Rapid Diagnostic Approaches in the Identification of Gram-Negative Bacterial Diseases of Fish

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Introduction
Increased interest in fish, both as food and as pets, has prompted an awareness of problems associated with their health. Bacterial diseases, particularly those involving gram-negative organisms, are a major cause of mortality. A number of schematic aids have been developed which offer assistance in the diagnosis of these diseases. Some of these aids do not take into account the increased occurrence of certain enteric organisms producing disease in fishes. At present, at least two members of the Enterobacteriaceae are commonly considered as fish pathogens. This group of organisms will assume a greater role in fish disease problems concomitant with increased awareness of enteric organisms in the aquatic environment. It is essential for procedures to be developed which consider these organisms in the diagnosis of any bacterial fish disease. In diagnostic work, the ideal is to obtain accurate determinations with minimal time, effort and equipment. In most situations, rapid identification of the genera of etiologic agents provides the needed information to start therapy.

For purposes of this discussion, a comparison of two recently developed diagnostic aids seems appropriate. One scheme developed by the Fish Health Section (FHS) of the American Fisheries Society employs conventional non-inhibitory media for primary isolation and identification of fish pathogenic bacteria. A second scheme uses selective and differential media to provide rapid identification of pathogens.

Selection of Specimens
Regardless of the schematic aid followed, none are better or worse than the collection and diagnostic approach employed to obtain the material for culture. Diagnosis of fish mortality should not be based upon assessment of results from a single fish. Several typical moribund specimens should be examined; however, dead fish should never be used for diagnostic procedures because of the rapid postmortem migration of endogeneous bacteria into the tissues. The first objective in any diagnostic examination is to determine if bacteria are present in affected tissues. Gram-stained smears should be made if outward clinical signs are present. One must bear in mind that a number of types of bacteria may be observed in external lesions in addition to the suspected etiologic agent. If a septicemia is suspected, then stained smears from kidneys and other internal organs should be examined. The gram stain will establish if the disease problem is of bacterial origin and give insight into the morphology or group of organisms involved.

Diagnostic Approach
In bacterial fish diseases caused by gram-
negative rods, the relative width and length of the organism in question is important. This observation should separate short rods (1 to 3 μ by 0.5 to 0.8 μ) from long, thin rods (2 to 12 μ by 0.4 to 0.8 μ) characteristic of flexibacteria (formerly myxobacteria).

The observation of long, thin, gram-negative rods provides a basis for presumptive diagnosis of flexibacterial infection. Subsequent culture on Cytophaga agar and use of specific antisera are necessary for characterization of flexibacterial pathogens such as the columnaris and coldwater disease organisms.(2)

When culturing short gram-negative rods on non-inhibitory media such as trypticase soy agar, several points should be taken into consideration. First, if the sample is not carefully collected the resulting overgrowth of contaminating organisms may preclude the isolation of the suspected pathogen. Also, since most gram-negative rods grown on non-inhibitory media have similar colonial morphology, a statistical cross section of the colonies on the plate must be examined biochemically to assure isolation of the pathogen.

If one chooses to use non-inhibitory media isolation procedures, the incubation temperature should be maintained at 20–25°C—well out of the optimum mesophilic range—to retard as much contaminating growth as possible. With few exceptions, notably Aeromonas salmonicida and Pseudomonas fluorescens, most fish pathogens grow well at 37°C, while lower temperatures tend to slow down their growth rates. In most cases, at the end of 48 hours incubation there is sufficient growth for delineation of the bacterial growth into two major groups on the basis of cytochrome oxidase test. The cytochrome oxidase positive isolates most likely are members of the Pseudomonas, Aeromonas, and Vibrio groups. Hanging drop motility tests along with O/F glucose tests, pigment formation, and sensitivity to novobiocin and the vibriostat 0/129 (2,4-diamino-6,7-di-isopropyl pteridine) are then employed to separate these three groups of fish pathogens. By the 72nd hour incubation, most initial characterization media which were inoculated in a battery format at 2 days may be interpreted; unusual or conflicting results repeated for verification; or additional media sought and inoculated to complete the identification process. Although most schematic aids indicate straightforward reactions, variation in biochemical reactions may occur especially with carbohydrate reactions.

For the most part, the cytochrome oxidase negative group of gram-negative organisms encountered in fish diseases represent members of the Enterobacteriaceae. Using the diagnostic chart of the FHS, the two described enteric pathogens—Edwardsiella tarda and Enteric redmouth (ERM) bacteria—are separated using production of indole and reactions in triple sugar iron agar (TSI). Other enteric organisms which may cause fish diseases are not considered in this chart.

One should not rely too heavily on antisera to replace salient biochemical characteristics. Due to antigenic similarities, it is possible for closely related organisms such as those found in Enterobacteriaceae to agglutinate nonspecifically.

