STREPTOCOCCAL INFECTION IN

CULTURED TILAPIA *OREOCHROMIS MOSSAMBICUS* PETERS

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Dedicated to my

beloved Parents

CERTIFICATE

Certified that the dissertation entitled **"Streptococcal infection in cultured tilapia *Oreochromis mossambicus* Peters"** is a bonafide record of work done by **Mr. Sandeep Kumar Mukhi** under our guidance at the Central Marine Fisheries Research Institute during the tenure of his **M.F.Sc (Mariculture)** Programme of 1997-1999 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.

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केरल के वलप्पु के बहु संबर्धन तालाबों में संबर्धन किए जाने वाला तिलापिया ओरियोक्रोमिस मोसाम्बिकस से रोगकारक स्ट्रेप्टोकोकस बाक्टीरिया जातियों के 24 स्ट्रेन्स का विघटन किया गया । इस पर परीक्षण फरवरी से जुलाई, 1999 तक की अवधि तक किया गया था । स्ट्रेप्टोकोकेसिया कुटुम्ब के सभी स्ट्रेनों को पहचानने का प्रयास भी किया गया । स्ट्रेप्टोकोकेसिय को पहचानने को उनका पोषण और प्लेट में डालकर विघटन किया था । इसके लिए चयन माध्यम (*Selective media*) के रूप में कानल फीकल स्ट्रेप्टोकोकल ऐगार (*KF - Agar*) का उपयोग किया था । सभी अभिकारक (*reagents*) और हाई-माध्यम (*Hi Media*) के थे और इसके लिए क्रिया-पद्धति की सभी मानक रीतियों का अनुपालन भी किया था ।

सभी आइसोलेट ग्राम पोसिटीव (*Gram +ve*) थे । ये गतिशील नहीं थे । काटलेस (*catalase*) और ओक्सिडेस (*Oxidase*) का उत्पादन बिलकुल नहीं हुआ था । जिन आइसोलेटों ने इन्डोल (*Indole*) फोर्मेशन नहीं दिखाया था उन में उच्च शक्कर अपघटन क्रिया (*Saccharolytic activity*)दिखाई थी । शत प्रतिशत आइसोलेटों ने स्टार्च का जल-अपघटन *Hydrolysis*) दिखाया था । अधिकांश स्ट्रेन्स हाइड्रजन सलफाइट का उत्पादन करने में असमर्थ हुए थे । जब कि सिर्फ दो स्ट्रेनों ने इसका उत्पादन किया था । विविध स्ट्रेनों के नाईट्रेट रिडक्शन में विविधता दिखाई पडी, आँखों के आइसोलेटों ले उच्च प्रोटीन अपघटन(*proteolytic*) दिखाई था पर इनका शकर अनघटन कम था । आंत्र में पहले ही जीवित बाक्टीकियों ने लाक्टोस का सक्रिय किण्वन(*fermentation*) करने पर भी प्रोटीन अपघटन कार्यालाप में मंद चाल दिखाया । अधिकांश स्ट्रेनों ने बी हीमोलाइसिस(*B-Haemolysis*) से अपनी विषाक्तता प्रकट की प्रतिजैविकी-संवेदन पैटेर्न(*Antibiotic sensitivity pattern*ने आम्पिसिलिन के मददे शतप्रतिशत प्रतिरोधन और स्ट्रेप्टोमाइसिन(*streptomicin 10µgm*) के मददे शतप्रतिशत संवेदनशीलता दिखायी । इन स्ट्रेनों की विषाक्तता और रोगजनक कारक साबित करने के लिए कोश्च पोस्टूलेट्स (*Koch's postulates*) किया गया था ।

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1. INTRODUCTION

INTRODUCTION

Disease is one of the most limiting factors in aquaculture, as in aquatic medium all natural barriers that exists between a pathogen and the host are broken down whenever animals are cultured artificially from their natural environment the animals are really under stress. This makes them more easily susceptible to pathogens and other diseases. The culture ponds are stocked heavily which is another reason for the stress, but the sole purpose of aquaculture is to produce a maximum yield in minimum time as we are in need of protein. Owing to the widespread loss caused by various pathogens it is important to study the pathogens, the disease caused by them and the methods to eliminate them from aquaculture ponds.

Approximately 10 families of bacteria has been identified as having members that can produce diseases in cultured animals. These are:

Cytophagaceae	-	Flexibacter
Myxococcaceae	-	Myxococcus
Pseudomonadaceae	-	Pseudomonas
Vibrionaceae	-	Vibrio
Mycobacteriaceae	-	Mycobacterium
Enterobacteriaceae	-	Edwardsiella
Cornebacteriaceae	-	Renibacterium
Streptococcaceae	-	Streptococcus
Bacillaceae	-	Clostridium
Nocardiaceae	-	Nocardia

Streptococcus spp. have been described from diseased fish in both fresh water and salt water in Japan and USA and in rainbow trout (*Salmo gairdneri*) from South Africa. Colonies of *Streptococci* isolated from internal organs usually the kidney or lesions and grow on Brain Heart infusion Agar(BHI) at 30^0C for 2-3 days.

Morphologically, they are small, viscous, raised white colonies in BHI. They can cause haemorrhagic septicaemia of kidney and internal organs in rainbow trout. Tilapia, are susceptible to *Streptococcus* spp. following injury to body surface. When temperature rose from 25^{0}C to 30^{0}C in freshwater a significant difference in susceptibility was found. Highest mortalities occurred at 25^{0}C- 30^{0}C in fresh water when salinity rose from 15 to 30 ppt or at 30^{0}C when salinity rose from 0 to 15 ppt. Greatest mortality of tilapia due to *Streptococcus* species was recorded at higher temperature and at higher salinity.

Streptococcus spp can cause lesions consisting of meningocephalitis, endopthalitis and mild myocarditis, splenitis and ovaritis. Different strains can cause similiar infections in *O. niloticus*. Cataract is also found to be caused by *Streptococcus* spp. but these appeared to be secondary to *Streptococcus* infection.

As there is a general lack of information regarding the pathways of streptococcal infection in aquaculture ponds of our country and the factors influencing the spread of infection, the present study was aimed to investigate the *Streptococcaceae* present in *Tilapia* sp. and to know the effect of environmental and bacterial factors that determine the virulence of a strain as an essential step towards evolving a scientific basis for protection and disease management measures.

2. REVIEW OF LITERATURE

REVIEW OF LITERATURE

In view of the enormous volume of published work on *Streptococcus* spp. it might be supposed that *Streptococcus* spp. are very prevalent both in marine and fresh water sediments and sediments will indicate the water quality of aquaculture ponds. Pathogenic *Streptococci* have been isolated from both freshwater and salt-water fishes in Japan as well as in America. However there was differences between Japanese and American strains in different biochemical tests. The Japanese strain was identified as Lancefield group D, whereas the American strain was identified as *S. agalactiae*. (Kusuda *et al.* 1978 a). A second strain of *S. iniae* has been recovered from an Amazon freshwater Dolphin (*Inia geoffrensis*). This isolate differed from the first isolate in its ability to produce acid from lactose but not salicin and its inability to hydrolyse esculin. (Pier *et al.* 1978). An epizootic occurred among eels (*Anguilla japonica*) from April through June 1973, in few farms in Kochi, Japan and the causative organism was found out to be *Streptococcus* sp. (Kusuda *et al.* 1978 b).

Epidemiological study on Streptococcosis of cultured yellow tail (*Seriola quinqueradiata*) by Kitao *et al.* (1979) indicated that yellow tail have abundant opportunities to be infected in all seasons by *Streptococcus* sp from seawater and mud. A faecal *Streptococcus* of Lancefield group D but which could not be identified as belonging to any of the recognized species within this group, was isolated from the spleen, liver and kidney of affected trouts. The disease resembled a haemorrhagic septicemia and appeared to be associated with intensification and condition of stress (Boomker *et al.* 1979). The microscopical observation of the

smears prepared from eyes, spleen, and blood from the infected rainbow trout indicated the presence of *Streptococci*. (Barham *et al.* 1979).

The pathogenicity of cultured ayu (*Plecoglossus altivelis*) and amago (*Oncorhynchus rhodurus* var. *macrostomus*) was due to haemorrhagic lesions of body surface, especially operculum, abdomens and anus, when infected with an unknown species of *Streptococcus* (Ohnishi *et al.* 1987).

The literature from 1981, consists of reports from fresh water infection by *Streptococcus* sp. Kitao *et al.* (1981) reported that *Streptococcal* infections have been frequently observed in cultured freshwater fish, tilapia (*Tilapia nilotica*), rainbow trout (*Salmo gairdneri*) and ayu. (*Plecoglossus altivelis*) at farms in various districts of Japan. But the causative agent was not identical to any strains of *Streptococcus* previously reported . Vgalin (1983) recorded *Streptococcal* infection in ayu at freshwater fish farms in Tochigi prefecture, Japan during 1980's. The species infected showed haemorrhages in the anal region and ventral body surface and secretion of abnormal slime on the gill.

Shiomitsu (1982) isolated *Streptococcus* sp. from the brain of diseased yellow tail *(Seriola quinqueradiata)*. Exopthalmus, protrusive lesions on the inside surface of opercle and caudal peduncle, and epicarditis were the symptoms seen in diseased animals.

The comparison of biochemical and serological properties of 286 strains of *Streptococcus* sp. isolated from cultured yellow tail (*Seriola quinqueradiata)* at various parts of Japan during the period from 1980 were investigated by Kitao (1982). It was confirmed that these strains possess very similar characteristics

according to the biochemical tests. Some cultural characteristics of *Streptococcus* sp were found similar to those of *S. faecalis, S. faecium* . *Streptococcus* sp. were presumptively identified by bile. esculin medium (growth in 40% bile and hydrolyzed esculin), eosin-methylene blue medium (no ferment lactose), a modified NaCl medium by QADRI (no change in colour) and the rapid hippurate hydrolysis test by HWANG (Negative reaction).

The characterization of *Streptococcus* sp. pathogenic to yellow tail was done by Kusuda *et al.* (1982a). The isolates showed growth at a range (Optimum) of 10-45°C. (20-37°C), 0-7% (0%) of NaCl concentration and pH 3.5-10.0 (7.6). Pathogenic *Streptococcus* was detected in seawater, bottom mud, and the intestine of fish such as chub mackerel (*Scomber japonicus)* and black scraper (*Navodon modestus*). The intestine was considered to be the principal site for the progress of the infection. The causative organism at the Streptococcal infection in cultured yellow tail has biological and biochemical characteristics intermediate between *Streptococcus faecalis* and *S. faecium (*Kusuda 1982 b).

Streptococci belonging to serotype group D were first isolated from carp gills attacked by gill necrosis. By artificial infections the characteristic symptoms of the disease appeared only occasionally after the pathogen was smeared on the gill. The *Streptococci* spp. are found to be incidental bacteria, present on the gills damaged by myxobacterial gill disease. The physiological–biochemical properties of these bacteria are described by Farkas and Olah (1984).

The pathogenicity of a non-haemolytic group B *Streptococcus* sp. in gulf killifish(*Fundulus grandis*) was tested by both injecting and dip treatment in

bacterial suspension. The lethal dose (LD_{50}) in IP injections were 1.4 x 10^4 at 96 hr. and 7.5 x 10^4 at 168 hr. No death occurred among fish dipped in bacterial suspension for different time periods or when the fish were exposed to a 50 ppt hyperosmotic solution prior to dip. No death occurred with oral administration of the bacteria(Rasheed *et al. 1984)*

The pathogenesis of bacterial septicaemia was studied in cultured tilapia experimentally infected with *Streptococcus* sp. by means of immersion and oral inoculation (Liu *et al.* 1990). More than five days was required for showing significant but non-specific lesions of septicaemia with the bacterial concentration of 10^{4-5}cells/gm. in organs in groups of oral inoculation and single bacterial immersion.

A procedure established for the selective isolation of the species of *Streptococcus* responsible for rainbow trout. Streptococcosis in South Africa consisted of the inoculation of samples into nutrient broth which had been supplemented with 100 µg/ml of nalidixic acid, 160 µg/ml oxolinic acid or 200 µg/ml of Sodium azide. After incubation the sample was plated on to tetrazolium agar on which the rainbow trout pathogenic *Streptococcus* species grew as a real colony. (Bragg 1989).

Park *et al.* (1987) isolated pathogenic *Streptococcus* sp. from cultured yellow tail (*Seriola quinqueradiata*). The disease occurred among zero-group cultured yellow tail during months of September to October 1985 in the Southern Coast of Korea. The symptoms were petechiae on the inside of the opercula and congestion of the pectoral fins. The species showed optimum salt concentration for growth was 0% and growth occurred in the concentration of 0-6%, optimum pH for

growth was about 8. These strains were sensitive to chloramphenicol, tetracycline, carbenicilline and erythromrycin but resistant to sulfisoxazole.

The first documented epizootic of Streptococciosis has been recorded among cultured hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) in Saudi Arabia by Al-Harbi (1994). Affected species showed erratic swimming, melanosis, exophthalmia, haemorrhaging around the jaws and at the base of pectoral and dorsal fins and tail and presence of ascetic fluid in the abdominal cavity. The causative agent was characterised biochemically, and identified as an α–haemolytic *Streptococcus* sp.

The cause of an ongoing mortality of hybrid tilapias(*Tilapia nilotica* x *T. aureus)* in a Texas fish farm was found out to be a biotype of *Streptococcus iniae* (Perera *et al.* 1994). The identification was based upon classical biochemical and physiological analysis as well as ribosomal DNA gene sequencing. The causative agent, a β-haemolytic *Streptococcus* species, grew better at 37° than at 10, 25, 40°C and growth was inhibited at pH 9.6, in 6.5% NaCl, and 40% bile. The bacterium was resistant to ampicillin, and furazolidone, but was susceptible to several antibiotics including tetracycline, oxyteracycline and sulfadimethoxine–ormetoprim (5:1). The external symptoms in diseased tilapia were loss of orientation, exophthalmia, corneal opacity and petechia around the mouth, anus, and proximal cavity and enlargement of liver, spleen and kidney were observed.

