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ANAEROBIC BACTERIA ASSOCIATED WITH EPIZOOTICS IN GREY MULLET (*Mugil cephalus*) AND REDFISH (*Sciaenops ocellata*) ALONG THE TEXAS GULF COAST[1][2]

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Abstract: Anaerobic bacteria tentatively identified as species of *Catenabacterium* were recovered from brain, liver, kidney and blood of fish involved in a massive epizootic of grey mullet (*Mugil cephalus*) and redfish (*Sciaenops ocellata*). Pathogenicity was demonstrated for grey mullet (*M. cephalus*) and sea catfish (*Arius felis*) but not for channel catfish (*Ictalurus punctatus*) or white mice. Diseased fish were disoriented, weak and swimming at the surface of the water. Thioglycolate and salt bovine blood agar containing 40 μg/ml gentamicin were useful as selective culture media.

INTRODUCTION

During April, 1973, extensive mortality involving grey mullet (*Mugil cephalus*) and redfish (*Sciaenops ocellata*) occurred near Port Aransas, Texas. Just prior to death, fish would swim in an erratic, disoriented fashion at the surface of the water. When not swimming, the fish would attain a nearly vertical floating position with the head at the surface. Mortality appeared to be restricted to large (20-30 cm) fish. Water quality measurements with a galvanic cell oxygen analyzer, thermometer and conductivity meter[3] and a portable pH meter[4] indicated approximate concentrations of 10-11 ppm dissolved oxygen, salinity 28.6 o/oo and pH 8.0, 2.5 cm below the surface of the water. Within 6 to 8 weeks following the onset of this episode, the numbers of dead or dying fish increased in a pattern suggesting the involvement of infectious agents.

This report describes the isolation and identification of the etiologic agent involved in the epizootics, and the development of a selective medium for the recovery of the infectious agent from fish tissues.

MATERIALS AND METHODS

Culture media in test tubes were 'prereduced anaerobically sterilized' (PRAS) and processed as described elsewhere.[2,3] Solid media were freshly prepared just before use and incubated after inoculation at 20 C in GasPak[5] chambers.[2,3]

Moribund mullet and redfish collected near Port Aransas, Galveston and Orange, Texas were examined at necropsy. Samples of brain, liver, kidney and blood were cultured in brain heart infusion broth (BHI) and thioglycolate broth (TG) containing 1.0% NaCl, and

[1] Some of the work presented was conducted by the first author in partial fulfillment of requirements for the Master of Sciences degree.

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[3] Yellow Springs Instruments, Yellow Springs, Ohio.

[4] Beckman Instruments, Palo Alto, California.

[5] Baltimore Biological Laboratories, Cockeysville, Maryland 21030, USA.

streaked on bovine blood agar supplemented with 0.5% NaCl, (salt bovine blood agar, SBBA). Culture media were incubated 3 to 5 days at 20 C and turbid broth cultures streaked on freshly prepared SBBA. Cultures derived from BHI were incubated aerobically whereas those from TG were incubated anaerobically in GasPak chambers and at reduced oxygen tension in candle jars. Impression smears of brain, liver and kidney were gram stained.

Anaerobic isolants were examined for motility by darkfield microscopy. Following growth on SBBA and a clostridial sporulation medium[1] the bacteria were stained using Wirtz-Conklin stain[4] for evidence of sporulation. The isolants were screened using a PRAS basal medium prepared as described elsewhere.[2,3] Sterile glucose, lactose, sucrose, maltose, xylose and salicin were incorporated at a concentration of 1.0% in the basal medium.[2] Other tests included ammonia production from peptones, hydrogen sulfide production, gelatin liquefaction, lecithinase production, starch hydrolysis, litmus milk reaction, cytochrome oxidase production, catalase production, nitrate reduction to nitrite and gas-liquid chromatographic analysis (GLC) for alcohol and fatty acid production from peptone-yeast extract-glucose (PYG).[2,3] Base ratio determinations were performed as described elsewhere[5,7] by Dr. M. Mandel[6] and were expressed as molar percent guanine and cytosine (%GC).

