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COMPARATIVE SENSITIVITIES OF DIAGNOSTIC PROCEDURES USED TO DETECT BACTERIAL KIDNEY DISEASE IN SALMONID FISHES

Rocco C. Cipriano,[1] C. E. Starliper,[1] and John H. Schachte[2]

ABSTRACT: Kidney and spleen homogenates from each of 60 coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Salmo gairdneri*) were examined for detection of *Renibacterium salmoninarum*. The proportions of positives differed widely with the detection procedures used: in coho salmon, 5% were positive by the Gram-stain procedure, 10% by the direct fluorescent antibody test, 48% by bacteriological isolation, 65% by staphylococcal coagglutination, and 73% by counterimmunoelectrophoresis; in steelhead trout, 3% were positive by Gram-stain, 8.3% by fluorescent antibody, 17% by bacteriological isolation, and 67% by counterimmunoelectrophoresis. *Renibacterium salmoninarum* was not detected in either coho salmon or steelhead trout by immunodiffusion analysis.

INTRODUCTION

Fish health policies have been established in the United States relating to the certification of the disease status of certain populations of hatchery-reared fish. Certification programs were implemented to reduce the deleterious effects produced by infectious diseases among intensively cultured fishes. An effective certification program may prevent the spread of infectious disease into areas where the disease is not endemic, and limit the risks involved in stocking diseased fish into natural waters. Therefore, the success of disease certification programs depends on the ability of fish health specialists to use reliable, rapid, sensitive, and specific techniques to detect pathogens.

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum* (Sanders and Fryer, 1980), is particularly difficult to detect in asymptomatic fish. *Renibacterium salmoninarum* is a small (0.4×0.8 μm), nonmotile, Gram-positive diplobacillus that has extremely fastidious growth requirements. Despite the development of selective media for its culture (Evelyn, 1977; Austin et al., 1983), primary isolation of this bacterium requires at least 10 days at 15 C (Austin et al., 1983). Consequently, more rapid diagnostic procedures have been developed to detect this pathogen in fish. For example, the presence of small, Gram-positive diplobacilli in the kidneys of clinically diseased salmonids has been used to provide satisfactory diagnosis of BKD (McDaniel, 1979). However, the insensitive and nonspecific Gram-stain (Bullock and Snieszko, 1979) produces circumspect results. Because strains of *R. salmoninarum* are serologically homogenous (Bullock et al., 1974), serodiagnostic assays provide rapid and practical detection of this pathogen. Immunodiffusion tests (Chen et al., 1974; Kimura et al., 1978), fluorescent antibody tests (Bullock and Stuckey, 1975; Bullock et al., 1980), and staphylococcal coagglutination tests (Kimura and Yoshimizu, 1981) have each been modified and adapted to detect *R. salmoninarum*. The serodiagnostic assays have facilitated the diagnosis of BKD, but the detection and clinical significance of bacteria in asymptomatic fish remains a problem for fishery resource managers.

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In 1983, the New York State Department of Environmental Conservation and the U.S. Fish and Wildlife Service conducted collaborative studies to determine the comparative sensitivities of diagnostic procedures used to detect *R. salmoninarum*. In addition, the counterimmunoelectrophoresis (CIE) assay of van Oss and Bartholomew (1980) was adapted for the detection of *R. salmoninarum* and evaluated against existing methods.

MATERIALS AND METHODS

Mature coho salmon and steelhead trout return from Lake Ontario to spawn in the Salmon River (New York) in mid-October and mid-March, respectively. During the spawning runs in 1983, 60 coho salmon (4.5 kg) and 60 steelhead (3.6 kg) were collected by electrofishing in the Salmon River and transported to the state salmon hatchery at Altmar, New York. After the fish had been spawned, individual samples were taken for detection of *R. salmoninarum*.

Individual fish were necropsied under aseptic conditions, and primary isolation of *R. salmoninarum* was attempted by inoculating kidney material onto KDM-2 media (Evelyn, 1977). After inoculation, culture plates were incubated up to 21 days at 15 C. During incubation, each plate was examined and all pinpoint bacterial colonies were subcultured onto KDM-2 agar slants. These cultures were incubated at 15 C for an additional 10–14 days. Bacterial growth was washed from culture slants with sterile phosphate buffered saline (PBS) and allowed to react in a macroscopic slide agglutination test (Bullock et al., 1974) with rabbit antiserum to *R. salmoninarum* and normal rabbit serum. Sera used in these studies were obtained from the Biologics Section at the National Fish Health Research Laboratory. Specific antisera were produced by injecting New Zealand white rabbits with *R. salmoninarum* cells emulsified in Freund's complete adjuvant. Rabbits were injected intramuscularly (i.m.) with this suspension. After 21 days rabbits were given a booster injection (i.m.) of bacteria without adjuvant. Fourteen days after the booster injection, rabbits were bled and serum was collected. Normal serum was obtained from the control rabbits. Bacteria that were agglutinated by the specific rabbit antiserum but not by normal rabbit serum were further identified by the direct fluorescent antibody test (FAT) for *R. salmoninarum* (Bullock et al., 1980).

