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Microbial Diseases of Cultured Tilapia

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Microbial Diseases of Cultured Tilapia

BARD Project 1077

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Abstract	2
Objectives	3
Report Body	3
Background	3
Microbiology of tilapia vibrio isolates	3
Vibriosis in tilapia	4
Table 1. Characteristics of tilapia vibrio	5
Figure 1. Tissue concentration of vibrio following challenge	8
Table 2. Hematologic parameters of uninfected and infected tilapia exposed to vibrio	9
Table 3. Serum chemistry values of vibrio infected tilapia	10
Immunization	10
Antigens of <i>Vibrio anguillarum</i> recovered from diseased tilapia	10
Monitoring antigenic uptake	13
Figure 2. Antigen uptake following immersion	13
Figure 3. Antigen uptake following oral exposure	15
Immersion vaccination	16
Oral immunization	16
Establishing a challenge dose	16
Figure 4. Comparative immune response via immersion/oral routes	17
Combined immersion-/oral immunization	18
Production of biologic and proposed immunization protocol	19
Evaluation of Research Achievements	20

Abstract

Agents associated with tilapia vibriosis were identified as *Vibrio anguillarum*. Histopathologic studies on infected fish suggest that the organism is sequestered in select tissues following an initial septicemic infection phase. It is hypothesized that a chronic state is established following initial infection and that secondary septicemia and subsequent mortality is dependent upon factors which compromise host defenses. During the acute phase, hyperglycemia along with reduced electrolyte and liver function is suggestive of alterations of carbohydrate metabolism associated with gram negative endotoxicoeses. Reduced electrolyte- and plasma protein values are suggestive of renal dysfunction, a suggestion which correlates with the histopathologic picture.

An heat stable surface component (S- component) resembling S-layer protein was observed in virulent isolates of *V. anguillarum*. The component was insoluble in lauryl sarcosinate and electron microscopy studies revealed characteristic S- layer tetragonal patterns on bacterial surfaces and purified preparations of the S-component. The S component was believed to be the predominate antigenically active component in autoclave treated *V. anguillarum* cells.

Serologic- and protective immune responses were observed in fish immunized with autoclaved preparations of the agent. A production and recommended immunization protocol involving the use of immersion bath- followed by oral booster of autoclave preparations is presented.

Objectives of the proposal were two:

1. To obtain a clinical and pathological description of vibriosis in tilapia
2. To assess immunity of tilapia to heat denatured vibrio preparations resulting from oral- and immersion modes of administration

Report Body

Background. Tilapia have been extensively used as a species of choice for warmwater aquaculture on a world wide basis because of their adaptability, relative heartiness, and disease resistance. Periodically however, serious losses have been observed in market sized fish. These fish were observed to be particularly susceptible to two groups of bacterial diseases. Initial project emphasis focused upon vibriosis, but later observations revealed that a previously undescribed streptococcal disease was responsible for extensive losses in intensive culture systems in Taiwan, Japan, South Africa, the United States, Egypt and Israel. The research plan was re-oriented such that U. S. efforts would focus on vibriosis primarily, while those of Israel would primarily focus upon streptococcus. The present report reflects U. S. efforts.

Microbiology of tilapia vibrio isolates. Sixty isolates recovered from diseased fish from four locations in Texas were studied. In addition to the tests used to characterize the isolates to generic level, cultures were examined for salt tolerance, fermentation of adonitol, cellobiose, mannitol, maltose and sucrose, presence of the decarboxylases of ornithine and lysine, arginine dihydrolase, gelatinase, amylase and urease,

indole formation, nitrate reduction, citrate utilization, methyl-red and Voges-Proskauer reactions. Characteristics of the isolate recovered from the diseased tilapia were compared to reference cultures of *V. anguillarum* (ATCC 19264); *V. parahaemolyticus* (ATCC17802); and *V. alginolyticus* (ATCC 19108).

Organisms recovered from lesions and tissue samples of diseased fish were Gram-negative, somewhat pleomorphic rod-shaped organisms, motile by 1-3 polar flagella. Enrichment cultures yielded small (2-4 mm) convex, translucent colonies surrounded by a zone of alpha hemolysis after overnight incubation on bovine blood agar at 25 C. The organisms were identical in physiologic and biochemical characteristics and possessed features similar to ATCC 19264 *V. anguillarum* (Table 1).

