Isolation of a Rickettsiales-Like Organism from Diseased Coho Salmon (*Oncorhynchus kisutch*) in Chile*1

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(Received April 13, 1990)

A rickettsiales-like organism was isolated in cell culture from coho salmon (*Oncorhynchus kisutch*) reared in seawater net pens in the vicinity of Puerto Montt, Chile S. A. and experiencing an epizootic with accompanying mortality. The organism, observed in fish and isolated in cell culture, was Gram-negative, pleomorphic, predominantly coccoid in shape, and approximately 1 μm in diameter. It replicated, producing cytopathic effect (CPE) in cell lines from four species of salmonids, but failed to grow in any of eleven standard bacteriological media. The optimal temperature for replication was 15–18°C based on the production of CPE. Streptomycin, gentamicin, and tetracycline were inhibitory for the organism, but it was not sensitive to penicillin.

The isolated organism stained with Giemsa and Pinkerton's stains and with a modified Gimenez stain. It did not react with a monoclonal antibody against the group-specific LPS chlamydial antigen. On the basis of available information, we suggest this organism is a member of the order Rickettsiales and represents the first isolation of a member of this group from an aquatic poikilotherm.

Rickettsiales-like and chlamydiales-like organisms have been reported to infect many species of aquatic animals (Hoffman et al., 1969; Harshbarger et al., 1977; Johnson, 1984). These reports have been largely descriptive in nature with detailed accounts of the ultrastructure of the agents and the pathology produced in their aquatic hosts. Until now, however, attempts at in vitro culture of these intracellular microorganisms, observed in fish and shellfish, have been unsuccessful (Wolf, 1981; Bradley et al., 1988).

During 1989, coho salmon (*Oncorhynchus kisutch*) cultured in seawater net pens in Chile experienced an epizootic of unknown etiology. The disease was first observed at the Huito Channel, which is in the vicinity of Puerto Montt, and the site of a large number of salmon farms. The peak mortality occurred in May and was severe, with losses of up to 90% reported at certain locations (Bravo and Campos, 1989). The disease was only observed in coho salmon and not in chinook (*Oncorhynchus tsawytscha*) and Atlantic salmon (*Salmo salar*) or in rainbow trout (*Oncorhynchus mykiss*), although these species are also reared in the affected area.

Disease signs in moribund fish were not diagnostic. They included: lowered hematocrit, swollen kidney, enlarged spleen, and, occasionally, mottled liver. No infectious agents were isolated by Bravo and Campos (1989) who first described the disease, but they did observe, by both light and electron microscopy, an unidentified parasite in the blood and internal organs of infected fish.

As part of an effort to determine the cause of this continuing epizootic, kidney tissue from diseased fish was inoculated onto an established chinook salmon cell line, CHSE-214 (ATCC CRL 1681, Lannan et al., 1984), which is routinely used in the diagnosis of salmonid viral diseases. A rickettsiales-like agent was isolated in these cell cultures.

*1 This was an invited paper, presented as a special lecture at the annual meeting of the Japanese Society of Fish Pathology, Tokyo, March, 1990.
Materials and Methods

Isolation

Two year old coho salmon, 37 to 41 cm in length and 900 to 1200 g in weight, were collected on October 17, 1989 from a saltwater net pen at the Eicosal S. A. salmon farm on Isla Guar near Puerto Montt, Chile where an epizootic was in progress. The outer surfaces of the fish were sanitized with 50 ppm iodophor, and samples were taken for bacteriological and virological analysis. At the same time, kidney tissue was aseptically removed and inoculated directly into 25 cm\(^2\) tissue culture flasks (Corning Glass Works, Corning, NY) containing a monolayer of CHSE-214 cells in antibiotic-free Eagle’s Minimum Essential Medium with Earle’s salts (MEM-10) (Automod, Sigma Chemical Co., St. Louis, MO), supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT). The cultures were kept at 4°C for transport to the Oregon State University Hatfield Marine Science Center Fish Disease Laboratory, Newport, Oregon USA. Upon arrival, the cultures were incubated at 15°C. When cytopathic effect (CPE) was observed in these cultures, aliquots of spent culture medium were transferred to fresh CHSE-214 cultures and incubated at 4, 10, 15, 18 and 21°C.