Pathogenicity studies were conducted in white mice, grey mullet, sea catfish (*Arius felis*) and channel catfish (*Ictalurus punctatus*) using 0.5 ml of a 72 hr TG culture in mice and 0.02 ml of a 72 hr TG culture in fish administered intraperitoneally. Mice were maintained under conventional laboratory conditions. Fish were kept in 160 L glass aquariums. Marine fish were maintained in artificial seawater (25 o/oo) and the channel catfish in dechlorinated tapwater. Water from each aquarium was circulated through filters containing 5 kg of charcoal and oyster shell (1:2 ratio) mixture/75 L water, at a rate of 6 L/minute. Each aquarium was equipped with an air supply. Fish were introduced into the aquariums and held one week for observation. Measurements for dissolved oxygen and ammonia content were begun prior to inoculation and continued daily throughout the experiment using reagents and protocol supplied with Hach water quality kit.[7]

Antibiotic susceptibility tests were performed by a broth-disc method.[2] Prereduced BHI broth was inoculated with one drop of culture from PYG. The appropriate number of commercial antibiotic discs was added to each tube while under the gassing cannula by the use of flamed forceps. The antibiotics and final concentration in BHI were: chloramphenicol (12 mcg/ml), dihydrostreptomycin (5 mcg/m), erythromycin (3 mcg/ml), furacin (20 mcg/ml), gentamicin (10 mcg/ml), kanamycin (12 mcg/ml), nalidixic acid (5 mcg/ml), neomycin (12 mcg/ml), novobiocin (12 mcg/ml), penicillin-G (2 units/ml), polymyxin B (60 units/ml) and tetracycline (6 mcg/ml). After incubation at 20 C, turbidity comparisons between cultures containing no antibiotics and those containing the various antibiotics were used as a basis for interpretation. Tubes with an obvious turbidity similar to the control were reported as resistant while tubes with a turbidity obviously less than the control were reported sensitive.

Since all of the anaerobic isolants tested were found resistant to gentamicin, efforts were made to develop an isolation medium using gentamicin as a selective factor. Gentamicin solution was diluted and incorporated into TG at 5 mcg/ml increments from 5 to 100 mcg/ml. To test the efficacy of the media in selectively delineating the anaerobes from other pathogenic bacteria frequently associated with fish diseases, the anaerobes,

[6] The University of Texas System Cancer Center, Department of Molecular Biology, Texas Medical Center, Houston, Texas 77025.

[7] Hach Chemical Company Ames, Iowa.

as well as other bacteria frequently recovered from diseased fish, were inoculated into TG containing the various levels of gentamicin. The additional test organisms, constituting part of the culture collection in the Department of Veterinary Microbiology, Texas A&M University, included: *Aeromonas hydrophila, Plesiomonas shigelloides, Pseudomonas fluorescens, Ps. piscicida, Vibrio alginalyticus, V. anguillarum* and *V. parahemolyticus.*

Direct fluorescent antibody (FA) studies were conducted with the anaerobic isolants using fluorescein isothiocyanate (FITC) conjugates[1] of antiserums developed in New Zealand white rabbits against two isolants, M.S. 841 and M.S. 1065. The specificity and reactivity of the two conjugates were evaluated using homologous and heterologous systems and specific inhibition tests.[9]

RESULTS AND DISCUSSION

Vibrio sp. and *Pseudomonas* sp. were recovered from a few of the liver samples of fish, but strict anaerobic bacteria were recovered from all brain samples and from the majority of the liver, kidney and blood samples of all fish examined.