Two new Streptococcal species causing a meningoencephalitis in *Tilapia* spp. and trout (*Oncorhynchus mykiss*) appeared in Israel in 1986. They were Gr+ve, nonsporulating, facultatively anaerobic chain forming cocci, catalase negative. They

were able to grow at pH 9.6 but neither at 10°C nor at 45°C nor in the presence of 40% (vol/vol) bile salt or in the presence of 6.5% NaCl (wt/vol). The species were identified to be *Streptococcus shiloi* and *S. difficile* (Eldar *et al.* 1994).

The disease was reproduced experimentally in both these fishes. The LD_{50} of *S. shiloi* and *S. difficile* strains cultured "*in vitro*" (Two to three passages on BHI medium) were 10^7-10^8 .The virulence of these strains was increased (LD_{50} 10^2-10^5) after three passages "*in vivo*" (brain to brain passage) in fish without culture on agar plates. High virulent strain did not differ from low virulent strain by any identifiable extra-chromosomal elements.

Epizootiological aspects of *Streptococcus iniae* affecting tilapia in Texas was studied by Perera *et al.* (1994). Bacterial growth in Brain heart infusion (BHI) broth was detected at 10, 25, 35 and 45°C, between pH 5.5 – 8.5 and at salinities of up to 30 ppt. After suspension of the bacteria in distilled water, viable cocci were recorded for up to 5 days at 5°C and <24 hr. at 25°C. The 96 and 168 hr. LD_{50} in 30-90 gm fish was found to be 4.9×10^5/gm and 3.15×10^5/gm colony forming unit (Intraperitonial injection). Artificial infection was achieved through oral administration as well as by dipping fish in water containing the bacterium. Greater rates of mortality were observed in artificially infected tilapia maintained at 20°C than those kept at 15, 25, 30 or 35°C and in water of pH 9 compared with fish maintained in more acidic water.

The *Streptococcus* spp. isolated from healthy and diseased tilapia hybrids (*Oreochromis niloticus* x *O. aureus*), healthy Carp (*Cyprinus carpio*), diseased mullet (*Mugil cephalus*) and striped hybrid bass were divided into two groups *i.e.*

1.α.haemolytic, mannitol +ve. 2. γ haemolytic, mannitol –ve. Later the disease was produced experimentally in tilapia hybrids to investigate the effect of low dissolved oxygen levels and high nitrate. The diseased tilapia had a significantly lower haematocrit ($P<0.05$) and higher buffy coat ($P<0.01$) than the healthy fish. *Streptococcus* spp. are opportunistic pathogens because they are widespread in the aquaculture environment and because of their dependence on stress to assert pathogenity (Bunch and Bejerano 1997).

Nile tilapia (*O. niloticus)* and Channel catfish (*Ictalurus punctatus*) were experimentally infected by immersion with three isolates of *Streptococcus* sp. from diseased fish. Histopathology of these fish revealed meningitis, polyserotitis of heart, liver, spleen, ovary and kidney, splitis ovaritis, and myocarditis. (Chang *et al.* 1996).

In summary, it can be stated that the knowledge of *Streptococcus* infection and pathogenicity in fish has accumulated slowly from captured fish to cultured fish and progressed like any other discipline. Periodic reports of the pathogenicity resulting from fortuitous discovery with "short-term" concentration of special haemolytic groups marked the general trend of historic development, of Streptococcal infections in fish which are prognostic to the future developments to be expected.

3. MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. AREA OF STUDY

The present study was conducted during March-July 1999. The study area is located along 9^0 55^1- 10^0 10^1 N and 76^0 10^1 - 76^0 20^1 E. (Fig.1). In the brackish water polyculture ponds, along with tilapia other finfishes like, mullets, milkfish, *Etroplus* and shellfish like *Penaeus indicus* are cultured. The culture practice followed is modified extensive method, in which fishes are fed naturally and water exchange is influenced by tidal influx and outflow. The ponds are stocked with seeds available in the backwater region.

The area of the ponds is around 0.8 -1 ha with 1.5 mts. depth having very fertile clayey soil.

3.2. COLLECTION OF SAMPLE:

Monthly samples were collected at 0800-1000 hr invariably in all sampling dates. The fish sample (tilapia) were caught by cast netting, and kept in sterile polythene cover in live condition. Water and sediment samples were collected for quantitative estimation of total heterotrophic bacterial number. Water sample was collected in a sterile plastic bottle. Care was taken to collect the sediment and water following aseptic procedure.

The samples were transported immediately to the bacteriology laboratory at Central Marine Fisheries Research Institute, Cochin for bacteriological investigation within 2hrs of sampling.

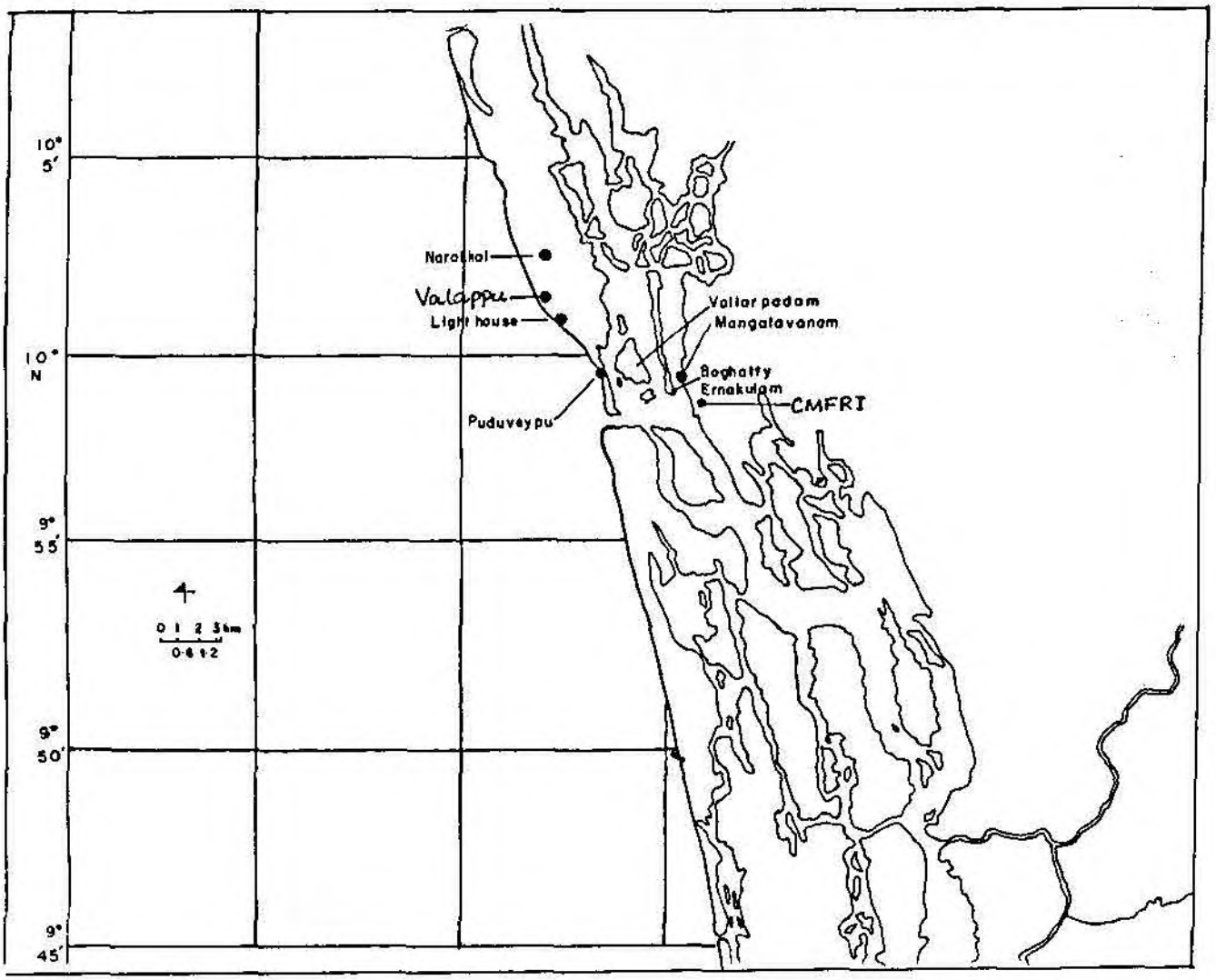


Fig. 1: Map showing the sampling station.

3.3. BACTERIOLOGICAL INVESTIGATION

Bacteriological investigations were carried out both for quantitative and qualitative analysis of heterotrophic bacteria as well as the pathogenic *Streptococcus sp.*

For isolation of total heterotrophic bacteria & pathogenic *Streptococci,* samples were taken from different parts of the diseased tilapia i.e. eye(exophlthalmic condition), gill, intestine, skin, (inflammation). Quantification of heterotrophic and pathogenic bacteria was done also from the samples of sediment and water.

3.3.1. QUANTITATIVE ANALYSIS

3.3.1.1. Quantification of Heterotrophic Bacteria.

Estimation of total viable heterotrophic bacteria was done by serial dilution technique and pour plating method (Rodina, 1972).

Approximately 1g of the sample (eye, gill, intestine, skin, sediment or 1ml of water sample) was aseptically transferred to a sterilised glass mortar, grounded well with pestle and mixed with 99 ml of sterile aged water. Then the various samples were transferred to conical flask containing aged sterile sea water and shaken for 30 minutes, at 160 rpm in a bacteriological shaker (ORBITEK). After thorough shaking serial dilutions were made according to the standard procedure. 1 ml of the inoculum was transferred to 10 cm diameter sterile glass petridishes and pour plated by using Zobell's Marine Agar (HiMedia). Then the plates were incubated at room temperature (RT) for 7 days.

3.3.1.2. Quantification of *Streptococcus* sp.

Quantification of pathogenic *Streptococcus* sp. was done by using a selective media. (Kennel Faecal Streptococcal Agar). The composition of KF-Agar is as follows.

Peptone – 1g; Yeast extract – 1 g; Sodium chloride – 0.5 g; Maltose – 2g

Sodium glycerophosphate–1 g; Lactose– 0.1 g; Na-Azide–0.04 g; Agar– 2g.

These ingredients were dissolved in distilled water by boiling and sterilised by autoclaving at 15 lb. for 10 minutes and aseptically added 1 ml of 1 % Triphenyl Tetrazolium Chloride (TTC) to the sterilised medium . The samples were streak-plated in KF agar and the number of colonies are counted after 48 hrs of incubation at RT.

3.3.2. ISOLATION OF PATHOGENIC *STREPTOCOCCI*

The isolation of *Streptococci* was done by three methods.

(I) Enrichment and streaking on selective media. i.e. KF agar

(II) Swab from the infected part on KF agar.

(III) Direct streak plating on KF Agar.

I . Samples from the different parts of the body were incorporated directly into the Streptococcus Enrichment Broth (SE-Broth) aseptically. The SE-Broth composed of, Casein – 2g; Beef extract –0.5 g; Bile Salt – 1g; Sodium chloride –0.5 g; Na-Citrate- 0.1 g; Na-Azide – 0.025g; D.W – 100 ml, sterilised at 15 lb. for 20 min. After 24 hrs of incubation a loopful of broth streaked over KF-Agar to get the isolated colonies.

II. Swab was taken from the infected part of the body and streaked over KF-Agar. The counts were taken in 24 and 48 hrs of incubation at RT.

III. Isolation of the pathogenic *Streptococcus* sp. can be done by following the method described earlier.

After isolation, selected bacterial colony was sub-cultured in peptone water and kept as stock culture. Subculture was also done at different stage of study to carry out different experiments.

3.4. MEASUREMENT OF HYDROLOGICAL PARAMETERS

3.4.1. TEMPERATURE

The temperature of water was measured in the field by using precision mercury thermometer with an accuracy of $\pm 0.1^0$C.

3.4.2. SALINITY

The salinity of the water sample was estimated by using the formula,

$$\frac{V_2 \times S}{V_1}$$

Where:

V_1 = Vol. of Silver nitrate for 10 ml of Standard Sea water.

V_2 = Vol. of Silver nitrate for 10 ml of sample.

S = Salinity of Standard Sea Water (35 ppt)

3.4.3. DISSOLVED OXYGEN (DO_2)

Dissolved oxygen was measured by Winkler's method. Using Winkler A (Manganese chloride) and Winkler B (Sodium hydroxide + Potassium iodide). The water sample was fixed at the site of sample collection by using Winkler A & Winkler B solution. The value of DO_2 was estimated by titration against Sodium thiosulphate solution.

$$\text{The amount of } DO_2 \text{ (ml/l)} = \frac{V \times N \times 1000 \times 8 \times 1.01}{1.429 \times 100}$$

Where

V = Titre value

N = The Normality of Sodium thiosulphate

8 = Equivalent weight of oxygen

1.01 = Correction factor

1.429 = Weight of 1 ml of oxygen.

100 = Volume of sample taken.

3.4.4. pH

pH of the water sample was determined by using pH electrode .

3.5. MORPHOLOGICAL CHARACTERISATION

3.5.1. SIZE

The approximate size of the colony was measured

3.5.2. ELEVATION:

The colonies were examined to see if they are elevated or flat.

3.5.3. COLOUR:

The colour of colonies was also noted.

3.5.4. MARGIN:

Margin of the colonies was noticed to find out, if it is uniform or wrinkled.

3.5.5. MOTILITY:

Motility of the bacteria was checked by "Hanging drop method".

3.5.5.1. Hanging Drop Method:

A drop of young culture was transferred to the center of the cover slip. A cavity slide was inverted over the cover slip so that the drop of culture will be

hanging in the center of the cavity. Microscopic examination was carried out to find the motility.