Vibriosis in tilapia. Although tilapia appear to be more disease resistant than many cultured species, bacterial diseases were suspected in a series of mortality associated with fish maintained in cooling ponds of hydroelectric facilities in several regions of Texas. Additionally, periodic tilapia mortality had been observed in fish being maintained on defined diets in various experimental ponds at Texas A&M. Processing most of the fish at that time resulted in the recovery of bacteria belonging to the genus *Vibrio*. Hence, initial project efforts focused upon vibriosis, although sporadic cases of disease fish, apparently chronically infected with gram positive cocci were also observed.

Gross lesions in diseased fish were observed in fins, skin and

musculature.

Lesions

included

frank

ulceration,

petechiae and

ecchymoses.

Ulcers often

extended into

the skeletal

muscle,

resulting in

necrosis.

Characteristic	Tilapia isolate	V. anguillarum	V. parahaemolyticus	V. alginolyticus
Growth in				
1% NaCl	+	+	-	+
3% NaCl	+	+	+	+
5% NaCl	+	+	+	+
8% NaCl	-	-	+	+
Susc. to 0/129	+	+	+	-
Susc. to				
Novobiocin	+	+	+	+
Indole	+	+	+	+
Methyl-red	+	+	+	+
Vog/Prosk.	-	-	-	+
Citrate	+	+	-	-
Nitrate Red.	+	+	+	+
Arginine dihydrolase	+	+	-	+
Lysine decarboxylase	-	-	+	-
Ornithine decarboxylase	-	-	+	-
Hemolysis	alpha	alpha	beta	none
Growth at:				
5 C	+	+	-	+
17 C	-	-	+	-
Chitinase	+	+	+	+
Amylase	+	+	+	+
Gelatinase	+	+	+	+
Fermentation of:				
Adonitol	+	+	-	-
Arabinose	-	-	+	-
Cellobiose	-	-	+	-
Glycerol	-	-	+	-
Lactose	-	-	-	-
Sorbitol	-	-	-	-
Sucrose	+	+	-	+

Table 1. Characteristics of tilapia vibrio

The vent was reddened and swollen in most of the fish. Internally, there were hemorrhages in the liver, spleen and kidney and, in most of the fish, the gut was distended and filled with a clear viscous fluid.

Blood samples and swabs of liver, kidney and peritoneal fluid were processed and the isolates characterized as a vibrio on the basis of Gram and flagella staining reactions, cellular morphology, the OF test of Leifson, anaerogenesis in glucose broth, the oxidase test and susceptibility to novobiocin and the vibriostat 0/129 (pteridine compound).

Exposure studies were conducted in ten groups of fish (ten

fish/group) maintained in 120-l aquaria at ambient temperature (22 C). Two weeks prior to initiating the study, fish were acclimatized in tap water adjusted to 10 S_{o/oo}(Instant Ocean). Half of the fish received a single 0.1 ml intramuscular injection of cells (ca 10⁵) from an overnight broth culture of one of the isolates recovered during the epizootic intraperitoneally (exposed group). The remaining fish (control group) received 0.1 ml sterile TSB intraperitoneally.

Three fish from the exposed group and three from the control group were sacrificed at 4-, 8-, 12-, 20-, 24-, 30-, 50- and 65 h after receiving the injections and processed to determine bacterial dynamics in the infected host. Twenty-four h after exposure, 20 fish from the exposed group and 20 from the control group were exsanguinated to measure key clinical parameters. Blood was collected from each fish by the hemal arch technique into heparinized 10-ml plastic syringes.

Blood smears, erythrocyte- and leukocyte counts, hemoglobin and erythrocyte fragility test were conducted on blood collected at that time. Plasma was separated by centrifugation and frozen until analyzed. Hemoglobin was determined by the cyanmethemoglobin method. Blood smears were stained with May-Grunwald-Giemsa. Total plasma protein was determined using the Biuret reagent and plasma albumin was measured colorimetrically with bromcresol green. Plasma chloride, osmolality and sodium and potassium concentrations were determined by standard techniques using a chloridometer, an osmometer and a flame photometer respectively.

