Corynebacterial Kidney Disease of Salmonids: Growth and Serological Studies on the Causative Bacterium

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Comparative growth studies of 10 isolates of the Corynebacterium of salmonid kidney disease were quantified on four media. Greatest cell yield was obtained from Mueller-Hinton medium with 0.1% L-cysteine hydrochloride. Serological tests, in which rabbit antisera used against typical bacteria strains showed all to be antigenically homologous, provided a reliable method of identification.

Bacterial kidney disease (KD) of salmonid fishes is a chronic to acute infection which often causes significant mortality among cultured species. The causative bacterium is now classified in the genus Corynebacterium, but a species has not been assigned. The organism has an absolute requirement for L-cysteine hydrochloride (2, 4) and grows slowly, often requiring one to several weeks to develop visible colonies on initial isolation. A synonym for the disease, symptoms and pathology produced, and additional characteristics of the bacterium were recently reviewed by Bullock et al. (1).

Slow growth and lack of either biochemical or serological methods for identification of the KD bacterium are among the most important problems associated with investigation of the disease. Diagnosis is based on clinical signs and microscopic examination of kidney tissue for the bacterium. This examination is fallible because other gram-positive rods (such as Listeria) that occur in trout may morphologically resemble this Corynebacterium (3). Not only is primary isolation of the causative agent time consuming, but most workers have found it necessary to add serum or blood to the growth medium. The serological relatedness of isolates from different geographical regions, which is not now known, must be determined before serological tests can be used for diagnosis in epizootiological studies.

The present work describes growth of strains of the KD bacterium isolated from salmonids from different parts of North America on media with and without serum, as well as the precipitin and agglutinin reactions of these stains.

MATERIALS AND METHODS

Cultures. Ten isolates of the KD bacterium used in the study were obtained from the West Coast, Inter-

mountain West, Midwest, and East Coast of the U. S.; and British Columbia (Table 1). Test cultures were identified as the Corynebacterium causing KD on the basis of their being small gram-positive diplobacilli isolated from clinical cases of KD and on their absolute requirement for L-cysteine. Three gram-positive isolates from fish, morphologically like the KD bacterium but incapable of inducing clinical kidney disease, were also used in serological tests.

Growth of KD strains. To determine if serum was essential for sustained growth of KD cultures, we compared cell yields of isolates grown on Mueller Hinton (MH) (Difco and BBL) plus 0.1% L-cysteine hydrochloride (MH + C-HCl) (5); Evelyn's medium; and tryp tic soy agar (TSA) (Difco). Evelyn's medium is a modification of Ordal and Earp's preparation, which contains serum instead of whole blood (4). The TSA contained neither L-cysteine hydrochloride nor serum.

All commercial media were prepared according to direction. 0.1% L-cysteine hydrochloride was added to the MH and Evelyn's media, the pH was adjusted to 6.5 (except for TSA, which was 7.2), and media were distributed in culture vessels and autoclaved at 15 pound (ca. 56.78 g (121 C) pressure for 13 min. Evelyn medium was prepared as above; after it was autoclaved and cooled, 6.5% fetal bovine serum was added. The glass or plastic culture vessels used provided 70 to 75 cm² of growth area.

 Cultures to be used as inocula for test media were grown on MH (Difco) + C-HCl slants for 14 to 21 days at 15 C. Growth from each slant was suspended in sterile Hanks balanced salts solution (BSS) and 0.5 ml was used as a standard inoculum. In all tests two flasks or bottles were used to determine growth of each isolate for each medium. All inoculated media were incubated at 15 C for 14 days before the cells were harvested. Cells were recovered by washing, and quantified in conical or Shevky-Stafford centrifuge tubes after centrifugation at 800 x g for 30 min.

Preparation of antisera. Rabbit antisera were prepared by Microbiological Associates, Inc., Bethesda, Md., using as antigens whole cells of strains 2 and 4 (Table 1) and whole cells and a sonicated
preparation of strain 3. The three strains were chosen because they produced clinical KD when injected into brook trout, grew slowly, required L-cysteine for growth, and originated from different geographic locations (Table 1). Cultures were grown in Blake bottles on MH (Difco) + C-HCl for 14 to 16 days at 15°C; the cells were collected from the medium, centrifuged at 800 × g for 30 min, and then washed three times in Hanks BSS. The approximately 1.5 ml of packed cells yielded by each bottle were suspended in 14 ml of Hanks BSS for injection of rabbits. A portion of the strain 3 antigen was cooled in a mixture of dry ice and ethanol and sonicated with 25 μm of glass powder for 12 min at 90 to 100 W on a Branson Sonifier, model W185D. Microscopic examination indicated that at least 90% of the cells were disrupted. Rabbits were immunized with standard laboratory procedures.

