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Title: CERTAIN ASPECTS OF THE IMMUNOLOGY AND CHEMOTHERAPY OF BACTERIAL KIDNEY DISEASE IN JUVENILE COHO SALMON (ONCORHYNCHUS KISUTCH)

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The detection and antigenic nature of the causative Corynebacterium of bacterial kidney disease and chemotherapy of this disease in juvenile coho salmon (Oncorhynchus kisutch) were examined.

Each of 207 yearling coho salmon collected from a population undergoing a severe epizootic of bacterial kidney disease were examined for the presence of anti-Corynebacterium precipitin or agglutinin antibodies, corynebacteria in Gram-stained kidney smears, and cultivable kidney disease Corynebacterium in kidney material inoculated onto cysteine serum agar. The presence of anti-Corynebacterium precipitin or agglutinin antibodies in the salmon does not serve as a suitable indicator of current infection by the kidney disease bacterium. Coho salmon anti-Corynebacterium antibodies appeared to have either cleared the bacteria from the fish or at least reduced the number of

kidney disease bacteria to a level not detectable by cultivation of or microscopic examination of kidney tissue.

By immunodiffusion and immunoelectrophoresis, two distinct antigens were detected in ammonium sulfate-precipitated material from phosphate-buffered saline extracts of whole Corynebacterium cells. On the basis of chemical analyses, Pronase and heat treatments, both antigens appeared to contain protein and carbohydrate. Three different isolates of the kidney disease Corynebacterium were antigenically similar.

Previous work indicating erythromycin as the drug of choice for treating this disease was confirmed. Erythromycin stearate fed at 100 mg per kg of fish per day for two 14-day treatment periods controlled the disease in experimentally infected juvenile coho salmon while Ampicillin and Pen V-K, fed either at 75 or 100 mg per kg of fish per day for two 14-day treatment periods, were ineffective.

Certain Aspects of the Immunology and Chemotherapy of Bacterial Kidney Disease in Juvenile Coho Salmon (*Oncorhynchus kisutch*)

by

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INTRODUCTION

Corynebacterial kidney disease is a chronic to subacute disease which may cause severe losses in hatchery-reared salmonids. It is difficult to control because sensitive methods of detecting low level infections are lacking and effective chemotherapy is not available. The causative agent of the disease is a slow-growing Gram-positive diplobacillus, presently classified as a Corynebacterium, which has an absolute requirement for cysteine. Because the primary isolation of the bacterium from diseased fish may require several weeks, diagnosis of the disease is currently based on clinical signs and the presence of Gram-positive diplobacilli in infected kidney and other tissues. This method of diagnosis is not entirely satisfactory since the kidney disease bacterium is often difficult to detect microscopically in low level infections.

This study was undertaken to test serological procedures for detecting corynebacterial kidney disease. These methods were compared with the standard procedures of culturing the bacterium from infected kidney tissue and microscopically examining Gram-stained kidney smears. The efficacies of two synthetic chemotherapeutic

agents, Ampicillin and Penicillin V-K, were compared to that of erythromycin for control of experimentally induced kidney disease. Erythromycin had previously been reported as the drug of choice in treatment of kidney disease (Wolf and Dunbar, 1959). In addition, studies to characterize a soluble Corynebacterium antigen were initiated.

LITERATURE REVIEW

The literature pertaining to corynebacterial kidney disease of salmonid fishes has been reviewed recently by Bullock, Conroy and Snieszko (1971). This review will summarize more recent findings and provide a perspective on the pathogenesis, diagnosis, and treatment of the disease.

Historical Background

Corynebacterial kidney disease was first reported, under the name of Dee disease, by the second interim report of the Furunculosis Committee (MacKie et al., 1933). This report described the pathology noted in Atlantic salmon (Salmo salar) from Aberdeenshire Dee and the River Spey in Scotland. Gram-positive bacilli could be demonstrated from lesions on the spleens of infected fish, but the bacterium could be cultured only on enriched media. Since brown trout (S. trutta) injected with splenic emulsions from infected salmon developed kidney disease and died, it was inferred that the observed bacilli were the cause of the disease.

The first published account of the occurrence of kidney disease in the United States was in 1935 (Belding and Merrill, 1935) in brook (Salvelinus fontinalis) and brown trout from a hatchery in Massachusetts. A serious epizootic of kidney disease was found in brook trout

in California about 1935, and the disease appeared in trout in California hatcheries in subsequent years.[1] Since 1950, bacterial kidney disease has often been detected in hatchery-reared salmonids in the British Isles, Canada, and the United States, and was recently reported to occur in salmonids in Japan.[2] Presumptive diagnoses of kidney disease in wild salmonids, based on the observation of characteristic Gram-positive diplobacilli in kidney smears from diseased fish, have been reported in Canada (Pippy, 1969) and the United States (MacLean and Yoder, 1970) and two confirmed diagnoses, involving isolation of the bacterium from wild salmonids, are on record (Wood and Wallis, 1955; Evelyn, Hoskins and Bell, 1973). There are no published reports of the occurrence of this disease in nonsalmonids.

Etiology of the Disease

The causative agent of bacterial kidney disease is currently classified as an unspeciated Corynebacterium. Since this organism is a small (0.3-0.5 μ by 0.5-1.0 μ), fastidious, Gram-positive (to Gram-variable), nonacid-fast, nonmotile, nonsporulating bacillus (Ordal and Earp, 1956; Smith, 1964), it shows similarities to

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[1] Personal communication, Joseph Wales, Oregon State University.

[2] Personal communication, G. L. Bullock, Eastern Fish Disease Laboratory.

Corynebacterium diphtheriae, the type species for this genus. However, information concerning phage susceptibility, cell wall composition, guanine-cytosine content, and antigenic composition is needed before the kidney disease agent can be classified as a Corynebacterium with certainty (Barksdale, 1970). The kidney disease bacterium has not been reported to produce a toxin.

The Gram-positive diplobacillus isolated from trout by Belding and Merrill (1935) differed in some of the characteristics listed above. Their isolate was 1 to 4 μ long, motile, grew feebly at 37 C, and formed visible colonies on meat infusion agar in 24 to 48 hr. Recently characterized strains of the bacterium isolated from confirmed cases of the disease are no longer than 1.5 μ, do not grow at 37 C, and require a week or more to show visible growth (Smith, 1964; Evelyn, Hoskins and Bell, 1973). Possibly Belding and Merrill were not working with a pure culture of the kidney disease agent and the aberrant characteristics they observed were due to a contaminant.

Clinical Signs and Gross Pathology

Corynebacterial kidney disease is generally regarded as a chronic to subacute, usually systemic, infection of salmonid fishes. Though the disease may occur over a wide range of water temperatures, it is most severe during periods of warm water temperatures in the range 13 C to 18 C (Earp, Ellis and Ordal, 1953). The severity

of the disease has been related to water chemistry. Based on the results of a survey of 40 fish hatcheries in the United States, Warren (1963a) reported that as the hardness of the water decreased, the severity of the disease increased. Whether this relationship of severity of kidney disease and water hardness is a direct cause and effect is not known.