Plasma alkaline phosphatase were determined using p-nitrophenyl phosphate substrate. Lactate dehydrogenase (LDH) and glutamic-oxaloacetic transaminase were analyzed using automated clinical chemistry procedures.

Estimates of bacterial numbers were made from the blood samples and samples of liver and kidney tissues of experimental fish by plate count. Tissues collected aseptically were weighed, homogenized in Tem Broek tissue grinders and 10%(w/v) suspension in TSB prepared. The suspensions were diluted through a ten-fold dilution series in TSB to 10^{-9} .

Fish which had been injected with bacteria developed darker coloration and became quiescent six hours following injection. Gills were pale. The majority of the exposed fish succumbed to infection 24 - 78 h after exposure. Internally the principal pathologic sign was enlargement and liquefaction of the liver and kidney and petechiae of parietal and visceral peritoneum. Deep necrotic hemorrhages were observed within the myomeres, along with focal hemorrhages on the surface of the heart. Histologically, moderate to severe cardiac myopathy as well as renal necrosis. Acute hypodermal inflammatory foci extending deep into the muscle. At the point of injection and at various other areas of foci, severe myofibrillar necrosis was observed, the center of which contained agglomerates of sarcoplasmic debris, macrophages, nuclear basophilic remnants and fibrin, with bacteria scattered throughout. Necrosis of the kidney extended to the renal glomerulus, tubules and to the endocrine cells of the interrenal tissues.

Figure 1. Tissue concentration of vibrio following challenge

The largest number of organisms could consistently be recovered from tissues of exposed fish 8 - 12 h after exposure. At that time, organisms were most in blood, but 20 or more hrs after exposure, the bacterial numbers in the examined tissues were comparable.

With the exception of mean corpuscular hemoglobin value, the hematologic parameters of infected fish were significantly different from those of non infected fish (Table 2). Hematocrit values, total blood cells and leukocytes were lower in infected

fish.

Table 2. Hematologic parameters of uninfected and infected tilapia exposed to vibrio (20 determinations in each group of fish; mean with range in parentheses)

Parameter	Units	Uninfected	Infected	Significance ^b
Erythrocytes	10 ⁶ /ml	2.1 (0.92-2.4)	1.1 (0.8-1.6)	***
Leucocytes	10 ⁴ /ml	4.1 (3.2-4.4)	2.7 (1.1-2.9)	***
Hematocrit	%	30.1 (26.3-33)	18 (15-25)	**
MCH ^a	%	15.4 (12.2-16.3)	13.3 (10.1-14.1)	NS
Leucocyte differential				
Lymphocytes	%	78 (68-81)	72 (65-77)	*
Neutrophils	%	20 (18-24)	15 (12-18)	*
Monocytes	%	1 (0-3)	4 (1-4)	**
^a MCH = Mean corpuscular hemoglobin concentration				
^b Statistical significance				
*** p=0.001				
** p=0.01				
* p=0.05				
NS = not significant				

All clinical chemistry parameters of infected fish were significantly different from those of non-infected fish (Table 3). LDH and GOT activities were higher in infected fish, whereas total protein, albumen, chloride, sodium and osmolality were reduced. Considerable hyperglycemia was suggested with the plasma glucose values of infected fish 128% of the values of non infected fish.

In tilapia, vibrios are apparently sequestered in various tissues during initial stages of infection. Histologically microcolonies could be demonstrated in skeletal muscle, gills, in loose connective tissues and throughout the digestive tract. Skin and muscle tissues were necrotic in some heavily infected areas, but bacterial colonies without accompanying necrosis were also present. Vibriosis in tilapia would appear to be a disease in

which the agent is initially localized in select tissues -- perhaps to establish a chronic

