agents were tested with the salmonid rickettsia by IFAT. The sera came from dogs infected with *Ehrlichia canis* or canine granulocytic *Ehrlichia* (Ewing et al. 1971) and included both acute and convalescent sera from dogs infected with *Neorickettsia helminthoeca,* the "salmon-poisoning" disease agent. In addition, serum from a horse infected with *Ehrlichia equi* was also tested. These assays were conducted as described for titration of the anti-rickettsial serum, with a series of two-fold dilutions of each antiserum as the primary antibody and the cell-culture-grown coho salmon rickettsiae as the antigen. The second antibody used against the dog or horse serum was a commercially prepared, FITC-conjugated, rabbit anti-dog IgG or rabbit anti-horse IgG (Sigma), respectively.

Results

Antiserum Titers

The fluorescent antibody titer of the serum from one rabbit was about an order of magnitude greater than that of the other; therefore, following titration, all further tests were performed with the
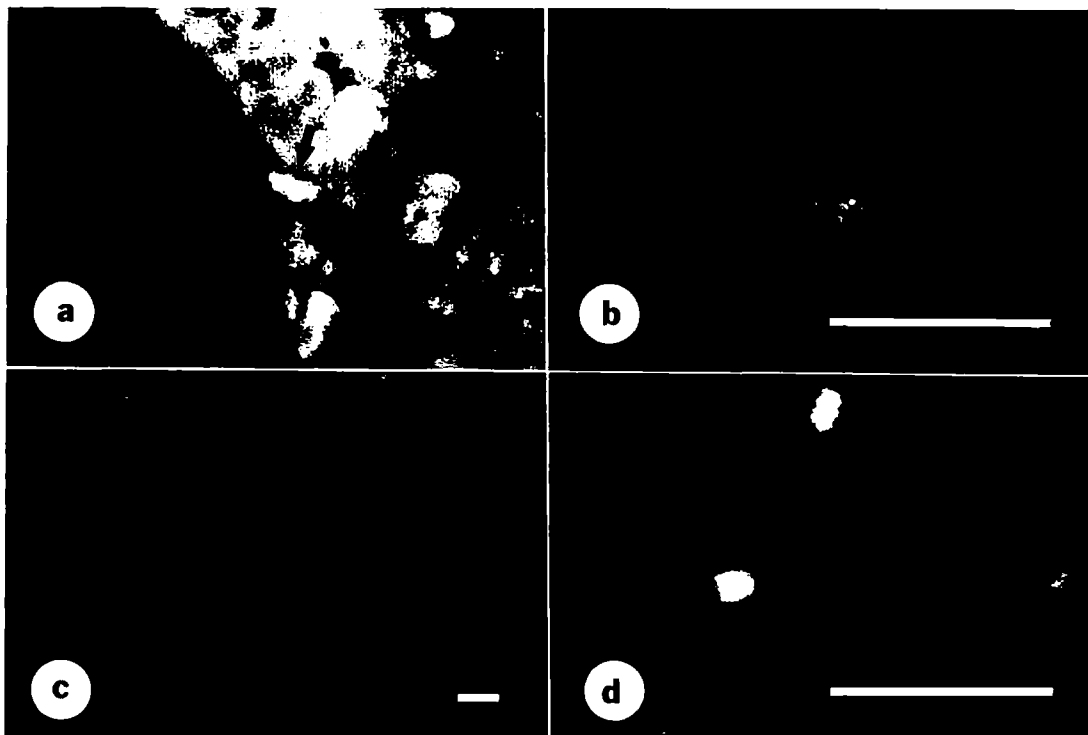
FIGURE 1.—The salmonid rickettsia. Indirect fluorescent antibody stain with a methyl green counterstain. Bars = 10 μm. (a) Intracellular rickettsiae (arrow) in a section of gonadal tissue from an experimentally infected coho salmon. The organisms occur within cytoplasmic inclusions in the host cells. (b) Enlarged detail from panel a. Note the ringlike staining of intracellular rickettsiae with fluorescence apparent only around the perimeter of each organism. (c) Rickettsiae free in a kidney smear from an experimentally infected fish. (d) Enlarged detail from panel c. Note the more uniformly stained appearance of these extracellular organisms.

higher-titer serum. The fluorescent antibody titer of the serum used was 16,384 (expressed as the reciprocal of the highest dilution producing 1+ fluorescence or higher), and the optimal dilution was 1:1,024. Thereafter, a 10^{-3} dilution of the serum was used as the primary antibody on blood films, tissue sections, and smears.

IFAT on Tissues from Infected Fish

Bright, specific fluorescence with little or no background fluorescence was observed on freshly prepared blood films and smears of kidney and liver tissue from rickettsia-infected fish. Preparations, fixed and stored at −20°C, also showed bright, specific fluorescence when tested 6–12 months after they were prepared, but tissue and blood smears stored at temperatures of 4°C or higher no longer reacted with the antibody after 3 months of storage.