Microscopy

Sterile 15 mm round coverslips (Bellco Glass, Inc., Vineland, NJ) were placed in 24-well flat bottom tissue culture plates (Corning). The plates were seeded with CHSE-214 cells, inoculated with the organism, and incubated at 15°C. At 24 hour intervals, coverslips were removed and rinsed in balanced salt solution (BSS). The cells were fixed in absolute methanol and stained with MAY GREENWALD (Allied Chemical Corp., New York, NY) and GIEMSA (J. T. Baker Chemical Co., Phillipsburg, NJ). The prepared coverslips were mounted on microscope slides with histological mounting medium (Permount, Fisher Chemical Co., Fair Lawn, NJ) for observation at 100× under oil immersion.

Cultures prepared for transmission electron microscopy were grown in 25 cm\(^2\) tissue culture flasks, and the cell sheet was fixed in place with 2.5% glutaraldehyde (Sigma) in BSS. The cells were scraped from the flask and centrifuged at 1000 × g for 5 min. The cell pellet was post fixed with osmium, dehydrated, and embedded in SPURR’s embedding medium (SPURR, 1969). The fixed cells were then sectioned, stained in REYNOLD’s lead citrate (REYNOLDS, 1963), and viewed with a Philips CM12/STEM at 60 kV in transmission mode.

Cells to be viewed by scanning electron microscopy were grown on coverslips in the same manner as those prepared for light microscopy. At selected intervals, the coverslips were removed from the plates and rinsed in BSS. After fixation in 2.5% glutaraldehyde, the cells were critical point dried, coated with 200 nm 60/40 wt% Au/Pd, and viewed with an Amray 1000A SEM operated at 20 kV.

In vitro culture and antibiotic sensitivity

The CHSE-214 cell line was used for isolation and routine propagation of the organism. Three additional salmonid cell lines and four cell lines originating from warmwater fish species were also inoculated with the organism and observed for the development of CPE. These cell lines were: CSE-119, from coho salmon; CHH-1 (ATCC CRL 1680) from chum salmon (Oncorhynchus keta) (both LANNAN et al., 1984); RTG-2 (ATCC CCL 55) derived from rainbow trout (Oncorhynchus mykiss) (WOLF and QUIMBY, 1962); EPC from the common carp (Cyprinus carpio), (FIJAN et al., 1983); FHM (ATCC CCL 42), originating from the fathead minnow (Pimephales promelas) (GRAVELL and MALSBERGER, 1965); BB (ATCC CCL 59) from the brown bullhead (Ictalurus nebulosus) (WOLF and QUIMBY, 1969); and BF-2 (ATTC CCL 91) derived from the bluegill (Lepomis macrochirus) (WOLF et al., 1966).

Tryptic soy agar, tryptic soy agar with 5% coho salmon blood, fluid thioglycolate, OF medium with 1% dextrose (all Difco Laboratories, Detroit, MI) MEM-10, Dorset egg medium, Petragnani medium (both Difco Manual, 1984), HERROLD’s egg yolk agar (U. S. Dept. of Agriculture, 1974) made up without malachite green, KDM-2 broth with 10% bovine serum (EVELYN, 1977), charcoal agar (Daly and STEVENSON, 1985); and mycoplasma medium (Mycotrim-TC, Hana Biologics, Inc., Alameda, CA) were inoculated with spent medium from cell cultures showing
extensive CPE. The inoculated media were incubated at 15°C and observed for 30 days for the appearance of growth.

Antibiotics at the concentrations indicated were added to 25 cm² flasks of CHSE-214 cells seeded in MEM-10: penicillin, 100 IU/ml; streptomycin, 100 μg/ml; gentamicin, 50 μg/ml; or tetracycline, 15 μg/ml (all Sigma). The flasks were inoculated with 0.1 ml of spent medium from a lysed culture, incubated at 15°C for 14 days, and observed for the appearance of CPE. After 14 days incubation, 0.5 ml of medium from each flask was inoculated onto fresh cell cultures prepared in antibiotic-free medium. These flasks were incubated at 15°C and observed for CPE for an additional 14 days.

Titration

The infectivity titer was determined by endpoint dilution assay (TCID₅₀) in 96-well plates with six wells per dilution or by plaque assay in 24-well plates with three wells per dilution. Dilution endpoints were calculated by the method of Reed and Muench (1938).

For the plaque assay, medium was removed from the plate and a 0.1 ml aliquot from the appropriate sample dilution was added to each of three wells. The organism was allowed to adsorb for one hour or 24 hours; then the infected cells were overlayed with MEM containing 5% FBS and 0.75% methylcellulose (Sigma). The cells were incubated for 14 days at 15°C, then fixed with 10% formalin and stained with 1% crystal violet.