Parameter	Unit	Uninfected	Infected	Significance ^a
Total Protein	g/dl	3.1 (2.6-3.3)	1.8 (1.6-2.2)	***
Albumin	g/dl	1.8 (1.3-2.2)	0.8 (0.6-1.3)	**
Chloride	mEq/L	120 (111-130)	110 (89-133)	*
Glucose	g/dl	88 (77-95)	110 (75-122)	**
Osmolality	mOsm	330 (290-345)	230 (225-280)	***
Potassium	mEq/L	4.1 (3.2-6.6)	6.2 (3.8-7.1)	***
Sodium	mEq/L	200 (180-220)	135 (121-192)	**
Alkaline phosphatase	IU/L	16.2 (11.4-17.1)	12.1 (7.2-14.3)	***
Lactate dehydrogenase	mU/ml	260 (199-320)	540 (415-600)	***
Glutamic oxalacetic transaminase	mU/ml	125 (100-151)	174 (115-220)	**

^aSee Table 2

Table 3. Serum chemistry values of vibrio infected tilapia (20 determinations in each group of fish; mean in parenthesis)

state. Septicemia and subsequent mortality may be dependent upon factors which compromise host defenses.

Hyperglycemia along with reduced electrolyte and liver function is suggestive of alterations of carbohydrate metabolism associated with gram negative endotoxemia (gram negative sepsis) as observed in human beings following acute septicemic bacterial infections. Reduced electrolyte- and plasma protein values are also suggestive of renal dysfunction, a suggestion which correlates with the histopathologic picture.

Immunization. Previous collaborative studies conducted at Texas A&M and the Hebrew University of Jerusalem have established that cultured tilapia are highly immunocompetent, hence immunoprophylaxis would appear an appropriate health management strategy for intensive tilapia production.

Antigens of *Vibrio anguillarum* recovered from diseased

tilapia. The development of an immunobiological for control of tilapia vibriosis proceeded along two lines in our studies: 1) characterizing antigenic profile- and 2) purifying antigenic components associated with virulence of agents involved in disease.

Antigenic profile. Cultures of the fish pathogenic agents were prepared in trypticase soy broth supplemented with 3% NaCl. After 20 h incubation 30 C, cells were concentrated 10 X by centrifugation in 0.85% saline and sub-divided into two aliquots. One aliquot was treated with formalin 2% (V/V final concentration of 40% formaldehyde) (formalin preparation) and the other autoclaved 120 C 30 min (autoclaved preparation). Rabbits and tilapia were immunized by parenteral injection with each of these preparations over a 5- and 12- week period respectively and the respective antisera used in crossed immunoelectrophoresis to identify antigenically active components. Isoelectric focusing and SDS-PAGE (Western Blotting) were used to characterize principal antigenic- and non antigenic components of the preparations. In general, formalin treatment resulted in fewer antigenic components in rabbits (3 components) and fish (1 component). The component which was antigenic for fish was similar to one of the components which was antigenic to rabbits. The autoclaved preparation yielded 5 components which were antigenic in rabbits and 3 of those five components were similar to components which were antigenic in fish. The formalin component which was antigenic in both fish and rabbit was identical to a component found in the autoclave preparation and had an isoelectric pH of 6.2 (common component). SDS-PAGE studies

suggested the molecular size of the common component to be approximately 56 kDaltons.

Virulence antigens. The virulence of many of the Gram negative agents are related to various colonization factors. Properties which facilitate the colonization event are associated with the cell surface. These bacterial properties can include the production of adhesions, outer membrane proteins, antiphagocytic carbohydrate capsules and paracrystalline arrays of proteins known as S layers on their surface. S layers are ideally situated to influence the outcome of a host-parasite relationship and S layers on pathogenic bacteria are being increasingly reported. During our preliminary antigenic studies of the tilapia vibrio, we observed a heat stable 56 kDalton antigenic component and hypothesized that the component may be analogous to S layer protein associated with the virulence of other pathogens.

Vibrio cells were harvested and washed once in 20 mM Tris hydrochloride (pH 7.4), then suspended in 10 ml cold Tris buffer. DNase and RNase were added, and the cells disrupted in a French press 18,000 lb/in². Unbroken cells were removed by centrifugation 5,000 x g 30 min 4 C. The cell envelope fraction was isolated by centrifugation at 32,000 x g 30 min 4 C. The envelope pellet was suspended in 1.0 ml 1% sodium lauryl sarcosinate in 20 mM Tris and incubated 20 min 23 C, centrifuged 32,000 x g 30 min 4 C and the process repeated 2 x (S- component). One aliquot of the S-component was compared electrophoretically to the common component by Grabar technique and another utilized to immunize rabbits.