The gross pathology of the disease has been described in detail by Bullock, Conroy and Snieszko (1971) and the histopathology of the disease was investigated by Wood and Yasutake (1956). Exophthalmos was the most commonly observed external symptom and external blebs and blisters above the lateral line occurred frequently. Internally, the hematopoietic organs were most frequently affected, though in extreme cases most organs and tissues were damaged. False white membranes covering the liver, spleen, gonads, and at times the swim bladder and peritoneal muscle were reported (Snieszko and Griffin, 1955). These membranes were present in diseased salmon taken from cold water (1.5 C to 8.3 C) but absent from fish collected from warmer water (Smith, 1964). Hunn (1964) reported that changes in certain physiological parameters occurred in diseased brook trout and that males were more susceptible than females. Reduced hematocrit and total plasma protein values were apparent in diseased fish and electrophoretic comparisons of serums from diseased and healthy fish indicated a large reduction in the

amount of the fastest migrating serum component from diseased fish. The influence of diet on the biochemical pathogenesis of kidney disease in juvenile coho salmon has been investigated (Wedemeyer and Ross, 1973). Experimentally infected fish fed a diet containing either corn gluten or cottonseed meal were observed for incidence and severity of the disease. The disease incidence was independent of the diet, however the nonspecific stress of infection was found to be more severe in fish fed the corn gluten.

Treatment of the Disease

The intracellular occurrence of the kidney disease Corynebacterium makes this disease perhaps the most difficult bacterial infection of fish to treat. Sulfamerazine, sulfadiazine, and other sulfa drugs have been used, alone or in combination, to treat kidney disease with varying degrees of success (Rucker et al., 1951; Snieszko and Griffin, 1955; Allison, 1958). In every instance, sulfa drugs only suppressed the disease temporarily, for when treatment was discontinued, the disease reappeared. Wolf and Dunbar (1959) tested 34 therapeutic agents in vitro and 10 agents in vivo and found that erythromycin, fed at 100 mg per kg of fish per day for 21 days, was the most effective agent for controlling the disease in experimentally infected brook trout. Their data suggested that erythromycin effected a cure of the disease in 50 percent of the treated fish. However,

toxic effects on fish have been attributed to erythromycin thiocyanate at both 100 mg per kg of fish per day (Piper, 1961) and 500 mg per kg of fish per day (Warren, 1963b).

Detection and Diagnosis

Since the kidney disease Corynebacterium requires 10 days to two weeks to show visible growth on culture medium, diagnosis of disease epizootics is based on clinical signs and microscopic examination of stained smears usually from kidneys or lesions from diseased fish (Bullock, Conroy and Snieszko, 1971). Because kidney disease has been found in fish also suffering from a lymphosarcoma (Ehlinger, 1963), furunculosis, and visceral granuloma (Bullock, Conroy and Snieszko, 1971), and the demonstration of the organism in kidney smears is often complicated by the presence of melanin pigment granules which resemble the bacterium, microscopic diagnostic procedures are not entirely satisfactory. A serological diagnostic method for the disease has been devised by Chen et al. (1974) which employs double diffusion in gel to detect specific kidney disease Corynebacterium antigens in mascerated tissue from diseased fish. When the antigen is present in the minced tissue, it is precipitated in the gel by rabbit anti-Corynebacterium serum.

Immunology of Bacterial Kidney Disease

Evelyn (1971) reported that sockeye salmon responded to an intraperitoneal injection of heat-killed cells in adjuvant. The agglutinin response was variable between individual fish, but some fish responded with final agglutinin titers up to 1:10, 240. Electrophoretic patterns of immune sockeye salmon serum indicated the presence of two populations of anti-Corynebacterium antibody since two peaks were lost when antibody was adsorbed from the serum with kidney disease bacterium cells.

There are no published reports concerning the immune response of naturally infected salmonids to the kidney disease bacterium. Thus, it is not clear whether fish respond to a natural challenge by this bacterium or if so, whether the immune response is protective against the disease.

MATERIALS AND METHODS

Commonly Employed Diluent and Bacteriological Media

Throughout this study, phosphate buffered saline (PBS) was a commonly employed diluent and cysteine serum agar (CSA) and Mueller-Hinton agar (MHA) were used for cultivation of the kidney disease Corynebacterium. These were prepared according to the following formulations.

Phosphate Buffered Saline, pH 7.2 (PBS)

	g/1000 ml	Molarity
NaCl	8.33	0.1425
KH_2PO_4	0.245	0.0018
Na_2HPO_4	0.809	0.0057
H_2O	to volume	

Cysteine Serum Agar (CSA)

	g/100 ml
Tryptose	1.00
Beef extract	0.30
Yeast extract	0.01
L-cysteine HCl	0.10
NaCl	0.50
Ionagar	1.50
Fetal Calf Serum	10-20%

Mueller-Hinton Agar (MHA)

	g/100 ml
Mueller-Hinton Agar[3]	3.80
L-cysteine HCl	0.10

Adjust to pH 6.7

Preparation of Commonly Employed Corynebacterium Antigens and Anti-Corynebacterium Serums

Culture of the Kidney Disease Corynebacterium

The strain of Corynebacterium used in this study was isolated from a diseased yearling coho salmon from the Nehalem River Salmon Hatchery in March 1973. At the outset of this work, cultures were maintained on CSA. Later, cultures were transferred and maintained on MHA. MHA is available commercially and does not include the complicating antigens introduced by the calf serum. All cultures were incubated at 18 C for 14-28 days. Henceforth, Corynebacterium cells will be referred to as KD cells.

Soluble Corynebacterium Antigen for Gel Diffusion Analysis

Antigen extracts of whole KD cells were obtained by the following methods. The cells were extracted with (1) 0.85 percent NaCl at

[3]Difco Laboratories, Detroit, Mich.

100 C for 30 minutes, (2) 5 percent trichloroacetic acid at 60 C for 20 minutes, (3) 10 percent trichloroacetic acid at 4 C for 18 hours, or (4) 0.20 N HCl at 100 C for 20 minutes. Prior to the extraction, the cells were washed three times with 0.85 percent NaCl and resuspended in the respective extraction medium to a concentration of 100 mg (wet weight) per ml. After the period of extraction, the cells were removed by centrifugation (1500 x g, 23 C, for 15 minutes). The pH of the supernatant was adjusted to neutrality and a final centrifugation (12,100 x g, 0 C, for 20 minutes) clarified the solution. The technique employing 0.85 percent NaCl was found to be the most satisfactory method and was used at the outset of this study. Later, a technique introduced to this laboratory by Dr. G. L. Bullock, Eastern Fish Disease Laboratory, was adopted for preparation of soluble Corynebacterium antigen. This method consisted of washing the KD cells from each 32 ounce bottle culture with 10 ml of PBS and removing the cells by centrifugation (13,200 x g, 0 C, for 20 minutes). No extraction was required since the antigen was present in the culture fluids.

Corynebacterium Whole Cell Antigen for Microtiter Analysis

Corynebacterium whole cell antigen was prepared in the following manner. The KD cells from each MHA culture were suspended

in 10 ml of PBS, microscopically checked for purity, and washed three times. Each wash consisted of suspending the cells in fresh PBS and centrifuging the preparation (4080 x g, 0 C, for 20 minutes). Cells from the final washing were resuspended in PBS to a concentration which when diluted 1:25 with PBS, had an optical density (O. D.) at 520 mμ of 1. 0 as measured with a Bausch and Lomb Spectronic 20 (13 x 100 mm cuvette). The undiluted suspension was placed in a 62 C water bath for 45 minutes and then cooled to 23 C. Formalin (37 percent) was added to the suspension to a final concentration of 0. 3 percent and the cells were stored at 4 C.