**Microtiter agglutination test.** We made agglutinin titrations for each isolate with each antiserum, using 25 μl of antiserum or antigen in twofold dilutions in microtiter “U” plates (Cooke Engineering Co., Alexandria, Va.). Antigens were prepared by suspending growth from a 16-× 125-mm slant culture of MH (Difco) + C-HCl in Hanks BSS to obtain a 0.9 optical density at 420 nm on a B & L Spectronic 20. We then sonicated each suspension at 60 W for 14 s to obtain a homogeneous suspension. Microtiter plates were incubated at 4°C overnight before examination. The final serum dilution showing agglutination was recorded as the titer.

**Precipitin reactions.** Using the Ouchterlony technique and a non-nutrient medium containing saline, 1% Noble (Difco) agar, and 1:10,000 Merthiolate, we carried out precipitin tests in 60-mm plastic culture dishes each containing 5 ml of medium. The pattern consisted of a 6-mm central antiserum well and six peripheral antigen wells of the same size. Precipitin reactions were determined with whole cells and a sonic extract of each culture against each of the antisera. Charged plates were incubated in a moist chamber at room temperature (25°C) for 4 days and examined daily.

**RESULTS**

**Growth of KD strains.** Serum was not essential for growth of the KD test strains. Average yields of packed cells were greater on MH medium (Difco and BBL) than on Evelyn’s medium, although yields were more uniform on Evelyn’s (Table 2). Cell yields from TSA, which contained neither serum nor L-cysteine, were only 12 to 50% as great as those from Evelyn’s or MH. Tests were also conducted with three additional lots of MH (Difco). Again, cell yields from all lots were, on the average, better than those previously obtained from BBL, MH, or Evelyn’s.

**Serological comparison of strains.** Agglutinin and precipitin reactions both showed all KD cultures to be serologically closely related. Heterologous agglutinin titers were often higher than the homologous titers; titers ranged from 1:256 to 1:4096 (Table 3). The three gram-positive isolates morphologically similar to the KD strains (no. 11 to 13, Table 1) failed to agglutinate with the test antisera at a dilution of 1:10.

As indicated by the bands of identity, the precipitin reactions also demonstrated homogeneity among KD isolates. All 10 strains formed one continuous band with the antisera within 4 days (Fig. 1); however, the strongest bands were obtained with antiserum prepared from strain 4. As in the agglutinin tests, the three gram-positive non-KD cultures failed to react.

**DISCUSSION**

In the growth tests all strains grew slowly on all test media. Higher cell yields from MH

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### Table 1. Corynebacterial strains and other gram-positive cultures used in growth or serological studies

| Strain no. | Source and date isolated |
|------------|--------------------------|
| 1          | Salmon (Oncorhynchus), Pacific Northwest, 1950 |
| 2          | Steelhead trout (Salmo gairdneri), Mont., 1950 |
| 3          | Rainbow trout (S. gairdneri), Va., 1972 |
| 4          | Rainbow trout, B. C., 1971 |
| 5          | Sockeye salmon (O. nerka), Vancouver, 1972 |
| 6          | Coho salmon (O. kisutch), Ohio, 1972 |
| 7          | Brook trout (Salvelinus fontinalis), Va., 1972 |
| 8          | Chinook salmon, Ore., 1967 |
| 9          | Chinook salmon, Idaho, 1972 |
| 10         | Rainbow trout, Ariz., 1972 |
| 11         | Gram-positive rod, trout, midwestern, U.S., 1956 |
| 12         | Gram-positive rod, trout, eastern U.S., 1950 |
| 13         | Gram-positive rod, trout, western U.S., 1972 |

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### Table 2. Comparative growth of seven strains of the kidney disease bacterium on four media

| Medium          | Packed cells (ml) | Average | Range |
|-----------------|-------------------|---------|-------|
| MH (Difco)*     | 0.21              | 0.16–0.25 |
| MH (BBL)*       | 0.11              | 0.07–0.20 |
| Evelyn’s*       | 0.10              | 0.09–1.13 |
| Tryptic soy agar| 0.02              | 0.01–0.05 |