Freeze-thaw

Spent medium from a cell culture in which CPE was complete was titered by endpoint dilution assay. Aliquots of the remaining medium were frozen at −70°C with no cryopreservative or with the addition of either 10% glycerol or 10% dimethyl sulfoxide (DMSO) (both Sigma). After 6 days, frozen cultures were rapidly thawed in a 20°C water bath and the titer again determined.

Serological and staining reactions

A fluorochrome-labeled monoclonal antibody commercially prepared against the group-specific (genus-specific) chlamydial LPS antigen (California Integrated Diagnostics, Inc., Benicia, CA) was used as a reagent to characterize the organism. Airdried smears of medium from a cell culture showing complete CPE were incubated with the monoclonal antibody and viewed with a fluorescence microscope.

Additional smears were heat-fixed and Gram-stained or fixed and stained in the appropriate manner for each of the following stains developed for the demonstration of rickettsiae and chlamydialae: Macchiavello (Macchiavello, 1937), Gimenez (Gimenez, 1964), or a modification of the Gimenez stain for staining Rickettsia tsutsugamushi (Bartolomev, 1981). CHSE-214 cells grown on coverslips were fixed in 10% neutral buffered formalin and stained with Pinkerton's stain (Simmons and Gentzkow, 1944).

Results

Isolation

No bacterial pathogens were isolated from the diseased fish, and no CPE was produced in cell cultures containing antibiotics. However, CPE in the form of clusters of rounded cells appeared in antibiotic-free CHSE-214 cultures after ten days incubation at 15°C (Fig. 1). When medium from infected cultures was transferred to fresh cells, CPE appeared in the monolayer within 5–6 days at 15 or 18°C, and the entire cell sheet was completely lysed by approximately 14 days. At temperatures above and below 15–18°C, i.e. 10 and 21°C, there was a delay in the appearance of CPE. It took 17 days for CPE to appear in these cultures, and it was 30 days before CPE reached completion. Cultures incubated at 4°C did not develop CPE during the 60-day observation period. Thus, the apparent temperature optimum for in vitro culture of the coho salmon organism was 15–18°C. All subsequent experimental cultures were incubated at 15°C.

Microscopy

Giemsa-stained smears of fluid from infected cultures contained large numbers of darkly-staining microorganisms. These microorganisms were pleomorphic, occurring as coccoid or ring forms, and frequently appeared in pairs. They varied in diameter from approximately 0.5 to 1.5 μm. Microscopic examination of fixed and fixed

Figs. 3–6. 3, Ultrathin section of an infected CHSE-214 cell. Rickettsiales-like organisms lie within membrane-bound vacuoles. Note the rippled cell wall and electron-lucent spherical structures. Bar = 1 μm. 4, A rickettsiales-like organism undergoing binary fission within a vacuole in the cytoplasm of an infected CHSE-214 cell. Bar = 1 μm. 5, Scanning electron micrograph of CHSE-214 cells 24 hours after inoculation with the rickettsiales-like organism. Organisms of varied sizes are attached to the exterior surfaces of the host cells. Bar = 1 μm. 6, Scanning electron micrograph of rickettsiales-like organisms free or being released from CHSE-214 cells eight days after inoculation. Note the ring form (arrow) and the variation in size among the coccoid forms. Bar = 10 μm.
stained cell cultures showed the microorganisms to be replicating within cytoplasmic inclusions in infected cells (Fig. 2).

Transmission electron microscopy revealed individual or paired organisms enclosed in membrane-bound vacuoles (Fig. 3). Each organism was bound by two membrane layers, an undulate outer membrane and a closely-apposed inner membrane. These membranes have been described for other Rickettsiales as a rippled cell wall and a plasma membrane (Anderson et al., 1965). Electron-dense areas containing ribosome-like structures were concentrated near the plasma membrane, and fibrillar DNA-like material was localized in the central region. Many of the organisms contained one or more electron-lucent spherical structures. Organisms apparently undergoing binary fission were frequently observed (Fig. 4).

When infected cells were examined by scanning electron microscopy after 24 hours incubation, irregular coccoid organisms of approximately 1 μm in diameter were found attached to the exterior surfaces of host cells (Fig. 5). After eight days incubation, the organisms were observed spilling from ruptured host cells or free in the intercellular spaces (Fig. 6). These organisms had highly folded outer membranes and varied in size and morphology.

**In vitro culture and antibiotic sensitivity**

The organism was cytopathic in all of the salmonid cell lines tested (Table 1), but, in cell lines from warmwater fish, the induction of CPE was variable. Cytopathic effect was induced in FHM and EPC cells, but none was apparent in BB and BF-2 cultures. No growth was demonstrated in any of the bacteriological media inoculated. These results suggested that the organism was an obligately intracellular parasite.