3.5.6. GRAM'S STAINING

Gram's staining is a differential staining procedure, requiring a primary stain and a counter stain. A thin smear of the culture prepared and air-dried and then heat fixed .The primary staining was with crystal violet followed by the addition of a mordant-iodine. It combines with the primary stain forming a crystal violet iodine complex. In the Gram staining procedure, Gram +ve organisms do not retain the primary dye, if iodine is omitted. After decolorizing with 95% alcohol, saffranin (Counterstain) added to the smear. By using this procedure, it is possible to divide the bacteria into Gram +ve, Gram-ve, Gram variable and Gram unreactive.

3.6.BIOCHEMICAL TEST

Biochemical tests were done for identification and characterisation of the bacteria. Pure cultures of the bacteria was maintained (after isolation from the selective media, KF-Agar) in peptone broth medium at RT. This pure culture helps in proceeding with different biochemical tests as follows .

3.6.1. CATALASE TEST:

Hydrogen peroxide (H_2O_2) was taken in a test tube, and a loop containing bacterial culture was dipped into it. The catalase +ve reaction shows a dense bubble comming out of the loop, whereas the –ve reaction fails to form the oxygen release.

3.6.2. OXIDASE TEST:

This test is done to detect the presence of certain respiratory enzymes like cytochrome oxidase in the bacteria that will catalyse the transport of electrons

between the electron donors in the bacteria and a redox dye . This dye is reduced to a deep purple colour.

For conducting the test, a filter paper strip in which a little reagent (freshly prepared 1% N^1,N^1,N^1 - tetramethyl paraphenylene diamine dihydrochloride) was poured, was streaked with a loop. Immediate appearance of a deep purple colour indicated positive reaction for the presence of oxidase enzyme.

3.6.3. *HAEMOLYSIS TEST*

This test was done to find out the haemolytic capability of the isolate. This experiment was done with goat blood, taking the basal medium either Blood-Base Agar or Tryptone Soya Agar.

3.6.3.1. Collection of blood

The goat blood was collected in a sterilised conical flask from slaughter house early in the morning . The anticoagulant used was Alsever's solution.

Composition of Alsever's Solution

NaCl	–	2.1 gm
Tri-Sodium citrate	–	4 gm
Citric Acid	_	0.275 gm
Glucose/Dextrose	_	10.25 gm
D.W.	_	500 ML

*500 ml Alsever's Solution in 200 ml of blood.

3.6.3.2. Media preparation & plating

The freshly collected blood was added to sterilise the medium (BBA/TSA), when it was at 45-50^{0}C at the rate of 7% v/v.

Pour plating or streaking of the pure bacterial culture was carried out in the sterilised petriplates. The plates were observed after 24 hr to find out the type of haemolysis.

Alpha haemolysis (α) : An indistinct zone of partial destruction of red blood cells around the colony.

Beta haemolysis (β) : A clear colourless zone around the *Streptococcus* colonies in which the blood cells have undergone complete dissolution.

Gamma haemolysis (γ): No apparent haemolytic activity or discolouration produced around the colony.

3.6.4. "MR-VP" TEST

"MR-VP" broth (HiMedia) was inoculated with the bacterium and incubated for 5-7 days at room temperature and then the 'Methyl Red' and 'Voges-Proskauer' reactions were carried out separately.

3.6.4.1 'Methyl Red' Test.

5 drops of 0.04% solution of methyl red are added to the cultured 'MR-VP', mixed well and read at once. A red colour is +ve while yellow colour signifies a –ve test.

3.6.4.2. 'Voges-Proskauer' Test.

0.6 ml of 5% solution of α naphthol in ethanol and 0.2 ml of 40% KOH was added to 1ml of 'MR-VP' cultured broth. Pink colour in 2-5 ml and deepening to magenta or crimson in 30 minutes indicates VP +ve & -ve if it remains colorless.

3.6.5. "OXFERM" (O/F) TEST / HUGH & LEIFSON'S TEST

This experiment was carried out in a media known as H & L medium, whose composition as follows. Peptone-1%; Sodium chloride-0.5%; Glucose-1%; Agar-agar-0.3%; Distilled water – 100ml; Phenol red – 1 cc/100 cc of 0.1% solution; K_2HPO_4-0.3%.

Fermentative reaction was carried out in anaerobic condition by closing the surface of the medium in the test tube with sterile liquid paraffin after inoculation. The positive reaction showed the colour change from red to yellow.

3.6.6. CITRATE UTILISATION

The citrate utilisation was found out by inoculating the cultures on slopes made from Simmon's Citrate Agar, having the compositon, Magnesium Sulphate-0.2 g; Ammonium Phosphate – 1g; Dipotassium phosphate – 1g; Sodium Citrate – 2g; Sodium chloride –5g; Agar-agar-15 g; Bromothymol blue –0.08g; pH 6.8 ± 0.2.

After incubation period at RT the positive reaction (utilisation of citrate) is shown by the development of the blue colour in the medium.

3.6.7. H_2S PRODUCTION TEST

L-cystine, the sulphur containing amino acid was prepared in liquid broth along with peptone, sodium chloride and beef extract for detection of H_2 S production. The test organisms were inoculated in the liquid medium and lead acetate paper impregnated with saturated lead acetate solution was kept at the top of the tube. The culture was incubated for 7 days at room temperature and blackening of the lead acetate paper was noted.

3.6.8. INDOLE PRODUCTION TEST

The tryptone broth medium was inoculated with one loopful of 24 hrs nutrient broth culture. Then the culture was incubated for 2-4 days. Two days are ordinarily sufficient if good growth is obtained. Then 5cc of Kovac's solution was added to the tube. The appearances of a deep cherry red ring in the reagent layer indicated the presence of indole.

3.6.9. NITRATE REDUCTION TEST

The ability to reduce nitrate was tested in an ordinary peptone broth containing 0.3% potassium nitrate and inoculated with the culture. Turbidity was checked after the incubation period and the nitrate reduction was tested with reagent, alpha naphthylamine, reagent A; sulphanilic acid, reagent B

3.6.10. TOLERANCE TEST.

3.6.10.1. Tolerance to salinity. (0% & 6.5%)

The bacteria was inoculated to the test tubes containing 0% and 6.5% Nacl in peptone broth (Peptone –1%) incubated for 24 hrs at RT. The cloudy appearance indicated the growth in that medium.

3.6.10.2. Tolerance to temperature (10^0C & 45^0C)

The isolate was inoculated into the peptone broth and incubated for 24hrs at different temperature i.e. for 10^0C it was incubated inside the refrigerator when regulated to that temperature and for 45^0C it was incubated in the BOD–incubator maintained at that temperature.

3.8.10.3. Tolerance to Bile salt & Na-Azohydrate .

Peptone water broth containing 40% Bile in one tube and 0.02% Na-Azohydrate in other tube were incubated with the bacteria and incubated for 24 hr at RT and the result was noted.

3.6.10.4. *Biophysical Characterisation:*

The optimum temperature, pH and NaCl concentration for growth were determined by finding the optical density (OD) in a spectrophotometer at 686 nm from the peptone culture broth. The different pH range used were 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, the salting range were 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7.0% (W/V) NaCl in peptone water broth. To determine optimum temperature the media was incubated at 5, 10, 15, 20, 25, 30, 35, 40, and 45^{0}C.

3.6.11. HYDROLYSIS TEST

3.6.11.1. Hydrolysis of Starch.

The starch medium (soluble starch – 0.2%; Beef extract –0.3%; Agar-agar-1.5%; Sea water-100 ml; pH –7.2) was prepared and sterilised and poured into plates . The test culture was streaked on the medium and incubated at RT. To conduct the test, the plates were inverted and a few iodine crystals were placed on the petridish cover and slightly warmed. Clear area in the medium showed the starch hydrolysis by extra-cellular enzyme production by *Streptococci.*

3.6.11.2. Hydrolysis of Gelatin.

The gelatin medium (peptone – 1%, Beef extract – 0.2%; Gelatin – 12%; Sea water – 100 ml) was sterilised by tyndallisation by keeping the medium in

streaming steam for three consecutive days. Then the medium was inoculated heavily with the test organism by stab-inoculation and incubated for 96 hrs.

After the incubation period the hydrolysis of gelatin was indicated by "liquefaction" of the gelatin . Test cultures incubated at RT were chilled until the control solidifies before observations on liquefaction were made.

3.6.11.3. Hydrolysis of Casein.

Sterilised casein medium (Peptic digests of animal tissue –10 g/lt.; Sodium chloride – 5g/lt; Casein-1%) was poured into petriplates and streaking of inoculum was done after the media solidifies. The 'halo' space surrounding the colony indicated the hydrolysis of casein, after 24 hrs of incubation.

3.6.11.4. Hydrolysis of (L) – Arginine

The medium (Peptone – 0.5g; Yeast extract –0.5 g; K_2HPO_4-0.2g; L-Arginine mono hydrochloride –0.3 g; Dextrose –0.05g, Sea water – 100 ml) was distributed in test tubes and sterilised at 10 lb. / 10 min. Then they were inoculated with the bacteria. After 48 hrs. incubation a few drops of Nessler's reagent were added. Brown precipitate indicated hydrolysis.

3.6.11.5. Hydrolysis of Aesculin

The Aesculin medium (peptone – 1g; Sodium chloride – 0.5 g; Aesculin – 0.1g; Ferric citrate – 0.05g; Distilled water – 100 ml) was distributed in tubes and sterilised at 10l/10 min. Then they were inoculated with the test culture. The blackening of the medium after 2-7 days of inoculation indicated hydrolysis +ve.

3.6.12. SUGAR FERMENTATION /ACID & GAS PRODUCTION TEST

The sugar medium (Andradeis peptone water, Hi Media) to which 1% of the respective sugar was added, was sterilised at 10lb/10min. After sterilisation the medium is inoculated and incubated for 24 hrs. The change of colour indicated sugar fermentation. The different sugars used in this experiment were, Lactose, Meso-Inositol, D-Raffinose, Mannitol, Maltose, D-Glucose, Inulin, Sucrose, L(+) Arabinose, Sorbitol, Trehalose, Rhamnose.

3.6.13. ANTIBIOTIC SENSITIVITY TEST

Antibiotic sensitivity study was conducted with young culture (24 hrs) to find out the resistance of *Streptococcus spp.* to various antibiotics. The test cultures were swabbed on the Muller-Hinton agar or Antibiotic agar media and the discs (Hi Media) were placed on the surface of the medium and the plates were incubated. The antibiotics discs were of different concentration i.e.

Amoxycillin (Am)-30 mcg; Ampicillin (A) – 10 mcg; Bacitracin (B)- 10 units; Chloramphenicol (C) – 30 mcg; Chlortetracyline (Ct) – 30 mcg; Erythromycin (E) – 15 mcg; Gentamycin (G) – 1 mcg; Kanamycin (K) – 30 mcg, Neomycin (N) – 30 mcg; Novobiocin (Nv) – 30 mcg; Oxacillin (Ox)-10 units; Oxytetracycline (O) – 30 mcg' Penicillin (P) – 10 units; Polymyxin B (Pb) – 300 mcg; Streptomycin (S)- 10 mcg; Tetracyline (T) – 30 mcg;

The antibiotics diffuses through the agar occupying a circular zone around the original spot. The bacteria grow on the agar surface in all places except in circular zone where the antibiotic is present. Size of the zone is related to concentration of the antibiotic.

The size of the 'halo' was noted and mentioned as Sensitive (S) or Resistant (R) after comparing with the standard recommended table.

3.7. CHALLENGE STUDY

3.7.1. EXPERIMENTAL SET UP.

To carry out the etiological study tilapia specimens of equal size (40-50 gm) were collected from uninfected ponds. Subsequently they were acclimatised to the artificial condition (plate No.11). The salinity was maintained at 14 ppt. They were stocked in plastic container at the rate of 2-3 fishes per each tank aerated properly, and fed with artificial feed and the faecal matter was removed everyday. Enough care was taken to give best suitable environment condition to the live specimens.

3.7.2. SAMPLE PREPARATION & INJECTION

Few strains of sub-cultured isolates in their logarithmic phase were centrifuged at 600 rpm and then washed 2-3 times by sterile saline. Each tilapia was injected intraperiotoneally with *Streptococci* at the rate of 10^7-10^9 cells/ml. Each strain was injected into each of 30 fish. Ten control fish were injected with sterile saline.

3.7.3. OBSERVATION

Infected fishes were examined, the time of death was noted. The gross symptoms at the time of death were also noted. The pathogenic *Streptococci* was again isolated from these infected fishes and biochemical tests were conducted to prove Koch's postulates.

4. RESULTS

RESULTS

4.1. SIGNS AND GROSS PATHOLOGY IN *Oreochromis mossambicus*.

Most of the infected fishes showed lesions in different parts of the body i.e. around the jaws and base of dorsal and anal fin. Fin erosion was one of the most occured symptoms in the infected fishes. Exophthalmic condition and corneal opacity were common features in the affected tilapia (Plate 1&2). Haemorrhaging of internal organs were not apparent in cultured fishes obtained for bacteriological investigation.

4.2.PHYSICO-CHEMICAL PARAMETERS

The physico-chemical parameters of the pond studied through out the experimental period is presented in the table (Table No.2)

4.3.QUANTIFICATION OF BACTERIAL POPULATION

4.3.1.Total heterotrophs:

The total plate count (TPC) of heterotrophic bacteria was retrieved using Zobell's agar (HiMedia) following standard procedure. The counts ranged in various parts of tilapia were as follows.

Eye	:	$(36\text{-}160)\times10^3$/gm
Gill	:	$(45\text{-}106)\times10^5$/gm
Intestine	:	$(52\text{-}98)\times10^7$/gm
Skin	:	$(42\text{-}76)\times10^6$ gm
Sediment	:	$(10\text{-}49)\times10^5$
Water	:	$(15\text{-}20)\times10^4$/500ml.