The rickettsia was detected in sections of naturally and experimentally infected fish and was observed in formalin-fixed coho salmon tissues collected from an epizootic in 1987 and stored for 4 years at ambient temperature. The rickettsial antigens remained stable in these tissues and displayed bright fluorescence when tested with the IFAT. Rickettsiae in these sections and within host cells in blood films and tissue smears appeared as rings, with fluorescence only around the outer edges (Figure 1a, b). However, extracellular rickettsiae present in tissue smears, as well as those in test slides of cell culture material, stained more uniformly (Figure 1c, d). In some smears, many of these extracellular rickettsiae had irregular outlines and appeared to have been damaged.

A small population of leukocytes present within liver tissues and in peripheral blood also reacted with the antibody against the coho salmon rickettsia. These cells were fluorescent in preparations from both infected and uninfected fish. Fluorescence was not reduced when the antiserum was adsorbed against CHSE-214 cells, and the leuko-

cytes did not react with preimmune rabbit serum. However, the large size difference between the leukocyte and the rickettsia prevents confusion of the two.

Reactions with Other Bacteria and Sera

There were no cross-reactions in the IFAT with any of the gram-negative or gram-positive bacterial fish pathogens tested. The section of the kidney smear that was positive for bacterial kidney disease showed 4+ fluorescence when tested with *R. salmoninarum* antiserum. However, there was no specific fluorescence observed in the anti-rickettsial test portion. There was also no specific fluorescence in any of the preparations made from cultured bacteria.

There was no apparent reaction between the coho salmon rickettsia and serum from animals infected with *E. canis, E. equi,* or *N. helminthoeca.* No specific fluorescence was observed in any of these tests. However, the serum from a dog infected with canine granulocytic *Ehrlichia* had a fluorescent antibody titer of 64 against the rickettsia from coho salmon.

Discussion

The IFAT described here provides a sensitive and specific method for detection of the coho salmon rickettsia. It was successfully used on blood films, tissue sections, and smears. Use of the IFAT avoids the problems associated with methods currently used to detect the rickettsia and diagnose the disease it produces. Unlike the Giemsa stain, the IFAT was specific, and rickettsiae were clearly differentiated from background material. With this technique, there were no reactions with four other bacterial pathogens that might be present in salmonid tissues. A low number of normal fish leukocytes were fluorescent in smears from either infected or uninfected fish, suggesting that one or more antigens are common to these cells and the rickettsial pathogen. However, the large size difference between the leukocytes and the rickettsiae made them easily distinguishable in smears of infected fish tissue.

The test was simple to perform, the results were rapidly determined, and there was no requirement for strict aseptic conditions. The circumstances associated with necropsy of fish in the field are rarely ideal, and when samples are collected for isolation of pathogens in cell culture, the chance of contamination is great. The requirement to conduct these assays in antibiotic-free medium makes isolation of the rickettsia difficult to accomplish. Even when contamination is avoided, 2–3 weeks of incubation at 15–18°C are required, on primary isolation, before the first appearance of a cytopathic effect.

Salmonids develop the rickettsial disease following transfer to salt water, but the source of the infection in the marine environment has not been determined. The IFAT provides a tool for elucidation of the infectious process through examination of organisms collected from the vicinity of net-pens where salmonids are reared.

Although the IFAT was used successfully on freshly prepared material, it is imperative that preparations not tested immediately be stored at or below −20°C. Rickettsiae in blood films and tissue smears could no longer be detected by the IFAT after 3 months of storage at temperatures of 4°C or higher, although rickettsiae in sections of formalin-fixed tissue reacted well in the IFAT after 4 years of storage at ambient temperature.

The fluorescence pattern of extracellular rickettsiae was different from that observed in organisms found within host cells. Intracellular rickettsiae appeared in the IFAT as rings, whereas fluorescence was observed over the entire surface of organisms that were attached to host-cell debris or free in the smear. Many of these organisms appeared to have been damaged, perhaps as a result of procedures associated with preparation of the smear.

To look for antigens common to the coho salmon rickettsia and members of the tribe Ehrlichieae, five sera from animals with ehrlichial infections were tested with the salmonid rickettsia in an IFAT. A low-level reaction was observed with serum from a dog infected with canine granulocytic *Ehrlichia,* suggesting a small number of shared antigens. There was no reaction with any of the other sera.

It was interesting to note that there was no reaction in the IFAT between the salmonid rickettsia and either acute or convalescent serum from dogs infected with *N. helminthoeca.* Until the rickettsia was isolated from coho salmon, the "salmon-poisoning" disease agent was the only rickettsia reported to have an association with fish. *Neorickettsia helminthoeca* is, however, a pathogen of canids, not fish. It does not replicate within fish cells, but is carried by a digenetic trematode, *Nanophyetes salmincola,* which is a parasite of salmonids in the Pacific Northwest of the USA. The IFAT has shown that the coho salmon rickettsia and *N. helminthoeca* are serologically unrelated.

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