Preparation of Anti-*Corynebacterium* Serums

Rabbit antiserum, prepared in young, female New Zealand white rabbits, and coho salmon antiserum, prepared in yearling coho salmon, were used in this study.

Rabbit anti-*Corynebacterium* serum was prepared as follows. KD cells were harvested in PBS from MHA cultures, centrifuged (1500 x g, 23 C, for 15 minutes), and resuspended in PBS to a concentration of 1. 75 ml of wet packed cells per 10 ml of diluent. The cell suspension was placed in a 62 C water bath for 45 minutes to kill the bacteria, cooled, and emulsified with an equal volume of Freund's Complete Adjuvant (FCA). Two rabbits were immunized according to the following schedule:

1st day - 2 ml intramuscular (i.m.) in each flank

16th day - 2 ml i.m. in each flank

33rd day - 2 ml i.m. in each flank

64th day - 30 ml blood collected from rabbit I

72nd day - 20 ml blood collected from rabbit II

84th day - 50 ml blood collected from each rabbit

The antiserum had an agglutination titer of 1:4096 and precipitated the Corynebacterium soluble antigen in gel diffusion tests.

Ninety yearling coho salmon obtained from the Nehalem River Salmon Hatchery and maintained in 13 C water at the Fish Disease Laboratory, Corvallis, Oregon, were immunized against the kidney disease Corynebacterium with the same antigen as used for rabbits except that cells were suspended to a concentration of 50 mg wet weight per ml prior to being heat-killed and emulsified in FCA. The salmon were immunized according to the following schedule:

1st day - 0.2 ml intraperitoneally (i.p.)

21st day - 0.2 ml i.p.

42nd day - approximately 1.0 ml of blood collected from each of 10 fish

136th day - approximately 1.0 ml of blood collected from each of 18 fish

The final agglutination titer of the pooled coho salmon antiserum was 1:256.

Rabbit and salmon blood were processed identically. Freshly collected blood was incubated at 23 C for 1 hr and then placed at 4 C for 12 hr for clot retraction. After the blood was centrifuged (455 x g, 23 C, for 15 minutes), the serum was harvested and merthiolate was added to a final concentration of 1:5000.

Rabbit anti-Corynebacterium serum also was obtained from Dr. G. L. Bullock for use in this study.

Serological Procedures Employed

Precipitin Analysis by Single Diffusion in Gels

A modification of the Oudin method was used to detect precipitin antibodies in coho salmon serums. Glass tubing having an internal diameter of 2 mm was cut in 5 cm sections, cleaned with dichromic acid, rinsed several times with distilled water, dried, and sealed at one end with a flame. Since this test was to be used to detect anti-Corynebacterium antibody, the soluble Corynebacterium antigen was incorporated into the gel. A 0.6 percent solution of Ionagar in PBS was prepared, cooled to 45 C, and placed in a 45 C water bath. An equal volume of the soluble Corynebacterium antigen preparation was mixed with the agar solution and 50-100 µl of the mixture pipetted into each glass tube and allowed to solidify. After layering 25-50 µl of fish serum over the agar-antigen mixture, tubes were incubated at 23 C. The tubes were examined after 24 hr for the presence of precipitin bands.

Precipitin Analysis by Double Diffusion in Gels

Ouchterlony double diffusion in gel was used for the detection and analysis of soluble Corynebacterium antigens. A solution containing 0.5 percent Agarose[4] in PBS and 1:5000 merthiolate was prepared and 15 ml poured into each disposable 100 x 15 mm Petri dish. A pattern consisting of a central 5 mm antiserum well and six surrounding antigen wells (each 5 mm) was used. The centers of adjacent wells were 13 mm apart. After the wells were filled with test solutions and antiserum, the plates were incubated at 23 C and results read in 18 to 36 hr.

Microtiter Determination of Agglutinating Antibody

The microtiter determinations were performed by the method of Conrath (1972). Twenty-five µl volumes each of test serum and Corynebacterium whole cell antigen were used. The plates were incubated at 23 C and results read after 18 hr. The microtiter was read as the final dilution of serum that showed visible agglutination of the whole cell antigen.

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[4]Agarose, VWR Scientific, San Francisco, Calif.

The Comparison of Serological Methods with the Standard Methods of Detection of Kidney Disease

Experimental Fish

In March 1973, approximately 400 yearling coho salmon were transported from the Nehalem River Salmon Hatchery to the Fish Disease Laboratory, Corvallis, Oregon. These fish were taken from a population experiencing an outbreak of corynebacterial kidney disease. The fish were fed Oregon Moist Pellets (OMP) every other day and were held in 3-foot circular tanks which received flowing 13 C well water.

Examination of Fish

Blood was collected by tail-drip from 207 of the coho salmon and the serum from each fish was examined for precipitating and agglutinating antibodies as previously described. In addition, kidney material from each fish was aseptically inoculated onto CSA and a kidney impression smear was prepared, Gram-stained, and microscopically examined. The CSA cultures were incubated at 18 C for six weeks before they were interpreted as negative for Corynebacterium growth.

Antibiotic Efficacy Studies

All antibiotics were obtained from Mr. Oliver Scarvie, a sales representative for the Pfizer Co. The antibiotics included erythromycin stearate film-coated tablets, 500 mg; ampicillin trihydrate capsules (Ampicillin), 250 mg; and potassium phenoxymethyl penicillin tablets (Pen V-K), 250 mg.

Experimental Fish

In September 1973, 500 coho salmon, average weight 13 g, were transported from Alsea River Salmon Hatchery to the Fish Disease Laboratory, Corvallis, Oregon. The fish, held in 3-foot diameter circular tanks which received flowing 13 C well water, were fed OMP once a day prior to the experiment.

Incorporation of the Drugs into Food

Preliminary experiments indicated OMP was more palatable to the fish than was Oregon Test Diet. Thus, OMP was used in the experiment. The antibiotics were treated according to the form in which they were obtained. The film that coated the erythromycin tablets was scraped off and the tablets were ground to a fine powder with a mortar and pestle. The Pen V-K tablets were not coated; they also were ground to a fine powder with a mortar and pestle. Ampicillin was obtained in capsule form, so the antibiotic was merely

removed from the capsule.

The drugs were incorporated into the food by adding a known amount of antibiotics to a weighed amount of 1/8 inch OMP, mixing thoroughly, and repelleting the food into 3/32 inch pellets. This work was done at the Oregon Fish Commission Laboratory, Clackamas, Oregon. The drugs were incorporated into OMP so that if the fish ate the food at a rate of 3 percent of their body weight per day, they would receive the desired amount of antibiotic. Preliminary experiments indicated that 3 percent was a reasonable estimate of food consumption. Five groups of food were prepared to give the following dosages: (1) erythromycin, 100 mg per kg of fish per day, (2) Ampicillin, 75 and 100 mg per kg of fish per day, and (3) Pen V-K, 75 and 100 mg per kg of fish per day. The food was stored at -15 C.

Experimental Infection and Treatment of Fish

Three hundred fish were moved to 12 indoor tanks, 25 fish per tank, at the Fish Disease Laboratory. The 18-gallon tanks received constantly flowing 13 C well water. Fish in each of two tanks were assigned to the five antibiotic treatments and fish in the remaining two tanks, designated control tanks, received no antibiotic treatment.