Immunoelectrophoretic- and cross absorption studies utilizing common antigen preparations and rabbit anti-S- component serum revealed that the principal antigen of the common component was identical to the S- component.

Electron microscopy studies were conducted on whole cells and with S- component preparations. S- component preparations were concentrated by centrifugation 32,000 x g 30 min and washed once in Tris buffer. Whole cells were washed 2 X in Tris buffer. The preparations were drop mounted on carbon-Formvar coated grids and stained with 2% phosphotungstate (pH 6.8). On both whole cells and S- component preparations, sheet material with tetragonal patterning similar to that described for S- layers of pathogenic *Escherichia coli* and virulent *A. salmonicida* were observed. Hence, the S-component associated with the tilapia vibrio is similar to-, but differs from S-layer proteins observed with other fish pathogens in its larger size (56 kDaltons vs 50 - 52 kDaltons of other pathogens) and in its heat stability. We believe however, the vibrio S-component to be the principal component involved in the virulence of the pathogenic tilapia vibrio and one to which immunoprophylactic measures should be addressed.

Monitoring antigenic uptake. S-component was purified and mouse monoclonal antibodies prepared using Sp2 hybridoma methods (MCA). An indirect ELISA technique using the MCA was developed for detecting low levels of this immunogen in tilapia serum and body fluids. As little as 3 - 5 nanograms protein/ml of the antigenic component could be detected.

Figure 2. Antigen uptake following immersion

Two types of antigen uptake studies were conducted using the ELISA technique i.e. 1) bath and 2) oral antigen exposure. To assess uptake via bath exposure, fish were placed in a solution containing approximately 20 mg autoclaved bacterial protein/ L and absorption uptake dynamics studied over a 24 hr period. For this purpose, periodic serum samples obtained from the fish being bathed in the antigenic preparations were analyzed for levels of bacterial protein by the ELISA technique. Results suggest that significant

quantities of the immunogenic bacterial component is absorbed within one minute and maximum uptake occurs within ten minutes after being placed in the bath. Serum levels of the antigenic component were not shown to significantly increase even after 24 h in the bathing solution.

Figure 3. Antigen uptake following oral exposure

To assess uptake via the oral route, antigenic preparations consisting of heat killed bacterial suspensions were placed in gelatin capsules and administered orally to fish. Bacterial preparations microencapsulated with agarose and polyarabinose were also studied. Antigenic material could be detected in the serum 6 - 8 h after administering each of these preparations. Microencapsulation appeared not to have influenced the efficacy of antigen uptake. Studies thus focused upon incorporating killed bacterial preparations directly in tilapia rations to assess

efficacy of the preparations for immunizing fish orally.

Immersion vaccination. Thirty-, fourth generation tilapia (*S. mossambica*), originating from the Aquatic Animal Medicine Laboratory Texas A&M University 14- 16 cm total length were transferred into 400 L plastic maintenance units (Living Stream) and acclimatized two weeks prior to immunization. Bacterin was prepared from overnight cultures of the *V. anguillarum* originating from diseased tilapia used in antigenic characterization studies. Cells were harvested by centrifugation, washed 2X in saline and autoclaved 30 min 120 C. The water level of the fish holding maintenance units was lowered to approximately 40 L and the bacterin concentration added to a final concentration of 2 mcg/L. Fish were kept in the solution 30 min, the bacterin drained and 100 L fresh water added and drained 2X. Serum samples were obtained from 6 tagged fish/week for 16 weeks and the agglutinin titers ascertained via microtiter agglutination using fresh autoclaved washed cells of the homologous antigen.

Oral immunization. Preliminary studies revealed that autoclaved preparations at a concentration of approximately 5 mg antigen protein/Kg feed were not rejected by fish, whereas higher concentrations were rejected. A group of 30 fish were fed rations ad libitum 20 mg/Kg fish for 1 day. Afterwards, serum samples were taken on a weekly basis for 16 weeks from 6 fish/week. Serum was tested for agglutinins by microagglutination as described previously.

Figure 4. Comparative immune response via immersion/oral routes

Establishing a challenge dose. Thirty six fish were subdivided into groups of 6 groups, 6 fish/group and placed individually into 80 L aquaria for a 14 day acclimatization period.