The intensity of bacterial count is presented in intensity chart (Fig.2)

TABLE –1

SERIAL DILUTION

Tube No.	1	2	3	4	5
Dilution	1/10	1/100	1/1,000	1/10,000	1/1,00,000
Vol. of original fluid (ml)	1	0.1	0.01	0.001	0.0001
Dilution Factor	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}

TABLE –2

PHYSICO-CHEMICAL PARAMETERS

PARAMETER / MONTH	TEMPERATURE (^{0}C)	SALINITY (ppt)	DO_2 (ml/l)	pH
MARCH	30	16.06	4.81	7.97
APRIL	31^{0}C	17.56	3.40	7.61
MAY	33^{0}C	18.79	4.9	7.59
JUNE	29^{0}C	3.13	4.02	8.12

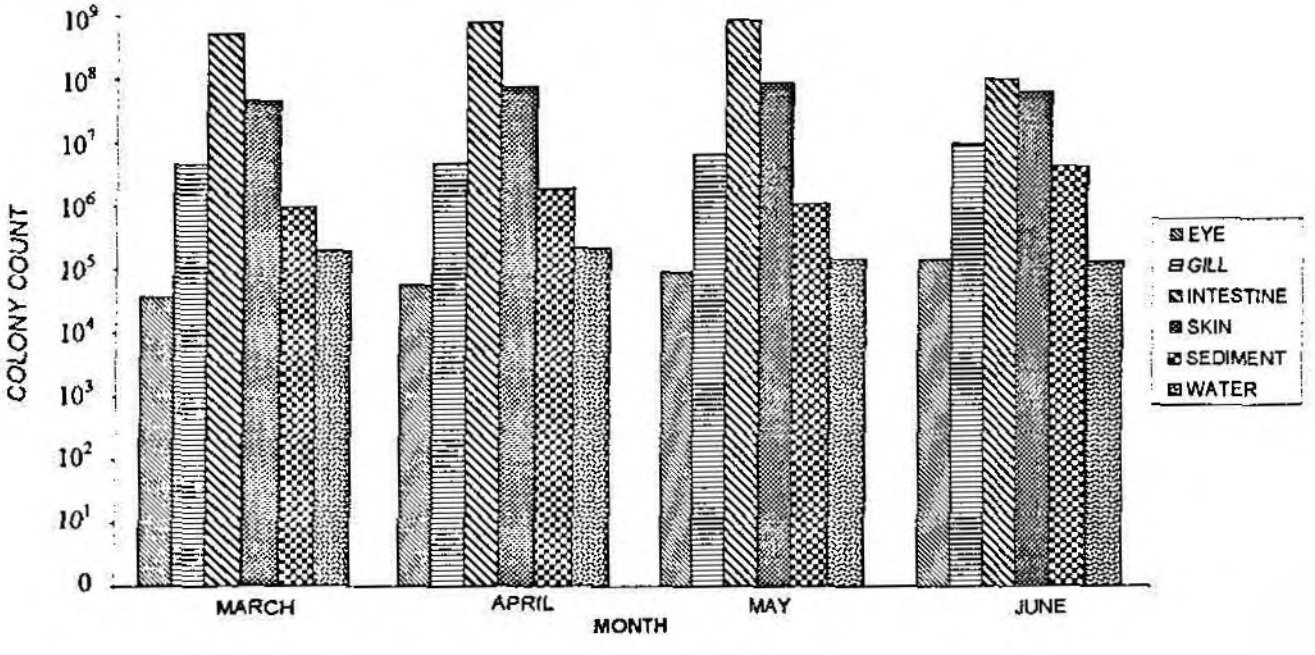


Fig No.2: Intensity chart of TPC during March to June '99 in various parts of tilapia.

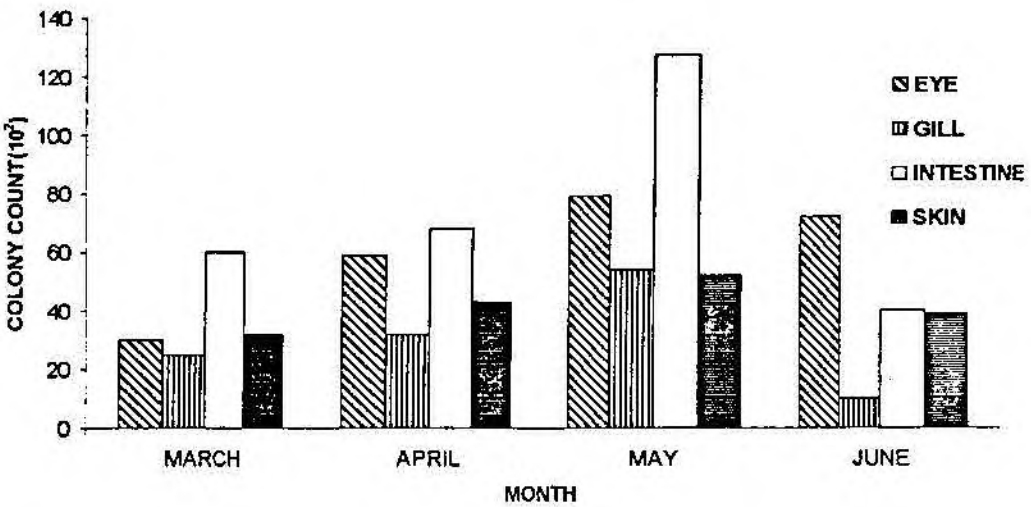


Fig No.3: Pathogenic *Streptococci* distribution during the months of March to June '99 in various parts of tilapia.

Plate – 1: Corneal opacity in tilapia due to Streptococcal infections

Plate – 2 : Fin rot in infected tilapia.

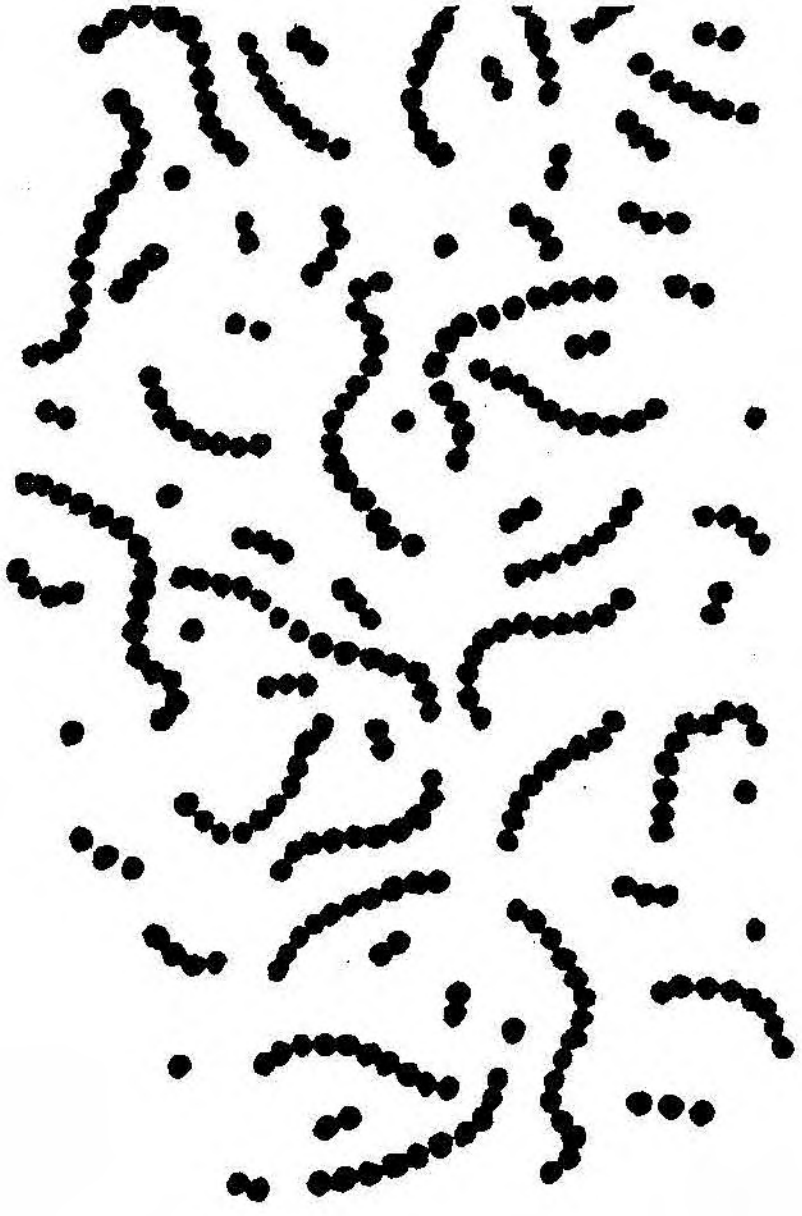
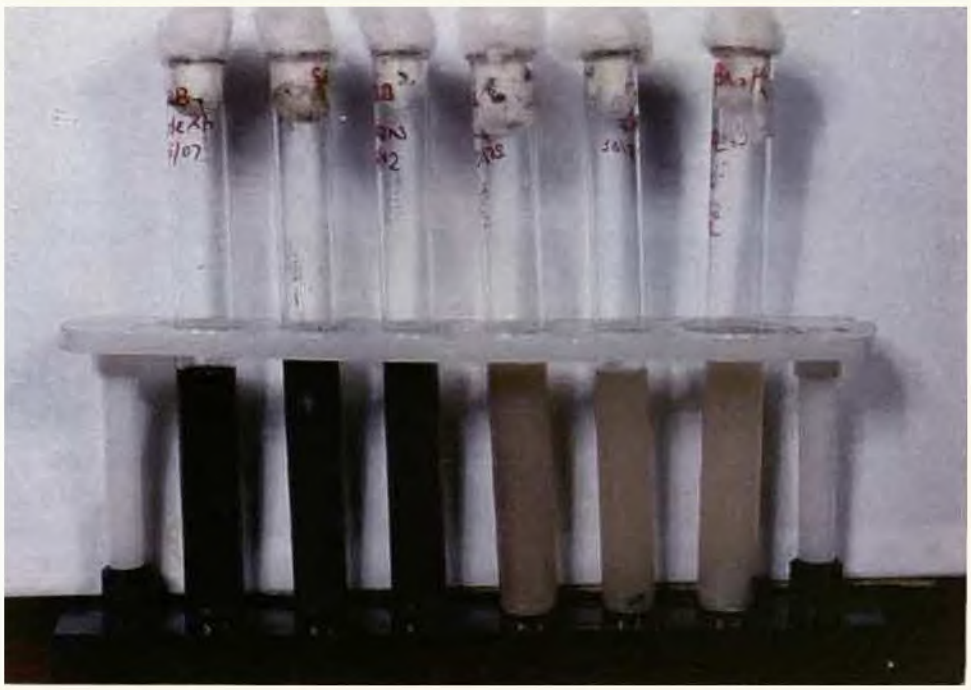
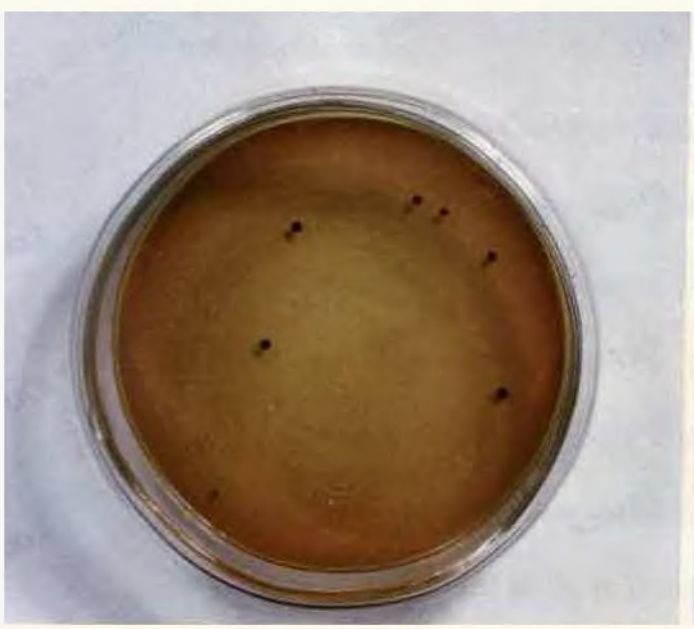


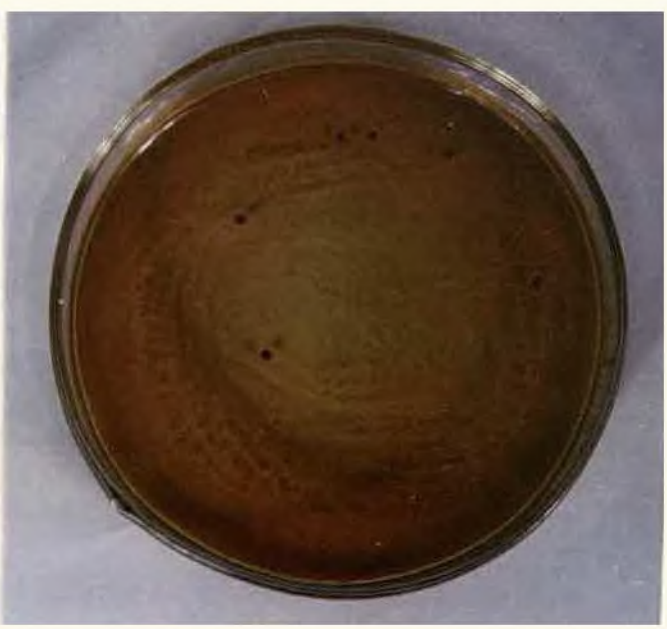
Fig. 4 : Streptococcal cell morphology

Plate 3 : Enrichment of samples in SE-Broth

Plate – 4 : Isolated colonies of *Streptococci* on KF-Agar.







4.3.2. Pathogenic *Streptococci*

The number of pathogenic *Streptococci* was enumerated using the selective media KF-agar during the month of March to June '99. The intensity of *Streptococci* in different parts of tilapia are.