Seven days after the fish were moved to the indoor tanks, each

fish was injected i. p. with 0. 1 ml of a suspension of viable KD cells. Prior to injection, the KD cells were washed three times in PBS and resuspended in PBS to an estimated concentration of 4.5×10^8 cells per ml as judged by a McFarland 3 turbidimetric standard.

The fish received two 14-day antibiotic treatments, the first beginning 15 days after injection of the KD cells, the second beginning 67 days post-injection. All the fish were fed unmedicated 3/32 inch OMP once a day except during periods of drug treatment; fish in control tanks received unmedicated OMP during the entire experiment.

Throughout the experiment, fish deaths were recorded with respect to tank number and date of death. Kidney impression smears were prepared from each dead fish, Gram-stained, and examined microscopically for the kidney disease Corynebacterium.

The experiment was terminated 136 days post-injection. Blood was collected by tail-drip and a kidney impression smear was prepared from each surviving fish. Serums from salmon from each tank were pooled and examined for precipitin and agglutinin antibodies by previously described methods. Kidney smears were Gram-stained and examined microscopically for KD cells. In addition, kidney material from every fifth surviving fish was inoculated onto MHA. An immunodiffusion method introduced by Chen et al. (1974) was employed to test for the presence of Corynebacterium soluble antigen

in kidney material pooled from the fish in each tank. The kidney material from each group of fish was suspended in PBS and minced. A drop of the suspension from each group of fish was placed in an antigen well of a double diffusion plate and rabbit anti-Corynebacterium serum was placed in the center well. The plate was incubated at 23 C for 72 hr and examined daily for the presence of precipitin bands.

Preliminary Characterization of the Soluble Corynebacterium Antigens

It has been found both at this laboratory and the Eastern Fish Disease Laboratory that all tested strains of the kidney disease Corynebacterium produce at least one common antigen detectable by double diffusion in gel. Since an antigen of this type could be useful in detection of latent carriers, it was decided to attempt its purification and characterization.

Throughout these studies, the previously described Ouchterlony double diffusion method was used to assay for antigen activity.

Preliminary Concentration of the Soluble Corynebacterium Antigens

To determine whether ammonium sulfate precipitation or ethanol precipitation would be suitable for preliminary concentration of antigen, the following procedures were used.

Ammonium sulfate was added to a stirred soluble antigen preparation to a final concentration of 0.8 saturation (560 g per l) and the solution was placed at 4 C for 18 hr. The precipitate was then removed by centrifugation (5900 x g, 0 C, for 15 minutes), dissolved in physiological saline, dialyzed against several changes of saline, and then assayed for antigen activity.

Ethanol precipitation of the antigen was performed as follows. Six volumes of 95 percent ethanol and one drop of a saturated sodium acetate solution were added to one volume of the soluble antigen preparation and the solution placed at 4 C for 18 hr. The resulting precipitate was then dissolved in saline and dialyzed several times against saline. Antigen assay was performed as previously described.

Heat Sensitivity

To test the heat-stability of the soluble antigen preparation, 1.0 ml of the preparation was pipetted into a 10 x 75 mm serological tube and placed in a 60 C water bath for 60 minutes. An assay for antigen was then performed.

Pronase Sensitivity

To determine the sensitivity of the antigen to Pronase digestion,

0.1 mg of Pronase[5] was added to 1.0 ml of the soluble Corynebacterium antigen preparation and the solution was placed at 37 C for 1 hr. Formalin then was added to a final concentration of 0.2 percent to inactivate Pronase enzymes. As a control test for formalin denaturation of the antigen, an equal concentration of formalin was added to an otherwise untreated soluble antigen preparation.

Chemical Analyses

Protein analyses of antigen preparations were performed by the Biuret method (Chase and Williams, 1968) and Folin-Ciocalteau reaction (Lowry et al., 1951) with bovine serum albumin as standard.

Total carbohydrate content of antigen preparations was determined by the phenol-sulfuric acid method (Dubois et al., 1956) and total hexose content by the anthrone reaction (Scott and Melvin, 1953).

Ion Exchange Chromatography

DEAE-Sephadex A-25 anion exchange resin was prepared according to instructions supplied by Pharmacia.[6] The swollen resin in tris buffer (0.05 M, pH 7.86) was poured into a column (1 x 20 cm) and 1.0 ml of the soluble Corynebacterium antigen was applied to the

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[5]Pronase, B Grade, Calbiochem, Los Angeles, Calif.

[6]Pharmacia Fine Chemicals, Piscataway, N.J.

resin. The material was eluted with a linear concentration gradient of 200 ml of 0.05 M tris (pH 7.86) and 500 ml of 0.05 M tris-1.0 M NaCl (pH 7.86) at a flow rate of 1 ml per minute. The 5 ml fractions were collected with an automatic fraction collector and each fraction assayed for antigen, protein, and total hexose content.

A CM-Sephadex C-25 cation exchange column was prepared in sodium phosphate buffer (0.05 M, pH 6.00). Preparation of the column, application, collection and analysis of the sample was identical to that described for the DEAE-Sephadex A-25 column except that the material was eluted from the column with a linear concentration gradient of 200 ml of 0.05 M NaH_2PO_4 (pH 6.00) and 500 ml of 0.05 M NaH_2PO_4-1.0 M NaCl (pH 6.00).

Sephadex G-200 Gel Filtration

A Sephadex G-200 gel filtration column (2.5 x 25 cm) was prepared in tris buffer (0.05 M, pH 7.86) according to instructions supplied by Pharmacia. Flow adaptors were employed to allow ascending type elution. A 1.0 ml sample of the soluble Corynebacterium antigen was applied to the column and eluted with tris buffer (0.05 M, pH 7.86) at a flow rate of 1 ml per minute. Fractions (5 ml) were collected with an automatic fraction collector and each fraction assayed, as previously described, for antigen, protein, total carbohydrate content, and total hexose content.

Immunoelectrophoresis

Immunoelectrophoresis of the soluble Corynebacterium antigen was performed by the microtechnique of Scheidegger (1955) with a commercial immunoelectrophoresis apparatus.[7]

A tris-glycine buffer (pH 8.3) was prepared by dissolving 6.0 g of tris (hydroxymethyl)-aminomethane (Sigma Trizma Base)[8] and 28.8 g of glycine (ammonia-free) in distilled water and adding distilled water to bring the solution to a final volume of 1000 ml. To prepare the gel, equal volumes of molten 2 percent Ionagar[9] and 2X tris-glycine buffer were mixed, merthiolate was added to a final concentration of 0.01 percent, and the solution was filtered by suction through Whatman No. 1 filter paper. The gel then was cooled to 55 C and poured and spread over microscope slides to form the agar layer. Wells and troughs were cut with a pattern cutter supplied with the immunoelectrophoresis apparatus.

Each well then was filled twice with an antigen preparation and electrophoresis was conducted for 3 hr at 4 C with a constant potential of 150 volts. After electrophoresis, undiluted rabbit anti-Corynebacterium serum was added to the troughs and the slides incubated in a

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[7]Shandon Scientific Co., Inc., Sewickley, Pa.

[8]Sigma Chemical Co., St. Louis, Mo.