Challenge material was prepared from an overnight broth culture of the agent. Cells were harvested by centrifugation, washed 2X in 0.85% and the turbidity adjusted to 1.0 O.D. units (595 mU wavelength). Ten-fold dilutions were prepared and the fourth-, fifth- and sixth- tubes diluted with equal volumes of saline. The number of bacteria/ml was estimated by placing 0.01 ml drops on the surfaces of trypticase soy agar plates, three drops

from each dilution.

Five tenths ml challenge material was administered intraperitoneally by injection. Fish were observed and deaths recorded for 10 days following challenge. Most of the deaths were observed by the seventh day, hence the LD₅₀ was established via cumulative mortality through the seventh day as 10^{4.6} cells/ml.

Combined immersion-/oral immunization. Thirty fish were immunized via immersion and six weeks later received oral antigenic preparations as described above. Serum was tested for agglutinins by microagglutination on a weekly basis from each of 6 fish.

Figure 5. Serologic response of tilapia resulting from immersion-followed by oral booster immunization

After 16 weeks (10 weeks following oral immunization) 30 "vaccinated" fish and 30 non vaccinated fish were challenged with 1- and 2 LD₅₀ live bacteria (1- and 2 x 10^{4.6} cells/ml respectively) via intraperitoneal injection and observed for 14 days.

Figure 6. Protective Immunity from immersion/oral booster protocol

Production of biologic and proposed immunization protocol. A proposed biologic production protocol consists of the following steps:

1. Prepare seed cultures in 3 - 5 ml trypticase soy broth and half strength trypticase soy broth on day one.

2. Six hrs after inoculating seed cultures (log phase culture), Gram stain, streak for purity and homogeneity. Transfer log phase cells to production broth and incubate 30 hrs 23 - 28 C.
3. Concentrate cells by centrifugation (yield = approx. 30 - 50 mg wet weight cells/L) resuspend in 0.02 original volume 0.85% saline.
4. Autoclave 120 C, 15 lb/in², 30 minutes.
5. Measure total protein concentration, adjust suspension to 200 mcg/ml.
6. Add and mix with rations to a ratio of 5 mg bacterin/Kg ration (=oral bacterin). Label, date and store under refrigeration for maximum of 2 months.
7. Dispense suspension in 100 ml aliquots (= 1 unit immersion bacterin) in sealed containers. Label, date and store under refrigeration for a maximum of 6 months.

A proposed immunization protocol consists of the following steps.

1. Add 1 unit (100 ml aliquot) immersion bacterin/ 10 L bath solution.
2. Fast fish 24 h prior to immunization. Transfer from hatchery into immersion tank containing sufficient bath solution to just cover fish.
3. After 20 minutes transfer fish to transport tank. Change bath solution after 5 cycles or sooner as needed. Flush with fresh water 2 X and repeat 1.
4. Six weeks after stocking, withhold rations 3 days and feed fish with oral bacterin at usual feeding rate.

Evaluation of Research Achievements. Project arose from the need to address a serious bacterial disease of one of the most important cultured food fish. The disease (vibriosis) was responsible for 20% - 40% losses of production fish in U. S. and Israel. Originally the etiology was believed to be *V. parahaemolyticus*, an agent also responsible for causing illness in

human beings. Subsequent studies of disease episodes in the U. S. and elsewhere revealed the etiology of tilapia vibriosis to be *V. anguillarum*. Clarification of the etiology of this serious disease was important both in terms of its significance to fish health and its implications to human health.

The observation that bacterial surface components may play a role in the virulence of tilapia vibrios and that the component(s) are apparently antigenic provide the basis for developing an immunoprophylactic control measure to the disease.

One of the shortcomings of currently available fish immunobiologics and associated immunization protocols relates to the fact that fish can only be immunized prior to stocking. The duration of immunity for many agents is probably not sufficient for the entire production season. Inasmuch as serologic responses were observed both with immersion- and oral immunogens, some efforts were directed toward the use of an oral "booster" after fish have been stocked. The study suggests the effectiveness of this approach.

General guidelines for preparation- and use of a tilapia vibrio bacterin were developed and presented in this report.