Eye	:	$(30\text{-}79)\times10^2$/gm
Gill	:	$(10\text{-}54)\times10^2$/gm
Intestine	:	$(40\text{-}127)\times10^2$/gm
Skin	:	$(32\text{-}052)\times10^2$/gm

The intensity chart of *Streptococci* during the observation is presented on Fig-3.

4.4. MORPHOLOGICAL CHARACTERS

The size of colony on KF-agar ranged from 0.5 to 6 mm after 48 hrs of incubation. They were pinkish red or deep red in colour, raised and circular (Plate-4,5&6). Some colonies were of entire margin and some were irregular margin. The cells were Gram-positive, cocci, spherical or oval cells arranged in pairs or chains (Fig-4) of varying length each cell approximately 1μ in diameter, non-motile and non-sporeforming.

4.5. BIOCHEMICAL CHARACTERS

Most of the isolates showed β-haemolysis(Plate-7&8) but few showed both α/β haemolysis(Plate-9&10) whereas 2 strains were non-haemolytic. All are highly oxidative and fermentative (O/F) in glucose utilisation and negative for citrate utilisation, H_2S production (except 2 strains) and indole production. The tolerance to salinity and temperature was variable for different strains, but all the strains were sensitive to 40% bile and could tolerate 0.02% Na-azohydrate. All the strains were found to be hydrolytic to starch, esculin (except 2 strains) and arginine. Few strains were able to hydrolyse

TABLE -3

1st.CYCLE(MARCH)

SL. NO.	TESTS	EYE	GILL	INTESTINE	SKIN	SEDIMENT	WATER
1	Gram Strain	+	+	+	+	+	+
2	Motility	-	-	-	-	-	-
3	Catalase production	-	-	-	-	-	-
4	Oxidase production	-	-	-	-	-	-
5	Haemolysis	β	β	β	β	β	β
6	MR-VP Test	+/-	+/-	+/-	+/-	-/-	-/-
7	O/F – Reaction	+/+	+/+	+/+	+/+	+/+	+/+
8	Citrate utilisation	-	-	-	-	-	-
9	H_2S production	-	-	-	-	-	-
10	Indole production	-	-	-	-	-	-
11	NO_3 Reduction	-	+	-	+	+	+
12	Urease	-	-	-	-	-	-
13	Tolerence Test						
	0% NaCl	N	N	N	N	N	N
	6.5% NaCl	+	+	+	+	+	+
	$10^0C/45^0C$	- / N	- / N	- / N	- / N	- / N	- / N
	9.6 pH	N	N	N	N	N	N
	40% Bile	-	-	-	-	-	-
	Na– Azohydrate (0.02%)	+	+	+	+	+	+
14	Hydrolysis of						
	Starch	+	+	+	+	+	+
	Gelatin	-	-	-	-	-	-
	Casein	-	-	-	-	-	-
	Esculin	+	+	+	+	+	+
	(L) Arginine	+	+	+	+	+	+
15	Acid production from						
	Lactose	+	+	+	+	-	-
	Meso inostitol	-	-	-	-	-	-
	D-Raffinose	-	-	-	-	-	-
	Mannitol	+	+	+	+	+	+
	Maltose	+	+	+	+	+	+
	D-glucose	+	+	+	+	+	+
	Inulin	-	-	-	-	-	-
	Sucrose	+	+	+	+	+	+
	L(+) Arabinose	-	-	+	-	-	-
	Sorbitol	+	-	-	-	-	-
	Trehalose	+	+	+	+	-	-
	Rhamnose	+	-	-	-	+	+
16	Sensitivity/Resistance test						
	Ampicillin	S	S	R	R	R	R
	Chloramphenicol	S	S	S	S	S	S
	Gentamycin	S	S	S	S	S	S
	Erythromycin	R	S	S	S	S	S
	Penicillin	S	S	S	R	R	R
	Streptomycin	S	S	S	S	S	S
	Tetracycline	S	S	S	S	S	S

TABLE – 4

2nd CYCLE (APRIL)

SL. NO.	TESTS	EYE	GILL	INTESTINE	SKIN	SEDIMENT	WATER
1	Gram Strain	+	+	+	+	+	+
2	Motility	-	-	-	-	-	-
3	Catalase production	-	-	-	-	-	-
4	Oxidase production	-	-	-	-	-	-
5	Haemolysis	β(L)	β(L)	β(L)	-	-	β
6	MR-VP Test	-/-	-/-	-/-	+/+	+/+	-/-
7	O/F – Reaction	+/+	+/+	+/+	+/+	+/+	+/+
8	Citrate utilisation	-	-	-	-	-	-
9	H_2S production	-	-	-	+	+	-
10	Indole production	-	-	-	-	-	-
11	NO_3 Reduction	-	-	-	+	+	+
12	Urease	-	-	-	+	+	-
13	Tolerence Test						
	0% NaCl	N	N	N	N	N	N
	6.5% NaCl	+	+	+	+	+	+
	$10^0C/45^0C$	-/+	-/+	-/+	-/+	-/+	-/+
	40% Bile	-	-	-	-	-	-
	Na – Azohydrate (0.02%)	+	+	+	+	+	+
14	Hydrolysis of						
	Starch	+	+	+	+	+	+
	Gelatin	-	-	-	+	+	+
	Casein	-	-	-	-	-	-
	Esculin	+	+	+	+	+	+
	(L) Arginine	+	+	+	+	+	+
15	Acid production from						
	Lactose	+	+	+	+	+(L)	-
	Meso inostitol	-	-	+(L)	-	-	-
	D-Raffinose	-	-	+	-	-	-
	Mannitol	+	-	+	+	+	+
	Maltose	+	+	+	+	+	+
	D-glucose	+	+	+	+	+	+
	Inulin	+(L)	-	+(L)	-	-	-
	Sucrose	+	+	+	+	+	+
	L(+) Arabinose	+	-	+	+	-	-
	Sorbitol	-	-	+	-	+	-
	Trehalose	+	+	+	+	-	-
	Rhamnose	-	-	-	-	-	-
16	Sensitivity/Resistance test						
	Ampicillin	R	S	S	S	R	R
	Chloramphenicol	S	S	S	S	S	S
	Erythromycin	S	S	S	S	S	S
	Penicillin	S	S	S	S	S	S
	Streptomycin	S	S	S	S	S	S
	Tetracycline	S	S	S	S	S	S

TABLE – 5

3rd CYCLE (MAY)

SL. NO.	TESTS	EYE	GILL	INTESTINE	SKIN	SEDIMENT	WATER
1	Gram Strain	+	+	+	+	+	+
2	Motility	-	-	-	-	-	-
3	Catalase production	-	-	-	-	-	-
4	Oxidase production	-	-	-	-	-	-
5	Haemolysis	α / β	β	α / β	α/ β	α/ β	α / β
6	MR-VP Test	- / -	- / -	+ / -	+ / +	+ / -	-/-
7	O/F - Test	+/+	+/+	+/+	+/+	+/+	+/+
8	Citrate utilisation	-	-	-	-	-	-
9	H_2S production	-	-	-	-	-	-
10	Indole production	-	-	-	-	-	-
11	NO_3 Reduction	-	-	-	-	-	+
12	Urease	-	-	-	-	-	-
13	Tolerence Test						
	0% NaCl	+	-	-	+	-	-
	6.5% NaCl	-	-	-	-	-	-
	10°C	-	-	-	-	-	-
	45°C	+	+	+	+	+	+
	9.6 pH	N	N	N	N	N	N
	40% Bile	-	-	-	-	-	-
	Na –Azohydrate (0.02%)	+	+	+	+	+	+
14	Hydrolysis of						
	Starch	+	+	+	+	+	+
	Gelatin	-	+	-	-	-	-
	Casein	-	-	-	-	-	-
	Aesculin	+	+	+	+	-	-
	(L) Arginine	+	+	+	+	+	+
15	Acid production from						
	Lactose	+	-	+	+	-	-
	Meso inostitol	-	-	-	-	-	-
	D-Raffinose	-	-	+	-	-	-
	Mannitol	+	+	+	-	-	-
	Maltose	+	+	+	+	+	+
	D-glucose	+	+	+	+	+	+
	Inulin	-	-	+	-	-	-
	Sucrose	+	+	+	+	+	+
	L(+) Arabinose	-	+	+	-	-	-
	Sorbitol	+	-	-	-	-	-
	Trehalose	+	+	+	+	+	-
	Rhamnose	-	-	-	-	-	-
16	Sensitivity/Resistance test						
	Ampicillin	R	R	R	R	R	R
	Chloramphenicol	S	S	S	S	S	S
	Erythromycin	S	I	S	S	S	S
	Gentamycin	S	S	S	S	S	S
	Penicillin	R	R	R	R	R	S
	Streptomycin	S	S	S	S	S	S
	Tetracycline	R	S	R	S	S	S

TABLE-6

4th CYCLE (JUNE)

SL. NO.	TEST	EYE	GILL	INTESTINE	SKIN	SEDIMENT	WATER
1	Gram stain	+	+	+	+	+	+
2	Motility	-	-	-	-	-	-
3	Catalase production	-	-	-	-	-	-
4	Oxidase production	-	-	-	-	-	-
5	Haemolysis	β	β	β	β	β	β
6	MR-VP Test	- / -	+ / -	+ / -	- / -	- / -	-/-
7	O/F - Test	+/+	+/+	+/+	+/+	+/+	+/+
8	Citrate utilisation	-	-	-	-	-	-
9	H_2S production	-	-	-	-	-	-
10	Indole production	-	-	-	-	-	-
11	NO_3 Reduction	+	-	+	+	+	+
12	Urease	-	+	+	-	-	-
13	Tolerence Test						
	0% NaCl	+	-	-	+	+	+
	6.5% NaCl	-	-	-	-	-	-
	10^0C	-	-	-	-	-	-
	45^0C	+	+	+	+	+	+
	9.6 pH	+	+	+	+	+	+
	40% Bile	-	-	-	-	-	-
	Na–Azohydrate (0.02%)	+	+	+	+	+	+
14	Hydrolysis of						
	Starch	+	+	+	+	+	+
	Gelatin	+	-	-	+	+	+
	Casein	-	-	-	-	-	-
	Aesculin	+	+	+	+	+	+
	(L) Arginine	+	+	+	+	+	+
15	Acid production from						
	Lactose	-	+	-	-	-	-
	Meso inostitol	-	-	-	-	-	-
	D-Raffinose	-	-	-	-	-	-
	Mannitol	+	+	-	+	+	+
	Maltose	+	+	-	+	+	+
	D-glucose	+	+	+	+	+	+
	Inulin	-	-	-	-	-	-
	Sucrose	+	+	-	+	+	+
	L(+) Arabinose	-	-	-	-	-	-
	Sorbitol	-	-	-	-	-	-
	Trehalose	-	-	-	-	-	-
	Rhamnose	+	+	+	+	+	+

Contd...

16	Sensitivity/Resistance Test						
	Amoxycillin	R	S	R	R	R	R
	Ampicillin	R	R	R	R	R	R
	Bacitracin	R	S	S	R	R	R
	Chloramphenicol	S	S	R	S	S	S
	Chlortetracyclin	S	S	S	S	S	S
	Erythromycin	I	S	R	I	S	S
	Gentamycin	S	S	S	S	S	S
	Kanamycin	I	S	S	I	I	I
	Neomycin	S	S	S	I	R	R
	Novobiocin	R	S	R	I	R	R
	Oxacillin	R	R	R	R	R	R
	Oxytetracycline	S	S	S	S	S	S
	Penicillin-G	R	R	R	R	R	R
	Polymyxin-B	S	S	S	S	S	S
	Streptomycin	S	S	I	S	S	S
	Tetracycline	S	S	S	S	S	S

L = Low

N = Not done

R = Resistance

S = Sensitivity

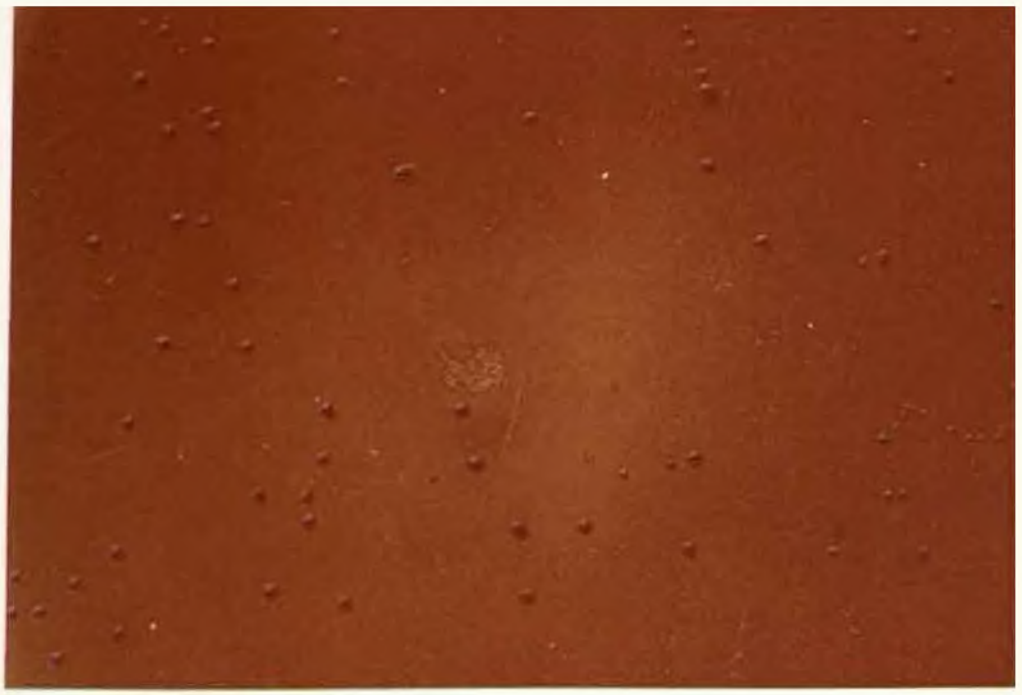
I = Intermediate

+ = Positive or presence

- = Negative

Plate – 5 : Isolation of *Streptococci* from corneal opacity of tilapia after 72hrs of incubation.