[9]Ionagar No. 2, Consolidated Laboratories, Inc., Chicago Heights, Ill.

moist chamber for 24 hr to allow formation of precipitin bands. The slides then were immersed for 24 hr in 1 percent NaCl, 1 hr in distilled water, and dried. To prevent distortion, the slides were covered with a wet Whatman No. 1 filter paper before being placed in a 37 C incubator to dry. The slides were stained with amido black by the method described by Shang (1966).

RESULTS

The Comparison of Serological Methods with Standard Methods of Diagnosing Corynebacterial Kidney Disease

This experiment was devised to detect low levels of corynebacterial kidney disease in hatchery-reared salmonid fishes. A technique suitable for detecting coho salmon anti-*Corynebacterium* precipitin antibodies was tested. Since it was hoped that the test, if proved effective, could be employed as a field diagnostic tool for detecting the disease, the antigen, rather than the antiserum, was incorporated into the gel. The field operation thus would have entailed collecting serum from the fish and layering this into previously prepared tubes containing the antigen-gel mixture.

The precipitin bands obtained with rabbit and coho salmon anti-*Corynebacterium* serums are diagramed in Figure 1. These bands generally began to appear after 1 hr of incubation and were readily visible after 24 hr. Nonimmune coho salmon or rabbit serums did not form precipitin bands when layered over the antigen-gel mixture.

The agglutinin and precipitin tests used in this study were not suitable for detecting corynebacterial kidney disease in yearling coho salmon (Table 1). The mean agglutinin titer of the 207 fish sampled was 9.2, and only 22 (10.1 percent) of the fish had precipitin antibody activity against the kidney disease *Corynebacterium*. In general, the

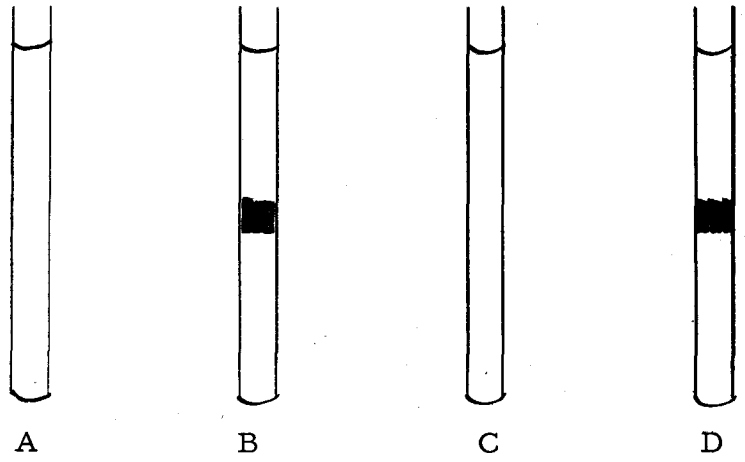


Figure 1. The detection of anti-Corynebacterium precipitins using a modified Oudin method: serum over antigen. A. Nonimmune coho salmon serum; B. Coho salmon anti-Corynebacterium serum (agglutinin titer = 1:256); C. Nonimmune rabbit serum; D. Rabbit anti-Corynebacterium serum (agglutinin titer = 1:4096).

Table 1. Comparison of agglutinin titer with other methods that indicate exposure of the coho salmon to the kidney disease Corynebacterium.[a]

Agglutinin titer^{-1}	Number of fish with titer	Number of fish at each titer showing exposure to Corynebacterium by the following: Kidney smear	Culture on cysteine serum agar	Precipitin test
0	28	1	0	0
4	49	5	1	4
8	50	0	0	0
16	43	1	2	4
32	12	2	2	1
64	6	3	3	3
128	4	0	0	2
256	15	1	1	8
Total	207	12	9	22
Mean titer[b]	9.2	11.3	34.6	51.3

[a] Each of 207 yearling coho salmon was examined for the presence of: (1) precipitating and agglutinating antibodies against the kidney disease Corynebacterium, (2) kidney disease corynebacteria in kidney smears, and (3) culturable kidney disease corynebacteria from kidney material inoculated onto cysteine serum agar.

[b] Mean titer $= \frac{\Sigma (\log t)(f)}{n}$ where t = agglutinin titer^{-1}, f = the frequency of occurrence of titer in the sample, and n = the number of fish in the indicated group.

precipitin activity correlated with higher agglutinin titers, though some variability was noted. In some instances precipitin activity was detected in serums with relatively low agglutinin titers while other serums with high agglutinin titers failed to show precipitin activity. Antibody heterogeneity was suggested by these data.

Salmon with agglutinin titers of 1:128 or greater generally did not harbor the kidney disease Corynebacterium as detectable by kidney smear or culture techniques (Table 2). The coho salmon anti-Corynebacterium agglutinins seemed to be protective since only one of the 19 fish in this group was shown to harbor the bacterium.

Anti-Corynebacterium precipitin antibodies were detected in 22 of the 207 salmon examined (Table 3). The serums from this group of fish had a mean agglutinin titer of 51.3 and 10 of these serums had titers of 1:128 or greater. As is clearly shown, only three fish in this group harbored the bacterium as detectable by culture or kidney smear techniques.

The kidney disease Corynebacterium was isolated from nine (4.4 percent) of the salmon examined and kidney smears from each of these nine fish showed the presence of the kidney disease bacterium (Table 4). The mean agglutinin titer of the serums from these fish was 34.6 and anti-Corynebacterium precipitin activity was demonstrated in only two of these fish. This further showed that anti-Corynebacterium precipitin activity, as measured in this study,

Table 2. Detection of kidney disease in the 19 coho salmon which had an anti-*Corynebacterium* agglutinin titer of 1:128 or greater.[a]

Fish identification number	Agglutinin titer^{-1}	Detection of *Corynebacterium* by:		
		Culture on cysteine serum agar	Kidney smear	Precipitin test
1	128	-[b]	-	-
24	256	+	+	+
26	256	-	-	-
29	128	-	-	-
30	256	-	-	+
36	256	-	-	-
43	256	-	-	-
50	256	-	-	+
71	256	-	-	+
73	256	-	-	-
82	256	-	-	-
84	256	-	-	+
97	256	-	-	+
142	256	-	-	+
149	256	-	-	-
163	128	-	-	+
169	256	-	-	+
201	128	-	-	+
211	256	-	-	-

[a] A total of 207 yearling coho salmon were examined.

[b] + indicates detection of the kidney disease bacterium by culture or smear methods, or of anti-*Corynebacterium* precipitins; - indicates no detection of either the bacterium or specific precipitins by the respective methods.

Table 3. Detection of kidney disease in the 22 coho salmon which had anti-Corynebacterium precipitin antibodies.[a]

Fish identification number	Detection of kidney disease by: Precipitin test	Culture on cysteine serum agar	Kidney smear	Anti-Corynebacterium agglutinin titer^{-1}
21	+[b]	-	-	16
24	+	+	+	256
30	+	-	-	256
38	+	-	-	16
49	+	-	-	64
50	+	-	-	256
54	+	-	-	4
55	+	-	-	16
71	+	-	-	256
78	+	-	-	4
84	+	-	-	256
97	+	-	-	256
103	+	-	-	4
142	+	-	-	256
151	+	-	+	32
163	+	-	-	128
169	+	-	-	256
197	+	+	+	64
201	+	-	-	128
203	+	-	-	64
207	+	-	-	4
216	+	-	-	16

[a] A total of 207 yearling coho salmon were examined.