Stained tissue impression smears revealed gram positive rods of various lengths (Figure 1). Long chains of pleomorphic gram positive rods were recovered from TG subcultures of brain tissue (Figure 2). Darkfield observation indicated the organisms were non-motile and spores could not be detected. However, many of the rods possessed "ovoid bodies" (Figure 3) which were more numerous in cultures older than 7 days.[8]

Subcultures of TG streaked on SBBA and incubated in candle jars at 20 C did not grow. On streaked SBBA plates incubated in GasPak chambers, beta hemolytic, translucent, slowly spreading, flat

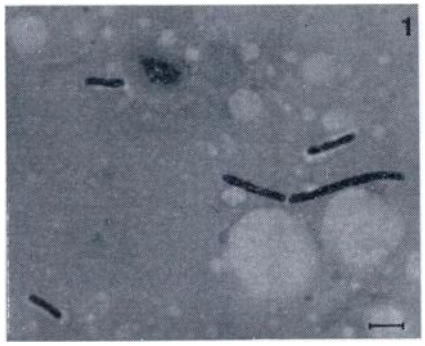

FIGURE 1. Gram positive rods in an impression smear of brain tissue from grey mullet (**Mugil cephalus**). Bar = 5 μm.

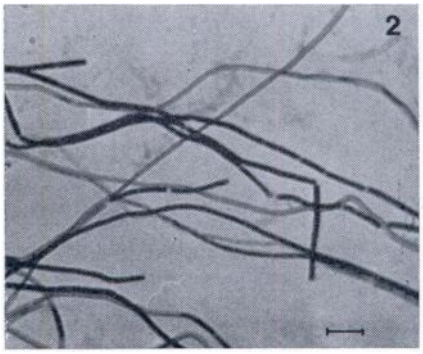

FIGURE 2. Anaerobic gram positive bacteria in long chains recovered from brain and liver tissues from grey mullet (**Mugil cephalus**) and redfish (**Sciaenops ocellata**) Bar = 5 μm.

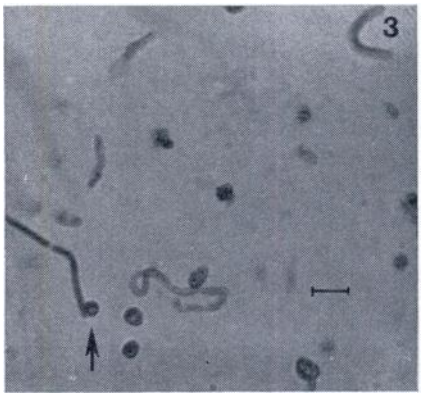

FIGURE 3. Ovoid bodies (arrow) associated with gram positive anaerobic bacteria recovered from brain and liver tissues from grey mullet (**Mugil cephalus**) and redfish (**Sciaenops ocellata**). Bar = 5 μm.

and contoured colonies with filamentous edges were observed. In TG the organisms grew yielding greyish-white growth whereas in PYG a viscid, mucoid sediment was observed (Figure 4). Deep agar colonies in liver infusion agar were arborescent with dense centers (Figure 5). In PYG, M.S. 1065 was observed to adhere to the bottom of the tube more so than M.S. 841. Limited aerotolerance was observed in M.S. 841 and M.S. 1065 in that exposure to oxidized medium yielded no growth but viable cells could be recovered.

All the anaerobic isolants possessed characteristics similar to either M.S. 841 or M.S. 1065. Results of biochemical tests of M.S. 841 and M.S. 1065 (Table 1) show that the two organisms are probably not the same organisms. The FA study demonstrated M.S. 841 and M.S. 1065 are antigenically distinct. The FITC conjugate prepared against M.S. 841 was specifically inhibited by unlabelled rabbit anti-M.S. 841 antiserum but not by rabbit anti-M.S. 1065 antiserum. Specific inhibition of M.S. 1065 FITC conjugate also was accomplished by unlabelled rabbit anti-M.S. 1065 antiserum but not by unlabelled rabbit anti-M.S. 841 antiserum.