Plate – 6 : The intensity of pathogenic *Streptococci* at log phase of growth



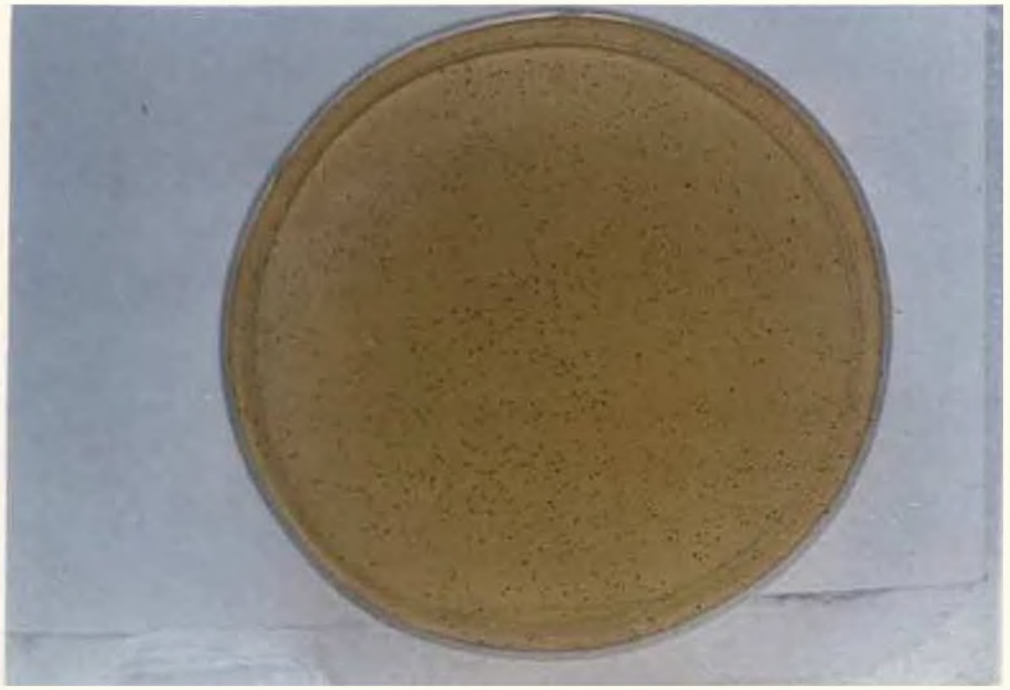
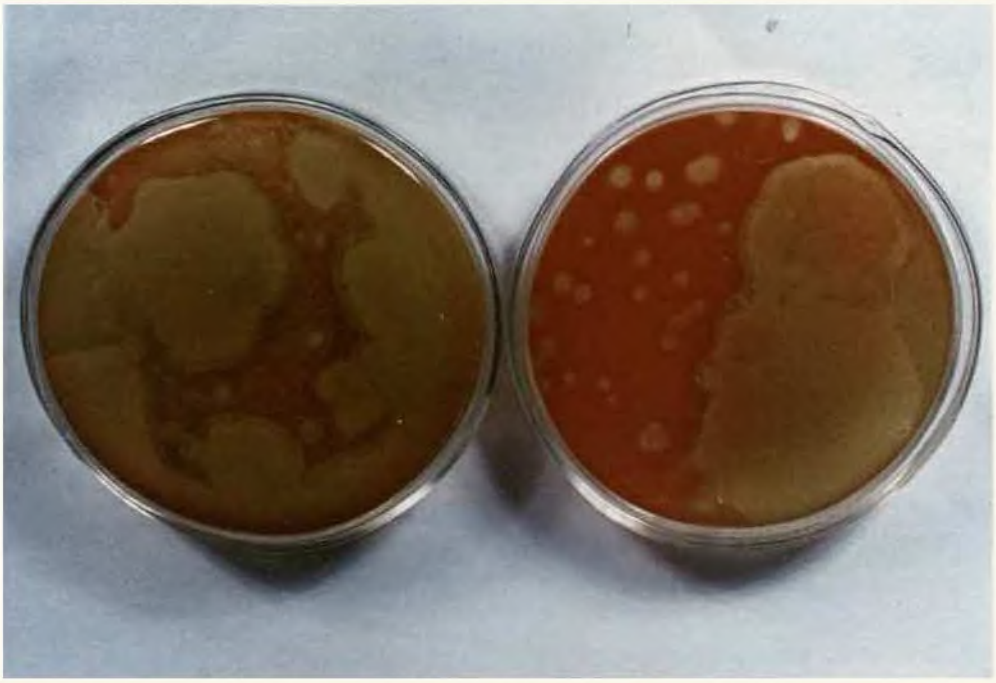


Plate – 7 : β-haemolytic activity of pathogenic *Streptococci* isolated from the corneal opacity of tilapia.

Plate – 8 : β- haemolysis on TSA media with 5% goat blood.



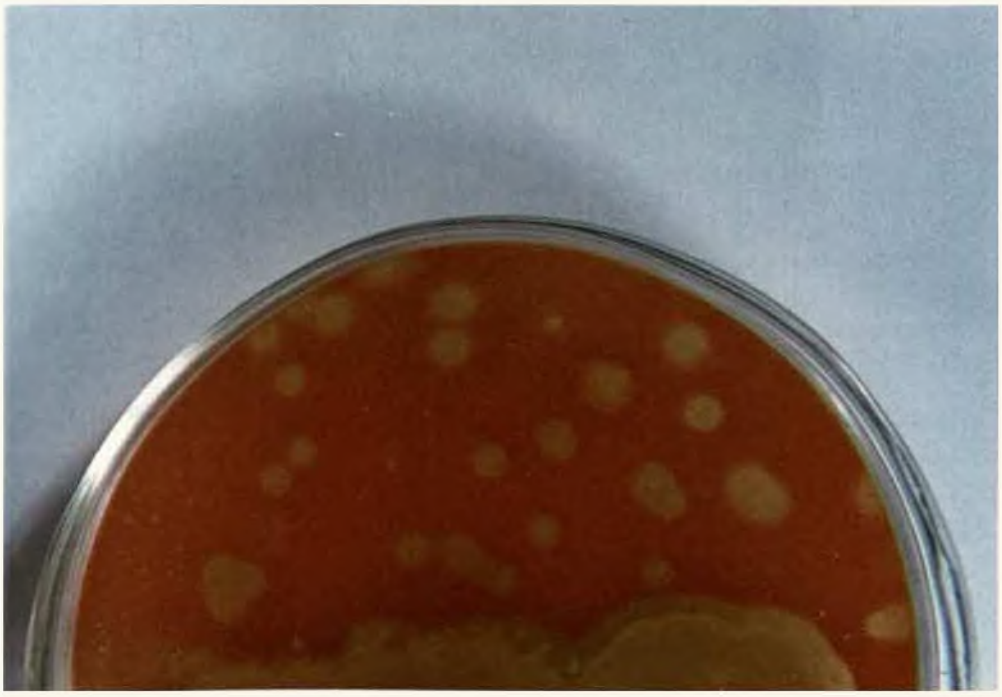
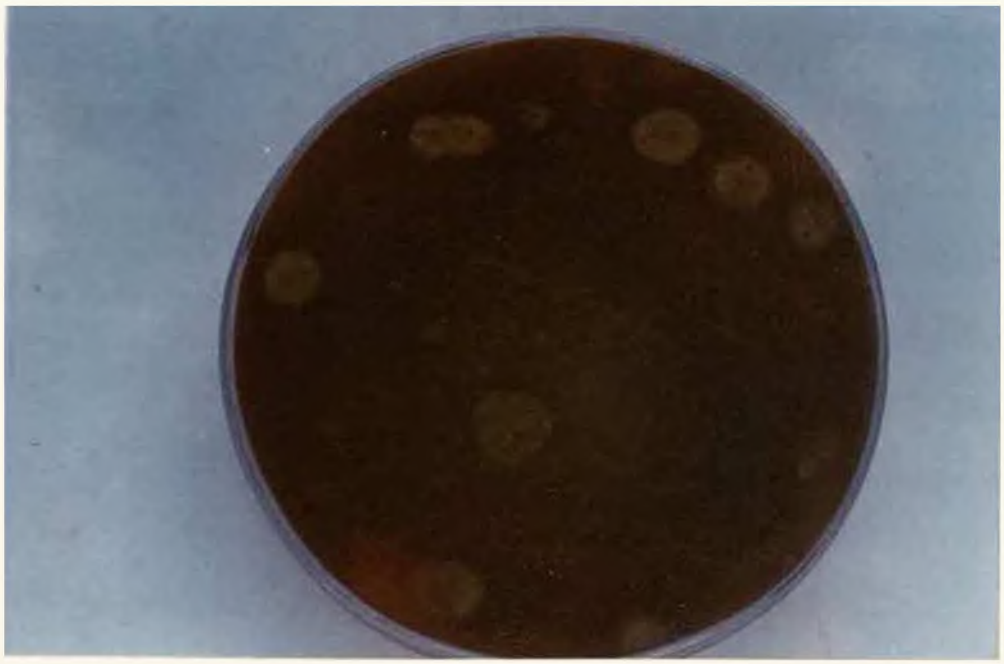
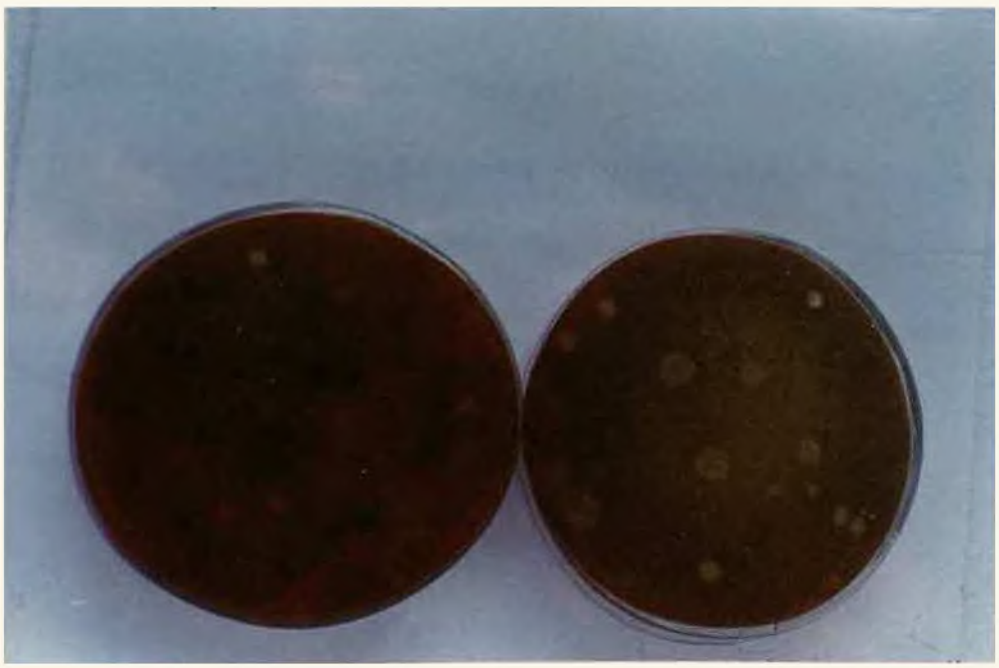


Plate-9 : β- haemolysis on Blood Base Agar medium

Plate – 10 : α / β - haemolytic activity of virulent *Streptococci*.





gelatin. Most of the strains were found out to be saccharolytic in nature but few were proteolytic. All the biochemical characters including the antibiotic sensitivity of the pathogenic *Streptococci* were listed in table. No. 3,4,5,6.

The biophysical characterisation was done for a single isolate. It was found that the strain grew in the temperature range of 15-45^{0}C, and optimally at 25-35^{0}C (Fig-5). The optimum pH for growth was 7.5-8.5 but growth occured between 6-9.5 (Fig-6). The isolate grow optimally a 3-4% (w/v) NaCl when maintained at RT (Fig-7).

4.5.CHALLENGE STUDY

100% mortality of tilapia was observed among the experimental fishes within 48 hrs of injection(I.P). After infection there was sudden decrease in feeding and the fishes showed erratic motion. Feeding was completely stopped after 12 hrs of infection. Whitish exudates in faecal matter which gave rise to a number of streptococcal colonies on KF-agar.

Dark body pigmentation (Plate-12) along with inflammation of kidney, spleen, liver and haemorrhagic heart were the symptoms of infected experimental fishes. The *Streptococci* isolated from, the heart, spleen, liver satisfied Koch's postulates. These isolates were characterised biochemically. The characteristics of these isolates were found to be of the same as that of the infectious pathogenic *Streptococci* spp.