During necropsy, the posterior kidney of each fish was excised and suspended in 1.0 ml of sterile PBS. The material was vortexed, heated for 60 min in a boiling waterbath, and centrifuged at 4,000 rpm for 10 min. The supernatant was placed in sterile microfuge tubes and stored at −70 C. These kidney homogenates served as a future antigen source for immunodiffusion, staphylococcal agglutination (CoA), and CIE tests. Additional samples of kidney from each fish were smeared onto glass microscope slides for Gram-stain and FAT analysis. Immunodiffusion was performed as described by Chen et al. (1974), direct FAT as described by Bullock et al. (1980), and CoA as described by Kimura and Yoshimizu (1981).

The CIE test was adapted to detect *R. salmoninarum* by a modification of the technique used to detect viral hepatitis B surface antigen, as described by van Oss and Bartholomew (1980). In this test, 1% high electroendosmotic agarose was dissolved in 0.05 M tris, 0.05 M sodium barbital, 0.014 M barbital buffer (pH 8.4). Agarose was poured onto glass plates to an approximate thickness of 3 mm. Opposing rows of wells (4 mm in diameter) were cut and spaced 8 mm apart. Within each row, antibody wells were oriented toward the anode side of the electrophoresis chamber and antigen wells toward the cathode. Migration of antibody was therefore against the current, whereas that of antigen (suspect kidney tissue) was with the current. Antigen wells filled with either PBS or BKD soluble antigen, and antibody wells filled with normal rabbit serum, served as controls. The electrophoresis chamber was filled with tris-barbital buffer and wicks were applied between buffer reservoirs and the agarose plates. Electrophoresis was performed for 60 min at 10 mA and bromphenol blue was used as a dye marker. The plates were then incubated at 18 C overnight in a moist chamber. A visible precipitin band between opposite antigen and antibody wells indicated a positive reaction (Fig. 1).

The same source of rabbit antiserum to *R. salmoninarum* was used with the different serodiagnostic assays in order to validate comparison of the relative sensitivities.

RESULTS

Renibacterium salmoninarum was detected in populations of both coho salmon and steelhead trout by the different diagnostic procedures. The use of several procedures enabled us to compare both the relative sensitivities of the procedures and the reproducibility of our results. We

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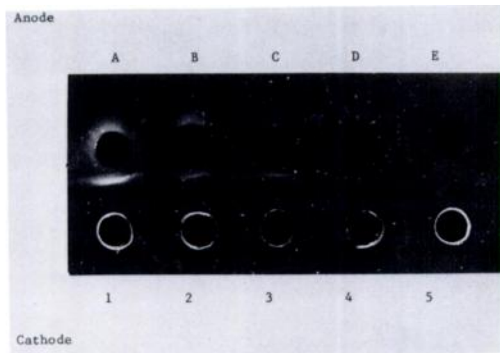

FIGURE 1. Results of a counterimmunoelectrophoresis assay conducted as described in the text. Antibody wells A, B, C, and D contained rabbit antiserum to *Renibacterium salmoninarum* and well F contained normal rabbit serum. Antigen wells 1 and 5 had a soluble extract antigen from *R. salmoninarum*, wells 2 and 3 had kidney homogenates of coho salmon infected with *R. salmoninarum* and well 4 contained a kidney homogenate from an uninfected salmon.

TABLE 1. Comparison of efficacy of diagnostic tests used to detect *Renibacterium salmoninarum* in 60 coho salmon (*Oncorhynchus kisutch*) and 60 steelhead trout (*Salmo gairdneri*).

Diagnostic test	Percent of fish in which infection was detected: Coho salmon	Steelhead trout
Gram-stain	5	3
Fluorescent antibody	10	8
Bacterial isolation	48	17
Staphylococcal coagglutination	65	NT[a]
Counterimmunoelectrophoresis	73	67
Immunodiffusion	0	0

[a] NT = not tested.

found that the relative sensitivities of the diagnostic tests in the two different populations of fish were similar and that the CIE test was the most sensitive method used to detect *R. salmoninarum* (Table 1).

