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MFR PAPER 1333

Anaerobic Bacteria as Possible Disease Agents in Fish

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There are currently 23 recognized genera of heterotrophic, obligately anaerobic bacteria. Within this group are gram-positive rods and cocci and gram-negative rods and cocci. Many of the genera have representative species which are clinically important pathogens of man and animals. The most familiar genus, *Clostridium,* contains the species *C. botulinum, C. tetani,* and *C. perfringens,* causing botulism toxicity, tetanus, and gas gangrene, respectively. Other genera of anaerobes such as *Propionibacterium, Fusobacterium, Bacteroides,* and *Eubacterium* are less familiar, but also significantly involved in human disease.

Although anaerobic bacteria have been studied since the beginnings of microbiology, it has been only recently that they have been identified as a major group of pathogens in animals and man. A number of reasons can be cited for this lag. Probably the most important was that many people did not believe that strict anaerobes could survive and proliferate in "oxygenated" tissues of the body. Lungs, brain, liver, and other vital organs were good candidates for facultative bacteria, but not for anaerobes. Frequent coinfections with facultative organisms further complicated the picture.

The development of standardized procedures for working with anaerobic bacteria has helped to dispell some of these beliefs. The popularization of the roll-tube method (Hungates' method) by workers at the Virginia Polytechnic Institute's Anaerobe Laboratory and commercialization of the Gas-Pak[1] (BBL) anaerobic jar have both greatly aided in standardizing and facilitating procedures for cultivating anaerobic bacteria. Recent advances in the procedures used to classify anaerobes have made the task of identifying these organisms somewhat less tedious. In particular, the use of gas-liquid chromatography to identify the major metabolic by-products from fermentable substrates allows rapid identification to genus and sometimes to species. Although other confirmatory tests are required, much time can be saved by knowing which tests to run. Many of these advances have come about within the past 3-5 years, and their adoption by clinical microbiology laboratories is still underway. It seems to me that in the not too distant future a number of fish disease laboratories will also adopt these procedures and develop new ones particularly suited to fish disease studies.

At the Fish and Shellfish Pathology Laboratory at the University of Miami, we have been involved with the study of anaerobes from marine fish for the past 2 years. Our studies began following the occurrence of several large fish kills in Biscayne Bay. In addition to the kills, moribund fish were present which exhibited a "twirling" symptom not unlike that observed in trout with whirling disease. Analysis of toxicological and parasitological as well as aerobic bacteriological data revealed no consistent cause of this symptom. We began a search for anaerobic bacteria in the brains (because of the likelihood of neurologic involvement) of fish

[1]Mention of trade names or commercial products does not imply endorsement by the National Marine Fisheries Service, NOAA.

showing these symptoms. At the time, only thioglycollate cultures were taken, but we were fortunate in isolating an anaerobe, in pure culture, from the brains of all the mullet, *Mugil cephalus*, sampled.

This bacterium has since been named by us as *Eubacterium tarantellus* sp. nov. (Udey et al., 1977). It is a gram-positive rod measuring 1.3-1.6 × 10.0-17.0 μm which upon initial isolation can occur as long filaments many times that length (Fig. 1). The organism does not form spores and is nonmotile. Colonies on BHI blood agar are transparent, filamentous, and often form a "pinwheel" design. Colonies are always surrounded by a large zone of beta hemolysis. The organism also produces lecithinase upon initial isolation, but may lose this characteristic upon repeated transfer. The minimum growth temperature of *E. tarantellus* is 15°C, which is probably a reflection of the fact that it was isolated in the tropics. Unless the organism can adapt to colder temperatures, its growth in many areas (outside the tropics) would be limited to summer months. It is interesting to note that *E. tarantellus* will not grow at salinities of greater than 20 ppt or above pH 8.0 (at least in laboratory media). This strongly suggests that the organism is an obligate fish pathogen, is limited to growth in estuaries, or is from exogenous sources such as land runoff or sewage effluents. Fortunately, *E. tarantellus* presents little threat to humans since it does not produce toxins; it is not pathogenic for guinea pigs. This is a particularly important consideration when working with anaerobes because numerous species of *Clostridium* produce potent toxins, making them a hazard in the laboratory.

To determine if *E. tarantellus* causes infection primarily of the central nervous system or a more generalized infection, an organ distribution study was undertaken. Organ samples from 38 fish belonging to 12 species were cultured on BHI blood agar. Sixty-six percent of the fish yielded positive brain cultures. Only 5 percent of the liver and kidney samples were positive, while 7 percent of the intestinal content samples contained *E. tarantellus*. In no case was the anaerobe isolated from an organ other than the brain if the brain was not also infected. This clearly indicates that this particular anaerobe has a definite tissue specificity. Of the 12 species sampled in this study, the bluefish, seatrout, menhaden, and snook all had a 100 percent incidence. In the past, the striped mullet also showed a 100 percent incidence. All strains which we have isolated to date appear to be closely related serologically (Fig. 2). Infections do not seem to be correlated with the type of feeding habits; menhaden and mullet are filter feeders, while bluefish and seatrout are carnivores. It seems likely that the fish are either all equally exposed to the source of infection or that it is being transmitted up the food chain. More work will be needed in this area in the future.