Plate – 11 : Experimental Set up for challenge study

Plate – 12 : Dark body pigmentation showing stress condition after intraperitonial injection

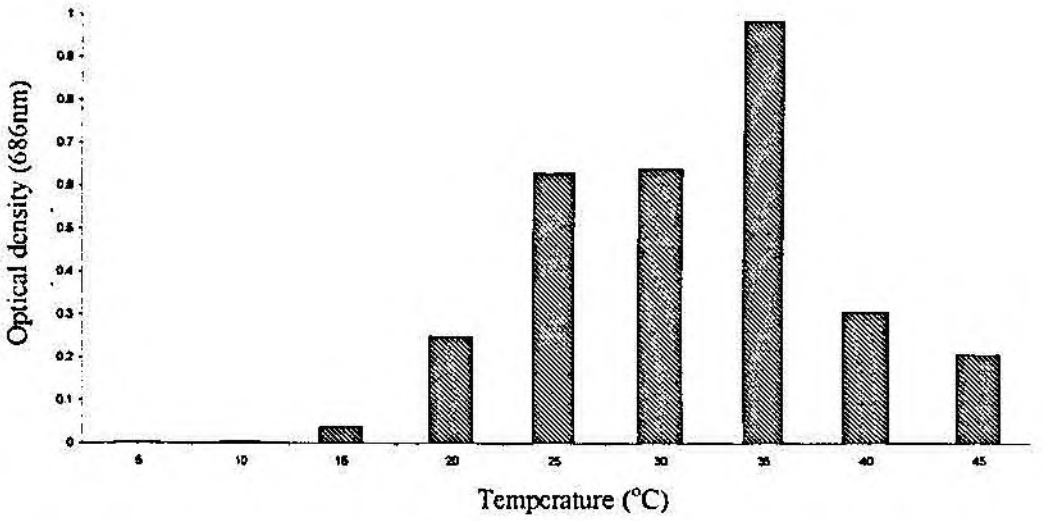


Fig.5 Effect of temperature on growth of *Streptococcus* sp. Bacteria was determined by optical density at 686 nm after 24 hrs.

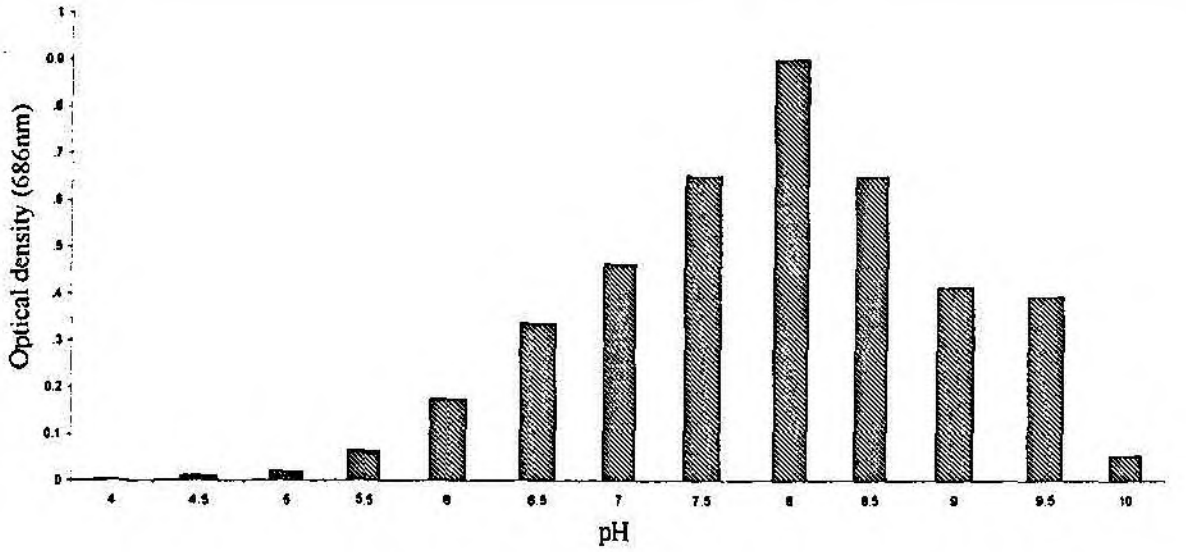


Fig. 6 Effect pH on growth of *Streptococcus* sp. Bacteria was determined by optical density at 686 nm after 24 hrs.

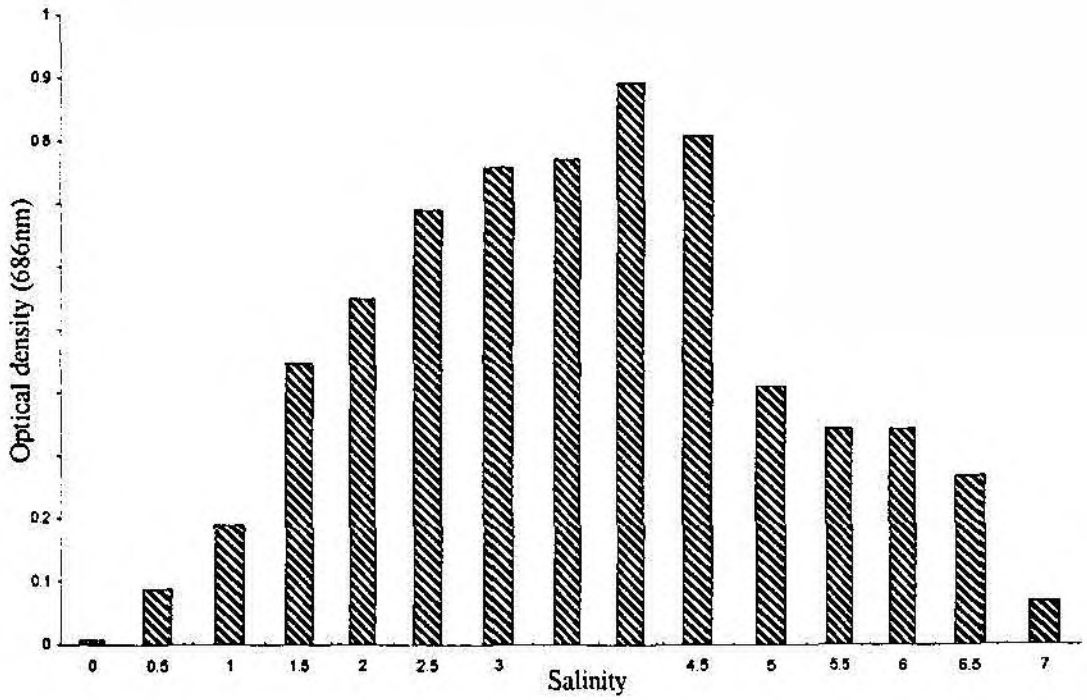


Fig.7 Effect of NaCl on growth of *Streptococcus* sp. Bacteria was determined by optical density at 686 nm after 24 hrs.

5. DISCUSSION

DISCUSSION

The principal interest of this investigation was to isolate virulent strains of *Streptococcus* spp. as there is limited definitive evidence for Streptococcosis in tilapia, even though there has been some indication that Streptococcal infection occurs in variety of species. Monthly occurrence and distribution of *Streptococci* was done during March 99 to June '99. Totally 4 observations in bacterial parameters were made in this study along with the environmental parameters. Biochemical analysis of 24 pathogenic Streptococcal strains has been done along with antibiotic sensitivity giving importance to haemolysis. As *Streptococci* are very well known for their haemolytic activity the significance of this biochemical test was to find out the pathogenicity of the strains. So far, from Valappu Perrenial pond *Tilapia* spp were unexplored for pathogenic *Streptococci.* This study was aimed to monitor the presence of *Streptococci* as fish pathogen in Valappu aquaculture ponds near Cochin.

Streptococci are opportunistic pathogens. Whenever they are not pathogenic they opt for other type of nutrition. Kitao *et al.* (1981) and Miyazaki *et al.* (1984) reported outbreaks of *Streptococcus* sp in Nile tilapia *Oreochromis niloticus* in Japan caused by β and γ haemolytic strains respectively. The present study showed haemolysis and isolates showing α and β haemolysis both simultaneously.

The discussion is presented in the following headings.

- ❖ Quantitative and qualitative distribution of pathogenic *Streptococci* in various parts of *Tilapia* sp. collected from Valappu .

- ❖ Relationship of environmental parameters to the occurrence of pathogenic *Streptococci.*
- ❖ Biochemical tests.
- ❖ Antibiotic sensitivity
- ❖ Haemolytic activity.
- ❖ Challenge study

In present observation most of the infected fishes showed lesions in different parts of the body. Perera *et al.* (1994) has reported in *Tilapia nilotica* and *T. aureus* disease signs characteristic of septicaemia caused by *Streptococcus iniae* in Texas fish farm. Fin erosion was one of the most occurred symptoms in diseased fishes, whereas fluid accumulation and bilateral exophthalmia and corneal opacity was observed by Perera *et al.* (1994). Ram Bhasker *et al.* (1991) reported corneal opacity and fin erosion among fishes in Visakhapatnam Harbour waters polluted with heavy metal oil and grease. Haemorrhaging of internal organs were not apparent in this study whereas abdominal swelling and haemorrhage of internal organs. Dermal and epidermal lesions were reported in tilapia (Perera *et al.* 1994) and cultured trout (Boomker *et al.* 1979). Excessive mortality was also experienced in the rainbow trout culture due to haemorrhagic septicaemia by pathogenic *Streptococci* and appears to be associated with culture intensification and conditions of stress, (Shiomitsu, 1982; Kott, 1982; Ugajin, 1981; Kitao *et al.* 1981). Physico-chemical parameters of the pond studied during this period showed a steady increases in temperature and salinity from the month of March '99 to May '99 and there was sudden decline in both the parameters due to South-west

monsoon rains. It was observed that the other parameters like dissolved oxygen and pH fluctuated within the tolerance limit. The biophysical characterisation showed the optimum temperature being 25-35^{0}C. (Fig-5) and the optimum pH 7.5-8.5 (Fig-6) and salinity optimal concentration ranged between 3-4% (w/v) in experimental condition maintained at RT (Fig-7). Both heterotrophic bacterial and Streptococcal count was positively correlated with the increase in temperature and salinity in field condition . Kitao *et al.* (1979) has reported that pathogenic *Streptococci* can be found in environment throughout the year with higher numbers found in sea during summer months in mud samples. These organisms grow at high incubation temperature implies a mammalian origin and may have importance when one is considering the zoonotic potential of the bacteria.

Streptococcus spp associated in diseased *Tilapia* spp was isolated in selective media (KF Agar) after enrichment in SE-Broth. Fujioka *et al* (1984) has reported that marine bacteria naturally present in coastal water of Hawaii can produce false positive colony on KF-Agar and capability of this media was questioned by this author as the primary medium to recover faecal *Streptococcus* from marine water in tropical climate. But in present study it has been found that KF-Agar is suitable for isolation and enumeration (Plate-4,5,6) and it is suggested that it can be used as selective media for isolation of pathogenic *Streptococci* from diseased fish in tropical climate. Oragui & Mara (1984) has suggested 'membrane filtration' of water samples to retrieve *Streptococcus* as it is very rare in water column. In the present study the enumeration of water sample was done by

'membrane filtration' method using whatman GF/C-filter as sample preparation methods can enhance the efficiency of preliminary isolation procedures.

The genus *Streptococcus* are large spherical group of organism characteristically arranged in chains and distributed in nature (Hashimoto 1982). In the present observation, morphologically the cells were spherical, or oval arranged in pairs or chains (Fig-4) of varying length.

The biochemical characteristics used for diagnosis of pathogenic *Streptococci* are given in table 3,4,5,6. *Streptococci* lacks the enzyme catalase, capable of reacting with oxygen. The number and varieties of these enzyme determine the physiological relation of the organism to oxygen in the formation of flavoproteins by oxygen resulting in the formation of H_2O_2 as one of the major toxic compound. In addition, their oxidation produce more toxic free radicals superoxide O_2^- The accumulation of super oxide is prevented by the enzyme super oxide dismutase which catalyses its conversion to O_2 and H_2O_2.

$$2O_2^- + 2H^+ \xrightarrow{\text{Super oxide dismutase}} O_2 + H_2O_2$$

The enzyme catalase or peroxidase decomposes H_2O_2 to O_2 and H_2O. i.e.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Some bacterial group are able to grow in presence of air but do not produce catalase. Pathogenic *Streptococci* comes in this group. Most of these organisms don't accumulate H_2O_2 since they decompose it by means of peroxidases. Super oxide dismutase, catalase, and peroxidase play a role in protecting the cell from the toxic effect of oxygen metabolism. Cytochrome oxidase was 100% negative in all the

strains isolated. Haemolytic activity in goat blood agar showed 3 types of haemolytic activity. The β-haemolytic activity was most frequently observed which showed 70% of total haemolysis. Whereas 5 strains showed α/β haemolysis and 2 were non-haemolytic.

Minami *et al.* (1979) isolated *Streptococcus* from diseased fish which was showing only β-haemolysis in Sheep blood agar.

Streptococci has also been reported to cause heavy mortality in cultured fish (Kusuda *et al.* 1976). The typical infection was due to α-haemolytic *Streptococci* in eye infection causing corneal opacity and exophthalmic and haemorrhagic abscess inside the operculum and at the base of caudal fin and pericarditis (Minami, 1979).

In order to know the virulence of the *Streptococci* isolates in the present study haemolytc activity was carried out in Blood Agar Base or TSA, containg 5% goat blood. The β-haemolytic activity was found to be 70% of total isolates whereas 20% showed α/β-haemolytic activity and rest were non-haemolytic. α/β-haemolytic strain was temperature sensitive as growth was absent at the elevated temperature of 45^0C but in other strains mild growth was observed . 6.5% NaCl was well tolerated by β-haemolytic strains whereas growth was absent in 6.5% NaCl in the α/β-haemolytic strains. Urease & H_2S was produced only by non-haemolytic strains but was absent in β and α/β-haemolytic strains.

TABLE – 7

% Composition of biochemical activity of *Streptococci* spp.

SL .NO.	Biochemical activity	Reaction	No. of isolates from 24 isolates	%composition of active isolates
1	Gram stain	+	24	100
2	Motility	-	24	100
3	Catalase production	-	24	100
4	Oxidase production	-	24	100
5	Haemolysis	β	17	70.83
		α/ β	5	20.83
		-	2	8.33
6	'MR' – Test	+	11	45.83
	'VP' – Test	-	21	87.5
7	O/F – Test	+ / +	24	100
8	Citrate utilisation	-	24	100
9	H_2S production	-	22	91.66
10	Indole production	-	24	100
11	NO_3 Reduction	+	13	54.16
12	Urease	-	22	91.66
13	Tolerance Test			
	0% NaCl	+	8 (14)	57.14
	6.5% NaCl	+	11	45.83
	10^0C	-	24	100
	45^0C	+	20 (20)	100
	40% Bile	-	24	100
	0.02% Na-Azohydrate	+	24	100
14	Hydrolysis of			
	Starch	+	24	100
	Gelatin	+	8	33.33
	Casein	-	24	100
	Aesculin	+	22	91.66
	(L) Arginine	+	24	100

Contd...