[b] + indicates detection of the kidney disease bacterium by culture or smear methods, or of anti-Corynebacterium precipitins; - indicates no detection of either the bacterium or specific precipitins by the respective methods.

Table 4. Presence of anti-Corynebacterium precipitins and agglutinins in serums from the nine coho salmon from which the kidney disease Corynebacterium was isolated or detected microscopically. [a]

Fish identification number	Detection of kidney disease by: Culture on cysteine serum agar	Kidney smear	Anti-Corynebacterium precipitin test	Anti-Corynebacterium agglutinin titer^{-1}
6	+[b]	+	-	32
10	+	+	-	16
13	+	+	-	16
14	+	+	-	64
24	+	+	+	256
41	+	+	-	64
146	+	+	-	32
197	+	+	+	64
200	+	+	-	4

[a] A total of 207 yearling coho salmon were examined.

[b] + indicates detection of the kidney disease bacterium by culture or smear methods, or of anti-Corynebacterium precipitins; - indicates no detection of either the bacterium or specific precipitins by the respective methods.

was not a suitable indicator of Corynebacterium infection in coho salmon.

The kidney impression smear technique demonstrated the presence of corynebacteria in 12 of the 207 salmon (Table 5). Serums from these 12 fish had a mean agglutinin titer of 11.3 and two of these serums had anti-Corynebacterium precipitin activity. The bacterium was cultured from six (50 percent) of the fish in this group, suggesting that the kidney smear technique was a more sensitive indicator of Corynebacterium infection than was the culture technique.

In summary, the tests for agglutinins and precipitins investigated were not suitable indicators of Corynebacterium infection in yearling coho salmon. Fish which had anti-Corynebacterium precipitin antibodies and high anti-Corynebacterium agglutinin titers generally did not seem to harbor the bacterium, and those fish which did harbor the bacterium usually lacked significant levels of anti-Corynebacterium precipitin and agglutinin antibodies.

Antibiotic Efficacy Studies

This experiment was conducted to compare the efficacies of two synthetic penicillins with erythromycin, the previous drug of choice, in treating corynebacterial kidney disease in experimentally infected juvenile coho salmon. A recent change in the patent status of erythromycin, which may make the use of this drug economically feasible,

Table 5. Serological and cultural detection of kidney disease in the 12 coho salmon in which corynebacteria were observed in kidney smears.[a]

Fish identification number	Detection of kidney disease by: Kidney smear	Culture on cysteine serum agar	Anti-*Corynebacterium* precipitin test	Anti-*Corynebacterium* agglutinin titer^{-1}
13	+[b]	+	-	16
14	+	+	-	64
41	+	+	-	64
146	+	+	-	32
151	+	-	+	32
167	+	-	-	4
179	+	-	-	0
182	+	-	-	4
197	+	+	+	64
200	+	+	-	4
206	+	-	-	4
210	+	-	-	4

[a] A total of 207 yearling coho salmon were examined.

[b] + indicates detection of the kidney disease bacterium by culture or smear methods, or of anti-*Corynebacterium* precipitins; - indicates no detection of either the bacterium or specific precipitins by the respective methods.

prompted a renewed interest in the drug. Similarly, Ampicillin and Pen V-K were considered to be relatively inexpensive antibiotics effective in treating infections caused by Gram-positive bacteria. Ampicillin inhibited *in vitro* growth of the kidney disease *Corynebacterium*.

Erythromycin was superior in treating corynebacterial kidney disease in juvenile coho salmon (Table 6). Pen V-K and Ampicillin, at the treatment levels tested, appeared to be relatively ineffective in controlling the disease since average mortality among salmon fed these drugs was higher than nonmedicated control fish (Table 6). A high percentage of the nonmedicated control fish in Tank 1 failed to die from the disease during the course of the experiment. No explanation is offered for this. All the fish received injections from the same suspension of KD cells and were maintained in a similar manner throughout the experiment. Statistical analysis of this data inferred, even with the variability noted, erythromycin was significantly effective in controlling the disease ($\alpha = .20$).

Since kidney smears prepared from each fish that died during the course of the experiment contained characteristic Gram-positive diplobacilli, it appeared that corynebacterial kidney disease was the cause of all deaths. The first death occurred 39 days post-injection, thus it seems unlikely that any fish died from the stress of the injection process.

Table 6. A comparison of the efficacies of Ampicillin and Pen V-K with erythromycin stearate in treating corynebacterial kidney disease in experimentally infected juvenile coho salmon.[a]

Antibiotic (mg/kg of fish/day)	Tank no.[b]	Percent fish surviving: 60 days	90 days	120 days	Average percent survival (120 days) at each treatment[c]
None	1	100	92	92	76
	2	88	60	60	
Erythromycin (100)	3	100	96	96	96
	4	100	100	96	
Ampicillin (75)	5	92	68	68	58
	6	80	60	48	
Pen V-K (75)	7	92	72	64	66
	8	96	68	68	
Ampicillin (100)	9	92	68	60	64
	10	92	76	68	
Pen V-K (100)	11	84	48	44	66
	12	100	96	80	

[a] Juvenile coho salmon were each injected with 4.5×10^8 kidney disease Corynebacterium cells. The fish received two 14-day antibiotic treatments, the first beginning 15 days post-injection, the second beginning 67 days post-injection.

[b] Each tank contained 25 fish.

[c] Least significant difference ($\alpha = .20$) calculated with arcsin transformed mortality indicated the number of deaths of erythromycin-treated fish was significantly lower than that of the control and penicillin-treated groups.

Serums collected from the surviving fish at the termination of the experiment had anti-Corynebacterium agglutinin titers of 1:64 or 1:128. Seven of the 207 kidney smears prepared from surviving fish contained Gram-positive diplobacilli characteristic for corynebacterial kidney disease while none of the MHA cultures inoculated with kidney material from these fish contained Corynebacterium colonies. The double diffusion analysis for soluble Corynebacterium antigen indicated the presence of the antigen in kidney material collected from surviving fish in Tank 5 (Ampicillin, 75 mg per kg of fish per day). When the kidney material collected from all the surviving fish was pooled and centrifuged (5900 x g, 0 C, for 15 minutes), precipitin bands were obtained indicating the presence of two distinct soluble antigens in the pelleted kidney material.

In summary, previous reports on the efficacy of erythromycin for the treatment of corynebacterial kidney disease were reaffirmed. Erythromycin stearate, fed for 14 days at the rate of 100 mg per kg of fish per day, was highly effective in treating the disease while Ampicillin and Pen V-K, at the rates of 75 and 100 mg per kg of fish per day for 14 days, were ineffective. Fish that survived the infection were shown to have responded immunologically to the bacterium and two soluble Corynebacterium antigens were detected in kidney material by a double diffusion method.

Preliminary Characterization of Soluble *Corynebacterium* Antigens

Ouchterlony double diffusion in gel with rabbit anti-*Corynebacterium* serum indicated ammonium sulfate precipitation of PBS extracts of whole *Corynebacterium* cells was superior to ethanol precipitation for the preliminary concentration of the soluble antigen preparation.

Two distinct antigens were detected with the rabbit anti-*Corynebacterium* serum (OSU antiserum) prepared at this laboratory whereas only one antigen was detected by the commercially-prepared rabbit anti-*Corynebacterium* serum (LT antiserum) supplied by Dr. G. L. Bullock (Figure 2). When the ammonium sulfate-precipitated antigens were treated with Pronase, no antigenic activity was detected by the LT antiserum, though reaction with the OSU antiserum indicated at least one of the antigens was degraded into two subunits which retained antigenic properties. This degradation by Pronase indicated protein was an integral part of both antigens.