* L-Cysteine hydrochloride (0.1%) added.
* Contains 0.1% L-cysteine hydrochloride and 6.5% serum.
Table 3. Agglutinin titers of 10 strains of the kidney disease Corynebacterium (1 to 10) and three gram-positive non-kidney disease cultures (11 to 13) with rabbit anti-kidney disease sera

| Culture | Cultures* |
|---------|-----------|
|         | 2 WC  | 3 WC  | 4 WC  | 3 SON |
| 1       | 1:4096 | 1:256 | 1:2048| 1:256 |
| 2       | 1:2048 | 1:2048| 1:4096| 1:2048|
| 3       | 1:512  | 1:1024| 1:512 | 1:1024|
| 4       | 1:1024 | 1:4096| 1:2048| 1:256 |
| 5       | 1:1024 | 1:2048| 1:1024| 1:256 |
| 6       | 1:512  | 1:4096| 1:1024| 1:256 |
| 7       | 1:512  | 1:4096| 1:2048| 1:4096|
| 8       | 1:2048 | 1:256 | 1:2048| 1:128 |
| 9       | 1:4096 | 1:4096| 1:4096| 1:1024|
| 10      | 1:2048 | 1:4096| 1:1024| 1:2048|
| 11-13   | <1:10  | <1:10 | <1:10 | <1:10 |

* See Table 1 for sources of cultures.
* For rabbit antisera preparations. WC, whole cell antigen; SON, sonicated antigen.

(Difco) than from the other media were probably the result of adaptation since this medium had been used to grow all strains of the bacterium for inoculation on test media. Satisfactory growth was also obtained on MH (BBL) and Evelyn's medium. The MH media have the advantage of being commercially available and not requiring the addition of blood or serum. Stock cultures of KD isolants have been maintained on MH for more than a year and the medium has also proven satisfactory for primary isolation. A further advantage of MH is the lack of competing serum antigens when the KD bacterium is grown for immunization.

As previously reported (2) growth was most abundant when surface moisture was present. In the present study the presence of moisture was ensured by horizontal incubation of culture vessels.

The described serological homogeneity

Fig. 1. Continuous precipitin band formed by five isolates of the kidney disease bacterium (wells 1 to 5) and rabbit anti-kidney disease serum (center well). The nonreacting well (6) contains a gram-positive isolate from trout, morphologically resembling the kidney disease bacterium.
among all KD isolates from a wide geographical range indicates presence of one or more common antigens. Therefore, serological procedures are applicable for diagnosis of epizootics, identification of isolated cultures, and possibly detection of carriers. There are only a few gram-positive bacteria pathogenic for fishes and the KD organism is the most frequently encountered. The failure of the three non-KD cultures to react with KD antisera is an indication of the specificity of the agglutinin and precipitin reactions of KD test strains. For identification of isolated cultures of the KD bacterium, a simple slide agglutination test would be the method of choice. For diagnosis of KD, however, we have found that a double-diffusion precipitin test with rabbit anti-KD serum and infected kidney tissue is rapid and accurate, and does not require isolation of the KD bacterium.

The serological homogeneity among selected KD isolants and the suitability of a commercially available medium for culture demonstrated here should help solve problems associated with KD and its diagnosis.

LITERATURE CITED
1. Bullock, G. L., D. A. Conroy, and S. F. Snieszko. 1971. Book 2A: Bacterial diseases of fishes. In S. F. Snieszko and H. R. Axelrod (ed.), Diseases of fishes. T.F.H. Publications, Inc., Neptune, N.J.
2. Earp, B. J., C. H. Ellis, and E. J. Ordal. 1953. Kidney disease in young salmon. Special Rep. Ser. 1, State of Washington Department of Fisheries, Olympia.
3. Gray, M. L., and A. H. Killinger. 1966. Listeria monocytogenes and listeric infections. Bacteriol. Rev. 30:309-382.
4. Ordal, E. J., and B. J. Earp. 1956. Cultivation and transmission of etiological agent of kidney disease in salmonid fishes. Proc. Soc. Exp. Biol. Med. 92:85-88.
5. Wolf, K., and C. E. Dunbar. 1959. Test of 34 therapeutic agents for control of kidney disease in trout. Trans. Amer. Fish. Soc. 88:117-124.