The presence of antibiotics, with the exception of penicillin, inhibited *in vitro* replication of the organism (Table 2). No CPE developed in cultures containing streptomycin, gentamicin, or tetracycline, and no infectious organisms could be subcultured from flasks containing these antibiotics.

**Titration**

Both endpoint dilution and plaque assay were used to determine the titer of the organism after growth in cell culture. Titters of 10^6–10^5 TCID₅₀/ml were detected in medium from CHSE-214 cultures that had undergone complete CPE. However, many of the agents remained associated with the cellular debris following lysis of the host cells. Therefore, this titer may be an underestimate of the actual titer because of the strong affinity of the organism for the cellular material. Titters comparable to those obtained by endpoint dilution were observed by plaque assay when the organism was adsorbed to the cells for 24-hours before the methylcellulose overlay was added. If the overlay was added after a one hour adsorption period, there was a ten-fold reduction in plaque titer.

**Freeze-thaw**

One cycle of freeze-thaw at −70°C decreased the titer of the organism by >99% (Table 3), and the addition of 10% glycerol caused an even further decrease in titer. However, the presence of 10% DMSO in the freezing medium provided a cryopreservative effect and increased survival of the

<table>
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<tr>
<th>Cell Line</th>
<th>Species of Origin</th>
<th>Cytopathic Effect Produced</th>
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<tbody>
<tr>
<td>CHSE-214</td>
<td>Oncorhynchus tshawytscha</td>
<td>yes</td>
</tr>
<tr>
<td>CSE-119</td>
<td>Oncorhynchus kisutch</td>
<td>yes</td>
</tr>
<tr>
<td>CHH-1</td>
<td>Oncorhynchus keta</td>
<td>yes</td>
</tr>
<tr>
<td>RTG-2</td>
<td>Oncorhynchus mykiss</td>
<td>yes</td>
</tr>
<tr>
<td>EPC</td>
<td>Cyprinus carpio</td>
<td>yes</td>
</tr>
<tr>
<td>FHM</td>
<td>Pimephales promelas</td>
<td>yes</td>
</tr>
<tr>
<td>BF-2</td>
<td>Leptomis macrochirus</td>
<td>no</td>
</tr>
<tr>
<td>BB</td>
<td>Ictalurus nebulosus</td>
<td>no</td>
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Table 2. In vitro antibiotic sensitivity of the rickettsiales-like organism

<table>
<thead>
<tr>
<th>Antibiotic Tested</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
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<tbody>
<tr>
<td>penicillin</td>
<td>100 IU/ml</td>
<td>no</td>
</tr>
<tr>
<td>streptomycin</td>
<td>100 μg/ml</td>
<td>yes</td>
</tr>
<tr>
<td>gentamicin</td>
<td>50 μg/ml</td>
<td>yes</td>
</tr>
<tr>
<td>tetracycline</td>
<td>15 μg/ml</td>
<td>yes</td>
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</tbody>
</table>

Table 3. Titer of the rickettsiales-like organism after six days storage at -70°C in the presence of selected cryopreservatives*1,2

<table>
<thead>
<tr>
<th>Cryopreservative Added</th>
<th>Titer (TCID₅₀/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>10⁵.⁰</td>
</tr>
<tr>
<td>10% glycerol</td>
<td>10⁴.¹</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>10⁴.³</td>
</tr>
</tbody>
</table>

*1: Starting titer was 10⁴.⁴ TCID₅₀/ml.
*2: Frozen cultures were rapidly thawed in a 20°C water bath.

Organism by a magnitude of ten over cultures frozen without additives.

Serological and staining reactions

The isolated organism was Gram-negative. It did not stain with the MACCHIAVELLO or GIMENEZ stains for rickettsiae and chlamydiae. It was stained positively by PINKERTON’s method and with a modification of the GIMENEZ stain developed to demonstrate R. tsutsugamushi. The organism failed to react with the anti-chlamydial LPS monoclonal antibody, a reagent used to identify organisms of the genus Chlamydia.

Discussion

Many of the infectious agents observed in cultured salmonids in Chile were introduced with the eggs shipped from salmon producing areas in the northern hemisphere. An example of this is Renibacterium salmoninarum, the causative agent of bacterial kidney disease. The disease currently affecting coho salmon, however, is believed to have had its origins in Chile. It has not been reported in fish held in fresh water, and the onset of the disease has not been observed until 6-12 weeks after the transfer of fish to saltwater rearing pens. It is possible the source of the microorganism responsible for this disease may be one or more native species of aquatic animal(s).