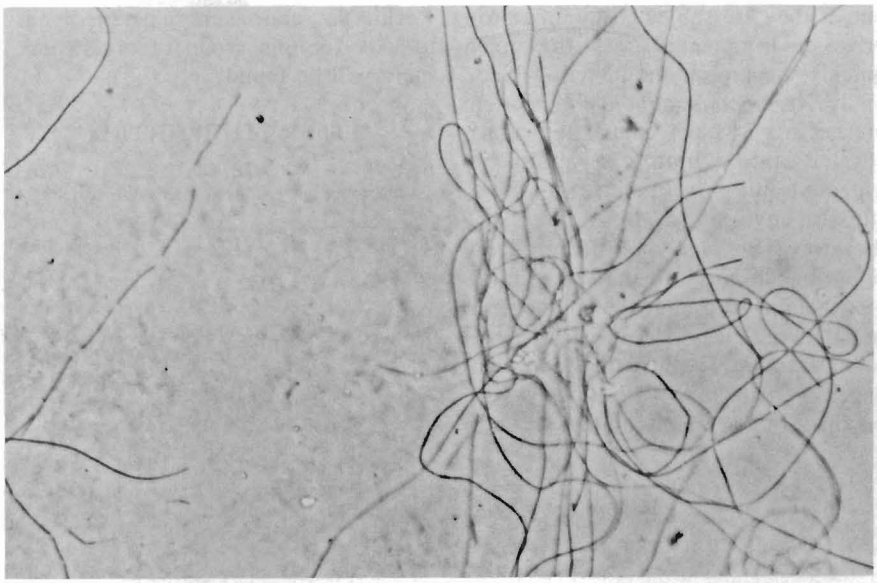

Figure 1.—Long filaments and shorter rods of *Eubacterium tarantellus* seen in initial cultures using Brewer's thioglycollate medium. Phase contrast, 400×.

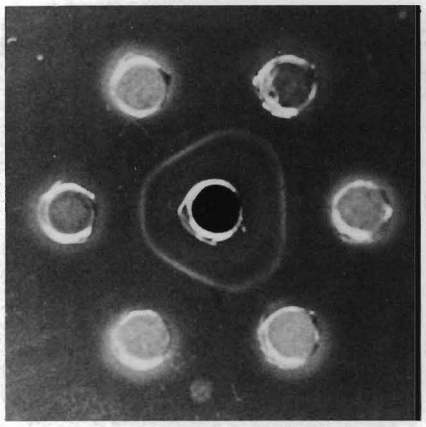

Figure 2.—Double immunodiffusion plate demonstrating the serological relatedness of *Eubacterium tarantellus* isolates. Outer wells: suspensions of sonically disrupted *E. tarantellus* cells of six strains isolated from different fish species. Center well: rabbit anti-*E. tarantellus* (strain 128) serum.

The question which arises most often in discussions of *E. tarantellus* infections is "What, if anything, is the organism doing to the large number of fish which seem to be infected?" This is a particularly germane question and one which is extremely difficult to answer for native fish populations. We are confident that, in the past, the anaerobe has been responsible for fish kills in Florida and in Texas (Henley and Lewis, 1976). If we can use two examples from the field of cultured fish diseases, the importance becomes more evident. The best example, perhaps, would be that of bacterial kidney disease (BKD). Fish can be infected with the BKD bacterium for several years and not show any outward symptoms,

but, if they are subjected to an appropriate environmental stress, they may quickly succumb to the infection. Secondly, *Aeromonas salmonicida* is often present in a large percentage of fish in a carrier state which can again be triggered into an active infection by a stressful environment. It is our feeling, therefore, that any significant deep organ infection (particularly the brain) is of major consequence to the fish population.

In the future, I believe that additional anaerobic bacterial fish pathogens will be discovered. Much of the groundwork is set, and, as more people begin to look for this group of organisms, more will be found.

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MFR PAPER 1334

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Recent Developments in Channel Catfish Virus Research

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Channel catfish virus (CCV) is well established as a highly virulent pathogen in some cultured channel catfish fingerling populations. This highly contagious disease of juvenile channel catfish occurs during the warm summer months. Although most epizootics have occurred in the southern states, the virus has been isolated from channel catfish fingerlings in several northern and western states. Mortality rates of CCV-infected populations may approach 100 percent. CCV is caused by herpesvirus of ictalurids (Wolf and Darlington, 1971). The virus has been isolated only from fish when fry or fingerlings are actually dying. Epizootics appear to be associated with some form of stress.

The brown bullhead (BB) cell line is normally used for isolation of CCV. A cell line is being developed from channel catfish ovaries (CCO) which is now in the 75th passage and approximately 20 months old. CCO cells appear to be more sensitive to CCV than the BB cells; CCO cells develop initial microscopic cytopathic effect (CPE) 12-24 hours before the BB cells, and total CPE occurs 24-48 hours in CCO cells before it does in BB cells. Also, the CCV titers in CCO cells are 0.5-1.0 log (base 10) higher than in the BB cells.

A fluorescent antibody (FA) technique has been developed for detecting CCV in vitro. Rabbit anti-CCV serum was conjugated with fluorescein isothiocyanate (FITC) using the method of Cherry (1974). CCO cultures in Leighton tubes were infected with CCV, and, at 2-hour intervals after infection, coverslip cultures were removed, fixed in acetone, and incubated with FITC conjugate. Infected CCO cells fluoresced at 4 hours postinfection (PI) with focal cytoplasmic fluorescence. Fluorescence increased with larger focal areas through 6 and 8 hours PI, until at 10 hours the entire cell sheet was fluorescing. The FA technique has not been applied to in vivo infections.

Confirmed CCV epizootics have been found only in farm-raised channel catfish populations. Different strains of channel catfish vary in their susceptibility to CCV (Plumb et al., 1975); however, data on susceptibility of other species of ictalurids to CCV are lacking. Young brown, black, and yellow bullhead catfish were exposed to CCV by incorporating the virus into the feed, intraperitoneal (IP) injection, and by cohabitation with CCV-infected channel catfish. Although some mortality occurred in all of these bullheads in each method of exposure, CCV was not isolated from any of the exposed fish. It is interesting to note that the BB cells support CCV replication but that the fish itself apparently does not.

In another study, blue and channel catfish fingerlings and their reciprocal crosses were compared for CCV