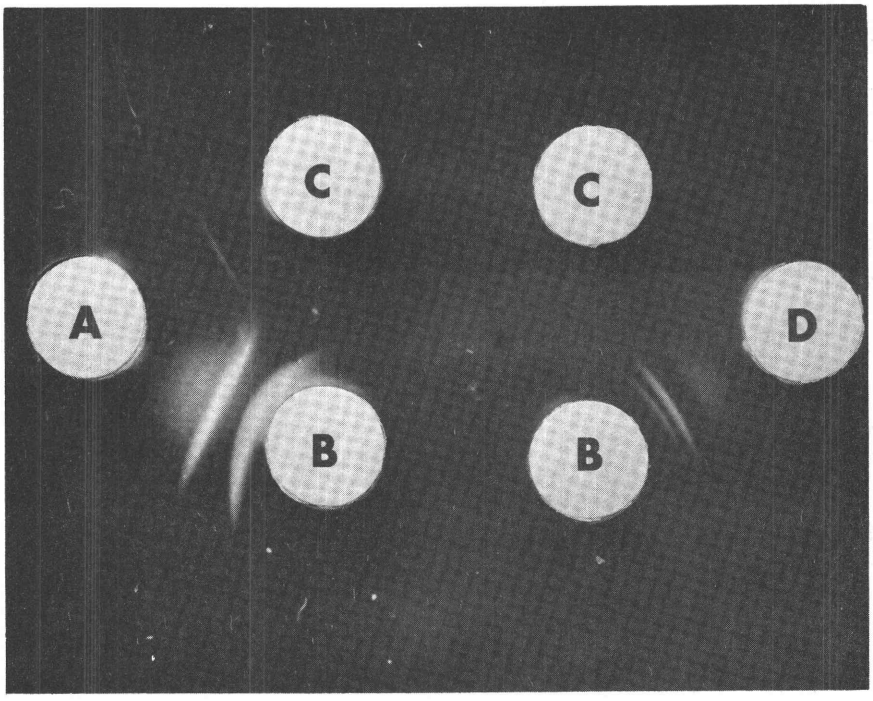
When the ammonium sulfate fraction was heat-treated, antigenic activity also was lost with the LT antiserum and retained with the OSU antiserum. However, no degradation into subunits was apparent after heat-treatment.

Chemical analyses of fractions collected from ion exchange columns and a gel filtration column indicated peaks of protein and

Figure 2. Ouchterlony double diffusion analysis of a soluble kidney disease Corynebacterium antigen preparation. A. Soluble Corynebacterium antigen preparation; B. OSU rabbit anti-Corynebacterium serum (agglutinin titer = 1:4096); C. Commercially-prepared rabbit anti-Corynebacterium serum (agglutinin titer = 1:4096); D. Pronase-treated (1 hr, 37 C) soluble Corynebacterium antigen preparation.

Figure 3. Immunoelectrophoretic analysis of soluble kidney disease Corynebacterium antigen preparations (anode to the left).

Upper well:	Corynebacterium antigen fraction eluted from DEAE-Sephadex 25 column
Center trough:	rabbit anti-Corynebacterium serum (agglutinin titer = 1:4096)
Lower well:	ammonium sulfate-precipitated Corynebacterium antigen preparation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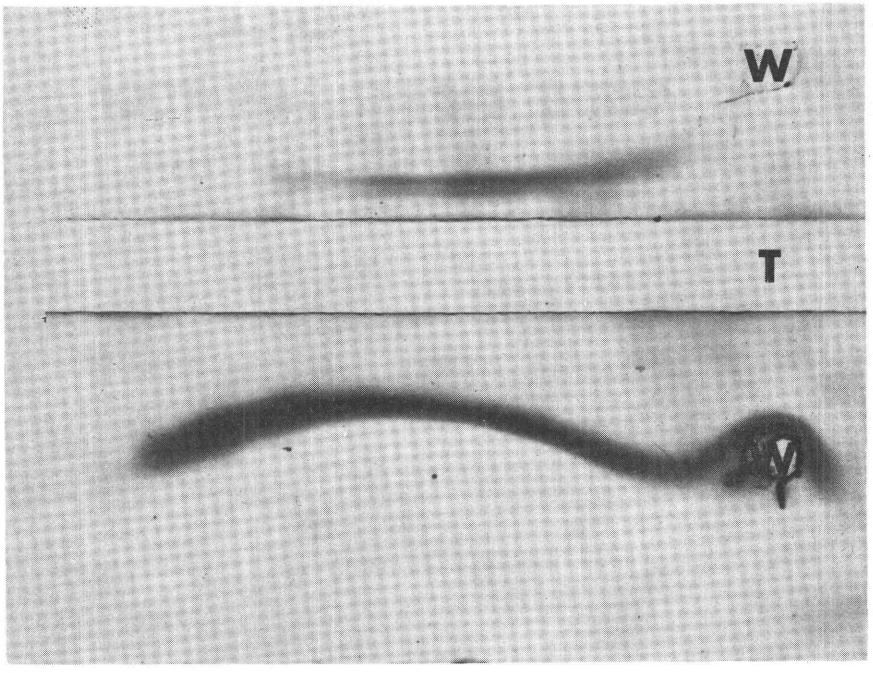
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carbohydrate content coincided with those fractions which contained antigenic activity. This suggested that the antigens contained carbohydrate, as well as protein.

Neither antigen detected by gel diffusion appeared to carry a net positive charge since no binding to the cation exchange column was noted. The most rapidly migrating antigen, as detected by gel diffusion, bound to the anion exchange column while the slower antigen was never detected in any fraction from this column.

Both antigens were excluded from the Sephadex G-200 gel filtration column, suggesting molecular weights of greater than 200,000 daltons for each component.

Immunoelectrophoretic analysis also indicated the presence of two distinct antigens (Figure 3). As expected, the antigen which bound to the anion exchange resin also showed anodic migration. The other antigen did not migrate in either direction.

In summary, two distinct antigens were precipitated from the PBS extracts of whole Corynebacterium cells. Both antigens appeared to have high molecular weight and on the basis of chemical analyses, Pronase and heat treatments, both antigens were shown to contain protein and carbohydrate. Neither bound to a cation exchange column while one bound to an anion exchange resin.

DISCUSSION

The three aspects of corynebacterial kidney disease investigated in this study were: (1) detection of the disease, (2) chemotherapy of the disease, and (3) antigenic factors from the causative agent.

Detection of low levels of corynebacterial infection in hatchery-reared salmonids continues to be a problem. Anti-Corynebacterium agglutinin and precipitin activities, as measured in this study, were not suitable indicators of corynebacterial kidney disease in yearling coho salmon. The presence of these antibodies in fish serum appeared to indicate the absence, rather than the presence of the kidney disease Corynebacterium. Indeed, of 31 fish in which anti-Corynebacterium precipitin activity or specific agglutinin titer of 1:128 or greater was detected, only three were shown to harbor kidney disease corynebacteria. This suggested that the coho salmon anti-Corynebacterium antibodies were protective, and when present in sufficient quantities, caused the elimination of corynebacteria from the fish. This is in agreement with another report concerning a different bacterial fish disease in which passive immunization studies showed that immune serum protected juvenile coho salmon against furunculosis (Spence, Fryer and Pilcher, 1965).