Rickettsiales-like and chlamydiales-like microorganisms have been observed in numerous aquatic animals. They have been found worldwide in molluscs (Buchanan, 1978; Morrison and Shum, 1982; Elston and Peacock, 1984) and have been reported to infect crustaceans (Johnson, 1984; Sparks et al., 1985; Brock et al., 1986), and amphibians (Desser and Barta, 1984); but few of these agents have been associated with fishes. A review by Wolf (1981) of the chlamydia and rickettsia of fish indicates that the only example in the literature of rickettsia in fish is an unconfirmed case report from Egypt in 1939 where small coccoid forms, staining pink with Giemsa, were found within monocytes and in plasma of blood smears from a dead tetradontid fish.

One rickettsia that is associated with fish but does not replicate in fish tissues is Neorickettsia helminthoea, the cause of the “salmon poisoning” disease of canines (Noonan, 1973). This rickettsia is carried by a digenetic trematode, Nanophyetus salmonicola, a parasite of salmonids in the Pacific Northwest USA (Milleman and Knapp, 1970).

One chlamydia-like agent that has been reported to infect fishes is the cause of epitheliocystis in numerous species (Hoffman et al., 1969; Morrison and Shum, 1983; Paperna and Alves de Matos, 1984; Rourke et al., 1984; Zimmer et al., 1984; Bradley et al., 1988). A possible second chlamydia-like organism, morphologically similar but substantially smaller than that causing epitheliocystis, was observed in lake trout (Salvelinus namaycush) by McAllister and Herman (1987). None of these pathogens of fish or shellfish has been propagated in vitro.

In the study reported here, a microorganism associated with the Chilean coho salmon disease was isolated, and preliminary characterization of the replication of this microorganism in cell culture was made. The microorganism was an obligately intracellular parasite, and replicated in cultured salmonid fish cells but did not grow in any of the standard bacteriological media tested. Growth of the microorganism was inhibited by streptomycin, gentamicin and tetracycline. Its apparent penicillin resistance was an unexpected observation as in vitro replication of the Chlamydiales (Moulder, 1984) and most species of the...
Rickettsiales-like Organism from Coho Salmon

Rickettsiales is inhibited by this antibiotic (WEISS and MOULDER, 1984). In vitro sensitivity of the organism to tetracycline suggests this drug may have application in the treatment of the infection. Tetracycline is an accepted therapeutant in fish culture.

The fact that no other fish pathogen was isolated from the coho salmon experiencing this epizootic suggests that the rickettsiales-like microorganism, observed in fish and isolated in cell culture, is the causative agent of the coho salmon disease. In vivo studies are planned to test this hypothesis. The potential pathogenicity of the organism, its apparent virulence, and the fact that it is not known to occur outside of Chile dictate that these studies be conducted in the area where the disease is endemic. For these reasons, the agent will be returned to Chile for infection experiments in coho salmon.

The exact taxonomic placement of this microorganism is yet to be determined. Although it was an obligate intracellular parasite and replicated in vitro within cytoplasmic inclusions in host cells, it did not share other characteristics of the Chlamydiales. It did not react with the monoclonal antibody against the group-specific LPS chlamydial antigen, and although polymorphic, did not appear to develop the small infectious elementary bodies and the large replicating initial bodies characteristic of these microorganisms. It did, however, contain the rippled cell wall and electron-lucent spherical structures described for certain rickettsial species (ANDERSON et al., 1965). For these reasons, and until precise placement can be determined, we suggest the organism be associated with the order Rickettsiales (tribe Ehrlichieae) rather than with the more limited and closely defined Chlamydiales.

Acknowledgements

The authors thank the following people for their contributions to this study: SANDRA BRAVO for the use of her laboratory facilities in Chile and for aid in obtaining the diseased fish examined; PATRICIA SALAS and JOHN CVITANICH for helpful discussions which led to the isolation of this microorganism; AL SOELDNER for assistance with the electron microscopy and MARGO WHIPPLE for her valuable technical support.

This manuscript was presented in its entirety by one of the authors (JLF) at the annual meeting of the Japanese Society of Fish Pathology, March 30, 1990, at the Tokyo University of Fisheries, Japan. The work was sponsored by the Oregon State University Sea Grant College Program supported by NOAA Office of Sea Grant, U.S. Department of Commerce, under Grant NA89AA-D-SG108, by the Salmon Growers Association of Chile, and by the Veterinary Sciences Faculty of the University of Chile. It is Oregon Agricultural Experiment Station Technical Paper No. 9170.

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