Using individual kidney samples from each of the 60 adult coho salmon, we found that 5% of the fish were positive for BKD by the Gram-stain procedure, whereas 48% of the fish were positive by bacteriological isolation. There was also a considerable variation among the sensitivities of the serodiagnostic techniques used. Although *R. salmoninarum* was not detected by immunodiffusion tests in these fish, prevalences of infection were 10% by FAT, 65% by CoA, and 73% by CIE. Samples that were positive in less sensitive tests were confirmed by other tests that had greater sensitivity.

Results obtained with steelhead trout were similar to those described for coho salmon. Among the 60 fish sampled, 3% were positive by the Gram-stain procedure, 8.3% by FAT, 17% by bacterial isolation, and 67% by CIE. As in coho salmon, no steelhead trout samples were positive by immunodiffusion analysis. Although the CoA test proved to be highly sensitive for *R. salmoninarum* in coho salmon, we did not use this test on steelhead trout because the end point reading was more subjective for this assay than for the other tests used.

DISCUSSION

Certain fish management decisions are made according to the prevalence(s) of a pathogen within a given population of fish. Consequently, low prevalences of infection often present sizable problems in understanding the significance of a pathogen in a population of fish. In fact, the estimated prevalence of infection may vary because of differing degrees of sensitivity of the assays used for examination. In the present study, for example, the prevalence of infection varied from 0 to 73% depending on which of six methods was used to detect *R. salmoninarum*. Primary bacteriological isolation, followed by serologic identification, was the most reliable method for definitive detection of *R. salmoninarum*. Thus, we were able to identify the occurrence of an asymptomatic carrier state in 48% of the coho salmon and in 17% of the steelhead trout ex-

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amined. All other tests produced speculative estimates of infection that could be compared with bacteriological isolation.

Subsequent results were not altogether dissimilar from those reported by other investigators. Bullock et al. (1980) reported that direct FAT was about twice as effective as the Gram-stain for detecting *R. salmoninarum*. This relationship was also apparent in our results. Only three kidney samples of coho salmon contained Gram-positive bacteria, but six were positive by FAT. In addition, two steelhead trout were positive by Gram-stain, but five were positive by FAT. Kimura and Yoshimitzu (1981) also noted that the staphylococcal coagglutination test was superior to Gram-stain for detecting *R. salmoninarum*, but did not compare the sensitivity of the CoA test with FAT. In our tests with coho salmon, the CoA test was about 13 times more sensitive than Gram-stain and six times more sensitive than FAT. However, because the agglutination end point of a CoA reaction sometimes required a subjective reading, more effort was expended toward developing the equally sensitive but more objective CIE assay. Unlike the Gram-stain, FAT, and bacteriological isolation procedures, which specifically detect bacteria or particulate antigens, the CoA, CIE, and immunodiffusion assays described here were designed to detect soluble protein antigens from *R. salmoninarum*. Results in coho salmon when either the CoA or CIE techniques were used were comparable, but the immunodiffusion assay did not detect BKD antigen in either population of fish. Although the three assays were constructed to detect similar antigens, the insensitivity of the immunodiffusion assay, compared with CIE tests, is understandable. It was calculated by van Oss and Bartholomew (1980) that more than 90% of antigens and antibodies in opposite wells of an immunodiffusion test do not interface because of the 360° radial diffusion of reactants from their respective wells. The electric current and electroendosmotic migration of antibodies used in CIE assays greatly increase the concentration of reactants between opposite wells, and thereby enhance the sensitivity of the test.

Because more samples were positive by CIE analysis than by actual bacteriological isolation, we wanted to be sure that the surplus CIE positive samples were not false reactions. In additional tests, these surplus CIE positive samples were placed in wells adjacent to soluble BKD protein antigen and again electrophoresed against rabbit antiserum to *R. salmoninarum*. Fusion of precipitin bands between the surplus CIE samples and the control BKD antigen, indicative of identity, provided additional evidence that the surplus CIE positive samples contained *R. salmoninarum* antigen.

In summary, we found that the estimated prevalence of infection in a population of fish was variable and depended on the sensitivity of assays used for examination. The comparative sensitivities of these assays under practical field use were similar as observed by results obtained with both coho salmon and steelhead trout. In order of descending hierarchy, CIE and CoA assays were comparable but more sensitive than bacteriological isolation, FAT, and Gram-stain for the detection of *R. salmoninarum*. Immunodiffusion assays were not suitable for the detection of *R. salmoninarum* in asymptomatically infected fish. In view of the differing sensitivities of the several assays in detecting *R. salmoninarum*, extreme caution should be exercised in attempting to correlate prevalence of infection with the significance of disease in the management of fishery resources.

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