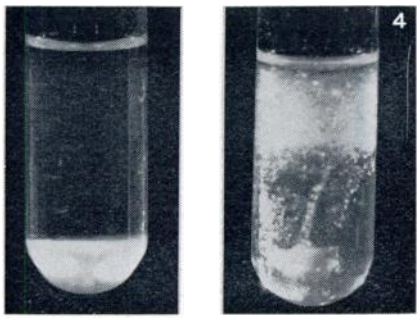

FIGURE 4. Anaerobic bacteria recovered from brain and liver tissues from grey mullet (**Mugil cephalus**) and redfish (**Sciaenops ocellata**) growing in peptone yeast glucose broth (left) and thioglycolate broth (right).

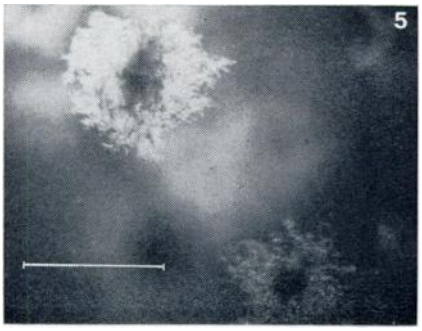

FIGURE 5. Arborescent colonies of anaerobic gram positive bacteria in liver infusion agar. Bar = 1 mm.

Pathogenic effects were not observed in white mice or in channel catfish during six weeks observation. However, signs similar to those oberved in the epizootic were produced in grey mullet and sea catfish challenged with either M.S. 841 or M.S. 1065. Signs of abnormal behavior began three to five days after challenge and within 30 days all fish were dead. Anaerobic gram positive rods were recovered in pure culture from their brains.

Antibiotic susceptibility studies revealed that M.S. 841 and M.S. 1065 were resistant to dihydrostreptomycin, gentamicin, kanamycin, nalidixic acid, neomycin, penicillin G and polymyxin B but were sensitive to chloramphenicol, erythromycin and tetracycline. M.S. 841 was resistant to furacin and sensitive to novobiocin but M.S. 1065 was sensitive to the former and resistant to the latter.

None of the gram negative organisms grew in media containing 30 mcg/ml or more of gentamicin but M.S. 841 and M.S. 1065 were able to grow at higher concentrations. Therefore, the optimum concentration of gentamicin used in the selective culture medium for M.S. 841 and M.S. 1065 was 40 mcg/ml, and, when incorporated into SBBA on TG, did not appear to influence cellular or colonial morphology.

Gram stain, cellular morphology, colonial morphology, motility, carbohydrate fermentation and GLC analysis indicates the two groups of anaerobic organisms may belong to the genus *Catenabacterium*. The organisms could be selectively cultured in TG or SBBA containing 40 mcg/ml gentamicin.

TABLE 1. Results of biochemical tests of anaerobic bacteria, M.S. 841 and M.S. 1065, recovered from marine fish tissues.

Test	M.S. 841	M.S. 1065
Motility	negative	negative
Spore production	negative	negative
Gas production from glucose	positive	positive
Oxygen tolerance (deep agar)	anaerobic	anaerobic
Carbohydrate fermentations		
Glucose	acid	acid
Lactose	acid	acid
Sucrose	no change	no change
Maltose	acid	acid
Xylose	no change	no change
Salicin	no change	no change
NH_4 production	positive	negative
H_2S production	positive	negative
Gelatin liquifaction	positive	positive
Lecithinase production	positive	negative
Starch hydrolysis	negative	negative
Litmus milk	acid, digestion	acid, clot, digestion
Cytochrome oxidase production	negative	negative
Catalase production	negative	negative
NO_3 reduction to NO_2	negative	positive
G.L.C.[8] analysis	ethanol acetic acid	ethanol acetic acid lactic acid
% G-C[9] content	30.0	30.6

[8] G.L.C. = gas-liquid chromatography.

[9] % G.C. = molar percent guanine-cytosine content.

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