In terms of the data obtained comparing the four detection

methods, four stages of the disease process may be hypothesized. In the first stage, corynebacteria, though present, are not demonstrable by either kidney smear or culture techniques and no significant anti-Corynebacterium antibody levels are present. In the second stage, corynebacteria are present in numbers sufficient to be detected by the kidney smear method, though not by culture techniques. When the disease process has reached this stage, the fish has received a sufficient antigenic stimulus to begin responding with anti-Corynebacterium antibody production. The mean agglutinin titer in fish from which positive kidney smears were obtained was 11.3, but Corynebacterium cultures were isolated from only 50 percent of these fish. In the third stage of the disease process, corynebacteria are demonstrable by both kidney smear and culture techniques and the immune response has progressed further. The mean agglutinin titer in fish from which Corynebacterium was cultured was 34.6. In the fourth stage of the disease, the immune response has progressed to the point at which a protective role seems apparent. In general, corynebacteria are not demonstrable by either kidney smear or culture methods in this stage of the disease process.

As earlier indicated, these proposed disease stages are hypothetical. The sample of coho salmon examined in this study contained fish in all stages of the disease and some variation was

observed in the relation of the immune state of the fish to the detectable presence of corynebacteria. Variability in the agglutinin response of sockeye salmon (O. nerka) injected with a heat-killed suspension of the kidney disease bacterium in adjuvant has been reported (Evelyn, 1971).

A sensitive method for detecting low level infections of corynebacteria in hatchery-reared salmonids is still needed. The kidney smear technique is not suitable when the bacteria are present in small numbers and the culture technique, which requires two weeks or more, may be complicated by the presence of faster-growing contaminants. A double diffusion method, introduced by Chen et al. (1974) for detecting soluble Corynebacterium antigen in fish kidney material may be superior to any of the methods tested in this study. This method demonstrated the presence of kidney disease corynebacteria in salmon that survived the antibiotic experiment. The kidney smear method demonstrated the presence of KD cells in only seven of the 207 surviving fish. Although anti-Corynebacterium agglutinin titers in serums collected from these fish were 1:64 or 1:128, the detection of soluble Corynebacterium antigen would seem to be a more direct inference of infection. The presence of anti-Corynebacterium agglutinins or precipitins only indicates exposure of the fish, at some time, to the kidney disease Corynebacterium. Detection of the soluble antigen indicates the presence of the

bacterium.

The agglutinin and precipitin tests used in this study, though of little value in detecting kidney disease, did provide valuable information on the immune response of juvenile coho salmon to a natural infection of the kidney disease Corynebacterium.

The antibiotic efficacy studies conducted reaffirmed the earlier observations of Wolf and Dunbar (1959). Erythromycin stearate was effective in controlling experimentally induced corynebacterial kidney disease in juvenile coho salmon while Ampicillin and Pen V-K were ineffective. Erythromycin thiocyanate has been reported to be toxic for rainbow trout at both 100 mg per kg of fish per day after six days (Piper, 1961) and 500 mg per kg of fish per day after three days (Warren, 1963b). No toxicity or palatability problems were encountered in this study. The reported toxicity may have been due to the thiocyanate salt rather than to the erythromycin. Thiocyanate, occasionally employed as an antihypertensive agent in human medicine, has been reported to cause toxic reactions in humans (Nickerson, 1970). The use of the stearate salt of erythromycin may eliminate the problem of toxicity for fish.

Further studies on the treatment of corynebacterial kidney disease with erythromycin stearate are required. Drug tolerance levels for the fish must be established and the possibility of erythromycin-resistant strains of Corynebacterium considered. Chemotherapy, though not the final answer in eliminating this disease from

populations of hatchery-reared fish, may control the infection for a sufficient time to allow the animals to respond immunologically to the corynebacteria. The coho salmon employed in this study responded to the bacterium in the course of the experiment, and evidence was provided earlier suggesting a protective role for coho salmon anti-Corynebacterium antibodies. The results of this study also indicate more replications of each treatment should be used. Though erythromycin was shown to be significantly effective at the 80 percent level, a higher degree of significance may have been attained if more replicates had been used. Additional replicates may have minimized the effects of the different survival results recorded from the two control tanks on the statistical significance of the data.

The fact that two distinct soluble Corynebacterium antigens were detected by the OSU antiserum while only one antigen was detected by the LT antiserum probably can be explained by the methods used for the preparation of each antiserum. For example, only one washing was employed in preparing the cells for production of the OSU antiserum while the cells used to produce the LT antiserum were washed three times. The additional washings may have removed antigenic material from the cells used for the production of the LT antiserum. Another possibility is antigenic differences between the two different kidney disease Corynebacterium isolates employed for the production of the two antiserums were present. However, all

isolates of the kidney disease bacterium examined showed antigenic similarity. The serological similarity of kidney disease cultures was further demonstrated by the immunodiffusion tests that employed both rabbit antiserums (Figure 2). Even though the cultures used for the preparation of each antiserum were isolated from geographically separated areas, a band of identity, which indicated a common antigen, was formed when the antiserums were tested against a soluble antigen preparation from a heterologous culture.

Information concerning basic physical and chemical properties of each of the two antigens has been obtained. Further studies to determine the exact composition of these antigens are required. In addition, knowledge pertaining to the roles of these antigens in the pathology and immunology of corynebacterial kidney disease in salmonids may aid in devising more sensitive methods of detecting the disease and serve as a basis for the development of a vaccine for preventing kidney disease.

SUMMARY AND CONCLUSIONS

1. The presence of agglutinin and precipitin antibodies against the kidney disease Corynebacterium in yearling coho salmon, though indicative of a prior exposure to the kidney disease bacterium, did not indicate a current infection in the fish.
2. The mean anti-Corynebacterium agglutinin titer from yearling coho salmon collected from a population of fish undergoing a severe natural infection of the kidney disease bacterium was low; however, the animals which showed the greatest immunological response appeared to have eliminated the bacteria or at least reduced the numbers of bacteria to a level not detectable by kidney smear or culture methods. This suggested the coho salmon anti-Corynebacterium antibodies were protective against the disease.
3. Erythromycin was confirmed as the drug of choice for treating corynebacterial kidney disease in experimentally infected coho salmon. Since the patent status of erythromycin has changed recently, the economic feasibility of employing this drug should be investigated.
4. Experimental infection of coho salmon with the kidney disease Corynebacterium was difficult to standardize. In experimental infections of this type, more than one replicate sample should be employed at each treatment to lend statistical significance

to the results.

5. Two distinct antigens detected in ammonium sulfate-precipitated material from PBS extracts of whole Corynebacterium cells appeared to contain protein and carbohydrate and to have molecular weights greater than 200,000 daltons. The role of these antigens in the pathology and immunology of the disease is under investigation.
6. Immunodiffusion comparison of two rabbit anti-Corynebacterium serums suggested that excessive washing of KD cells in preparation for injection into a rabbit may result in the loss of some antigenic material from the cell suspension. Three different isolates of the bacterium seemed to be antigenically similar.
7. The information obtained in this study will aid in devising new methods of detecting and treating corynebacterial kidney disease in hatchery-reared salmonid fishes.

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