Data available in most schemes designed for fish diseases are predicated on characteristics similar to those found in the above discussion. It is our opinion that these identification criteria should not always be taken as confirmatory because in many situations additional laboratory work may be necessary for definite speciation.

Utilization of a schematic aid advocating the use of selective and differential media allows for a more definitive approach to the diagnostic problem. Since the diagnostician is dedicated to saving time, we would suggest that a group of four plates be inoculated initially and incubated at different temperatures. These plates and their respective role in the diagnostic process are as follows:

1. Pseudosel—inoculated and incubated at 20°C for 24 to 48 hr. This plate con...
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1. Contains cetrimide, a bacterial inhibitor which precludes in general the growth of bacteria other than members of the genus *Pseudomonas*. The incubation temperature suggested further selects a psychrophilic population. Forty-eight hours incubation usually allows for the diffusion of fluorescein pigment, thus by the 48th hr the genus and species is presumptively available. Should the disease be cause by other psychrophilic pseudomonads, these would be selected at a genus level by the 48th hr.

2. Trypticase soy agar (Two plates)—inoculated and incubated at 20 and 35°C for 24 to 48 hr. These plates serve primarily as a point of reference for verification of this suggested selective media. This medium serves as a primary isolation medium for *Aeromonas salmonicida* and *Vibrio* sp. The appearance of brown diffusible pigment on the plate incubated at 20°C for 24 to 48 hr in conjunction with cytochrome oxidase activity and motility verify the presence of *A. salmonicida*. If novobiocin and/or 0/129 discs are placed on the plates at the time of initial inoculation, the presence of *Vibrio* sp. may be confirmed by the 24th hr.

3. Rimler-Shotts agar (9, 10)—inoculated and incubated at 35°C for 20 to 26 hr. The presence of yellow colonies on this medium is indicative of *Aeromonas hydrophila* (liquefaciens) at a 95% confidence level; complete assurance may be obtained if the colony is found to be cytochrome oxidase positive. If the colony is cytochrome oxidase negative, a possibility of either *Citrobacter* sp. or ERM bacterium exists. Inoculation of TSI and lysine decarboxylase resolve this differentiation by the 48th hr.

Other colonies, either bluish-green or black, indicate the presence of other enteric organisms. Each colony type should be inoculated into TSI in duplicate. All TSI cultures showing no gas forma-

Discussion

Either of the afore discussed diagnostic approaches will afford identification of bacterial fish pathogens with equal effectiveness. However, the use of selective media will, in most situations, result in a more rapid identification of known fish pathogens. It should be pointed out that without the inclusion of appropriate media to differentiate enteric organisms, one may spend considerable time in identifying the gram-negative organisms other than classic fish pathogens. The use of the second scheme (9) or some similar approach which includes the identification of enterics would aid in determining the etiology of unusual bacterial fish disease syndromes caused by enteric organisms.

The main purpose of rapid and accurate diagnosis of fish diseases caused by the gram-negative pathogens is to afford a rational basis of treatment for affected fish. Standard treatment procedures for incorporating drugs into diets or adding them to water have been described and the most commonly used treatments will only be summarized here. Infections caused by *A. salmonicida* have been controlled by a 10 to 14 day treatment using sulfamerizine at the rate of 20 g/100 kg of fish/day or oxytetracycline at 5.0 g/100 kg of fish/day (3). The latter treatment has also been used to control diseases caused by *A. hydrophila*, *V. anguillarum*, *Edwardsiella tarda*,
flexibacterial infections and Pseudomonas species\(^{(3,8)}\). Nifurpirinol (Furanace) used at 1.0 ppm in 1 or 2 daily 1 hr baths also effectively controls epizootics caused by flexibacteria\(^{(1)}\). Outbreaks of ERM may be controlled by a combination of a 5-day treatment with sulfamerazine followed by a 3-day treatment with oxytetracycline, both use at the above rates\(^{(3)}\).

References


Discussion

K. Wolf: would you want to relate your paper to control measures? e.g. What do you tell the hatchery owner at what point in the identification scheme?

E. B. Shotts: In most cases the hatchery owner could be given a preliminary report in from 18 to 24 hrs and often depending upon findings at that time a recommendation for therapy can be given. Certainly by 36 to 48 hrs the owner should have a report and be treating his disease problem.

J. L. Fryer: There is a problem to get fish pathologists to use the procedures you have presented. If in the course of their work they observed a Gram negative bacterium the treatment is the same regardless of which one it is and the same is true if a Gram positive bacterium is found. It seems most pathologists don’t want to go to this extra effort.

E. B. Shotts: The fish pathologist has only had to contend with problems associated with a small group of highly host adapted organisms in the past. It is our contention that this will be changing, as shown by the documented increased pollution of waters with sundry organisms of potential public health importance. A majority of these organisms are non host adapted species which may necessitate the fish pathologist having to proceed further for a diagnosis. The documented Enterobacteriaceae associated with fish disease currently are, in my mind, only forerunners of problems to come.