15	Acid production from			
	Lactose	+	13	54.16
	Meso-inositol	+	1	4.16
	D-Raffinose	+	2	8.33
	Mannitol	+	19	79.16
	Maltose	+	23	95.83
	D-Glucose	+	24	100
	Inulin	+	3	12.5
	Sucrose	+	23	95.83
	L (+) Arabinese	+	6	25
	Sorbitol	+	4	16.66
	Trehalose	+	12	50
	Rhamnose	+	8	33.33
	Total	+	138	48% Saccharolytic
16	Antibiotic Sensitivity Test	S		
	Amoxycillin	S	1 (6)	16.66
	Ampicillin	S	5	20.83
	Bacitracin	S	2 (6)	33.33
	Chloramphenicol	S	23	95.83
	Chlortetracycline	S	6 (6)	100
	Erythromycin	S	19	79.16
	Gentamycin	S	24	100
	Kanamycin	S	2 (6)	33.33
	Neomycin	S	3 (6)	50
	Novobiocin	S	1 (6)	16
	Oxacillin	S	0 (6)	0
	Oxytetracycline	S	6 (6)	100
	Penicillin	S	9	37.5
	Polymyxin	S	5 (6)	83.33
	Streptomycin	S	23	95.83
	Tetracycline	S	22	91.66

Generally proteolytic activity was found to be variable in β-haemolytic strains, negative in α/β-haemolytic strains but non-haemolytic strains were capable of liquefying gelatin. ASM (1981) has found that gelatin liquefaction was variable in *S. faecalis* and in a β-haemolytic strain (*S.iniae*) the enzyme gelatin was absent (Perera *et al.* 1994, Kusuda *et al.* 1976). The vitamin B and amino acid requirement was found extensive and because of this characteristics only they are usually cultivated on media containing peptone, yeast extract or other digested plant or animal material supplemented with a fermentable carbohydrate to provide an energy source. The present study, the aminoacid, arginine was utilised by all the 24 isolates. This finding was supported by various workers which is given in table no.8,9,10. Production of extracellular enzyme to hydrolyse starch was noted in all the isolates (100%). The hydrolysis of starch was also positive in the *S. iniae* isolated by Perera *et al.* (1994) from tilapia . Utilisation of aesculin was recorded as 100%.

TABLE – 8

COMPARISON OF MAIN CHARACTERISTICS OF ISOLATES WITH STANDARD ISOLATES

SL. NO.	CHARASTERIS -TICS	PRESENT ISOLATES		Cowan & Steel 1977			Perera *et al* 1994 *S.iniae*	Kusuda *et al* 1976 *S.iniae* in *S.quinqu-eradiata*	Carson *et al.* 1993 *S.iniai* from *O.mykiss*
				S.Pyroge-nes	*S.equisi-milis*	*S.faecium*			
1	Gram Stain	+	+	+	+	+	+	+	+
2	Motility	-	-	-	-	-	-	-	-
3	Catalase	-	-	-	-	-	-	-	-
4	Haemolysis	β	β	β	β	β	β	β	β
5	NO_3Reduction	v		ND	ND	ND	-	ND	ND
6	Citrate Utilization	-	-	ND	ND	-	ND	ND	ND
7	VP-Test	-	-	-	-	ND	-	-	ND
8	Growth at								
	10°C	-	-	-	-	ND	+	+	+
	45°C	+	+	-	-	-	+	+	-
	0% NaCl	-	+	ND	ND	-	ND	ND	ND
	6.5% NaCl	+	-	-	-	+	-	+	+
	9.6 pH	ND	+	-	-	-	-	+	ND
	40% Bile	-	-	-	-	+	-	+	-
9	Urease test	-	-	ND	ND	ND	-	-	ND
10	Hydrolysis of								
	Gelatin	-	+	-	-	-	-	-	ND
	Starch	+	+	ND	ND	ND	+	-	ND
	Arginine	+	+	+	+	-	+	+	+
	Aesculin	+	+	V	V	+	ND	ND	ND
11	Acid production *from*								
	Arabinose	-	-	ND	ND	-	-	-	-
	Glucose	+	+	ND	ND	ND	+	+	+
	Inulin	-	-	-	-	ND	-	ND	-
	Lactose	+	-	+	V	+	-	ND	-
	Meso inositol	-	-	ND	ND	ND	ND	ND	ND
	Maltose	+	+	+	+	ND	+	+	+
	Mannitol	+	+	-	-	-	+	+	+
	Raffinose	+	+	-	-	-	-	-	-
	Sorbitol	-	-	-	-	-	-	+	-
	Sucrose	+	+	+	+	-	+	-	-
	Trehalose	+	-	+	+	+	+	+	+
	Rhamnose	-	+	ND	ND	ND	-	ND	ND

TABLE – 9

COMPARISON OF MAIN CHARACTERISTICS OF ISOLATE WITH STANDARD ISOLATES

SL.NO.	CHARACTERISTICS	PRESENT ISOLATE	Cown & Steel 1977		ASM 1981
			S. agalactiae	*S. faecium*	*S. faecalis*
1	Gram Stain	+	+	+	+
2	Motility	-	-	-	-
3	Catalase	-	-	-	-
4	Haemolysis	α / β	α / β	α / β	α / β / γ
5	NO_3Reduction	-	ND	ND	-
6	Citrate Utilization	-	ND	ND	-
7	VP-Test	-	-	ND	ND
8	Growth at				
	10^0C	-	-	+	+
	45^0C	-	-	+	-
	0% NaCl	-	ND	ND	ND
	6.5% NaCl	ND	-	+	+
	9.6 pH	ND	-	+	-
	40% Bile	-	+	+	ND
9	Urease test	-	ND	ND	
10	Hydrolysis of				
	Gelatin	-	-	ND	V
	Starch	+	ND	ND	-
	Arginine	+	+	+	+
	Aesculin	+	-	+	+
11	Acid production from				
	Arabinose	-	ND	ND	ND
	Glucose	+	ND	ND	-
	Inulin	-	-	-	-
	Lactose	+	V	V	+
	Meso inositol	-	ND	ND	ND
	Maltose	+	+	ND	-
	Mannitol	+	-	V	+
	Raffinose	-	-	V	-
	Sorbitol	-	-	V	+
	Sucrose	+	+	ND	+
	Trehalose	+	+	V	ND
	Rhamnose	-	ND	ND	ND

TABLE – 10

COMPARISON OF MAIN CHARACTERISTICS OF ISOLATES WITH OTHERS

SL.NO.	CHARACTERISTICS	PRESENT ISOLATE	Cowan & Steel 1977 *S. mutans*	Collins & Patrica 1985 *S.thermophilus*
1	Gram Stain	+	+	+
2	Motility	-	-	-
3	Catalase	-	-	-
4	Haemolysis	-	- /α	-
5	NO_3Reduction	+	ND	ND
6	Citrate Utilization	-	ND	ND
7	VP-Test	+	+	ND
8	*Growth at*			
	10^0C	-	ND	-
	45^0C	+	-	+
	0% NaCl	ND	ND	ND
	6.5% NaCl	+	-	-
	9.6 pH	ND	-	-
	40% Bile	-	-	-
9	Urease test	+	ND	ND
10	Hydrolysis of			
	Gelatin	+	-	ND
	Starch	+		ND
	Arginine	+	-	-
	Aesculin	+	+	-
11	Acid production from			
	Arabinose	-	ND	ND
	Glucose	+	ND	-
	Inulin	-	ND	-
	Lactose	+	+	+
	Meso inositol	-	ND	ND
	Maltose	+	+	ND
	Mannitol	+	+	-
	Raffinose	-	ND	-
	Sorbitol	+	+	-
	Sucrose	+	+	ND
	Trehalose	-	+	-
	Rhamnose	-	ND	ND

ND = No Data

V = Variable

Lactose fermentation was recorded 54.16% in lactose media containing 1% peptone. The *Streptococcus* isolated by Perera *et al.* (1994) showed negative lactose fermentation but ASM (1981) showed lactose fermentation positive in various strains but in the present observation only 13 isolates was found positive for lactose fermentation. 100% glucose fermentation was obtained which was supported by the data reported in table No.8,9,10. Pathogenic bacteria ferment glucose rather than any other sugar, whereas bacteria of marine origin will ferment galactose & mannose found commonly in marine environment which supports the capability adaptive enzyme production by bacteria. Maltose and sucrose was fermented by 23 isolates out of 24 isolates studied. Mannitol was fermented by 79.16% of the total isolates. Trehalose and Lactose was fermented actively by 50% and 54.16% of total isolates respectively.

Rhamnose an Arabinose was fermented by 33% and 25% respectively. Meso-inositol, D-Raffinose, Inulin and Sorbitol was very poorly fermented by the pathogenic *Streptococci* isolated in the present study .

Antagonistic activity toward various antibiotics was tested and it was found chloramphenicol, Gentamycin and Oxytetracycline Streptomycin and Tetracycline were highly sensitive and gave 90-100% sensitivity range. 65% of the isolates showed multiple drug resistance (MDR) to oxacillin, novobiocin, amoxycillin, bacitracin, kanamycin and penicillin.

The present isolates showed the presence of plasmid by the MDR activity and it can be correlated with the resistance of the bacteria towards the antibiotics. Foo *et al.* (1985) has found no plasmids from the isolates and no relationship can be

established with the antibiotic resistance. The occurrence of plasmid with R+ factor with transferable drug resistance was very evident by the clear band obtained during plasmid isolation. The frequency with which the R+ markers recombine independently, form the basis for constructing the genetic map of plasmids

The pathogenicity of bacterial septicaemia in cultured tilapia was studied by experimentally infecting with *Streptococcus* by means of intraperitonial injection. The results indicated that mortality occurred within 48 hrs of infection but no specific lesions of septicaemia was recorded whereas inflammation of kidney, spleen, liver and haemolytic of heart were the symptoms of infected fishes. The *Streptococci* isolated satisfied Koch's postulates also. The characteristics of the isolates were found to be same of that infected *Streptococci.* Liv *et al.* (1990) found that significant infection was produced after 5 days of incubation in a single bacterial immersion study, whereas in the present study it took only 2 days for the mortality to occur as it was intraperitonial injection proving the pathogenicity of the isolated *Streptococci.*

The source of infection has not been established . However, they are all natural flora of eye and intestine-playing a role in causing the disease as opportunistic pathogens.

6. SUMMARY

SUMMARY

1. Gram positive bacteria have rarely been reported as the etiological agents of warm-water fish diseases. So the present study "Streptococcal infection in Cultured tilapia *Oreochromis mossambicus*" was carried out in fishes obtained from a polyculture pond. An attempt was also made to identify all the representative Streptococcal species occured in *Tilapia* sp. Challenge study was also done by intraperitoneal injection of the organisms which was found lethal to the cultured *Tilapia* sp.

2. The selective media, KF Agar was used to isolate the pathogenic *Streptococci* by enrichment with SE broth and direct streak plating. All fishes examined were found to harbour α/β, or β-haemolytic *Streptococcus* sp.

3. Inoculation -reisolation testing by Koch's postulates established the organism as the etiological agent of corneal opacity, skin lesions and bacterial septicaemia.

4. The pathogen was isolated from eyes, intestine and skin of diseased fish by swabbing on KF Agar (Plate No.4). Numerous punctiforms, flat, deep red or pinkish red colonies developed after 48 hrs of incubation at RT.

5. All reagents and media used were from Hi-Media and all standard methods were followed in isolation and identification procedures.

6. Total heterotrophs ranged from $15x10^4$ to $20x10^4$/500ml in sea water and $10x10^5$ to $49x10^5$/gm in sediments and $36x10^3$ to $98x10^7$/gm in eye and intestine of Tilapia.

7. Catalase and oxidase was found to be absent in all the isolates.

8. Most of the isolates were inert to produce hydrogen sulfide, ammonia, gelatinase, urease, and indole. From 12 carbohydrates tested, very high activity was found in glucose, sucrose, maltose & mannitol.

9. No growth occurred on 10 and 40% bile agar. Salinity tolerance was highly variable with these isolates.

10. The isolates from eyes were highly proteolytic and poor in lactose fermentation whereas the natural flora of intestine was actively fermenting lactose but exhibited poor proteolytic activity which showed that all the isolates were harbouring adaptive enzymes required to metabolise respective substrates available in their respective 'niche' from where they have been isolated.

11. Sensitivity to antibiotics was demonstrated with Hi-Media discs. 65% isolates showed multiple drug resistance (MDR) to Oxacillin (100%), Novobiocin (84%), Amoxycilin (83%), Bacitracin (66%), Kanamycin (66%) and Penicillin indicating the presence of R+ factors with transferable drug resistance.

12. 3 types of haemolysis was exhibited by the 24 isolates, they are β-haemolysis (70%), α/β -haemolysis (20%) and non-haemolysis (10%).

13. Challenge study by intraperitoneal(IP) injections were lethal to the cultured *Oreochromis mossambicus*.

14. The sources of infection in these fishes were from the fishes itself as they are natural flora of eyes and intestine. Due to stress by chemicals or overstocking they turn into opportunistic pathogens. However, similar strains of this organism has been isolated from bovine and human sources also.

7. REFERENCES

REFERENCES

Al-Harbi, A.H., (1994) First isolation of *Streptococcus* sp. from hybrid tilapia (*Oreachromis niloticus* x *O. aureus*) in Saudi Arabia. AQUACULTURE, VOL-128, no.314, pp.195-201, 1994.

Aoki, T., Takami, K., Kitao, T. (1989). Spread of drug resistant strains of *Streptococcus* sp. in yellowtail farms. THE SECOND ASIAN FISHERIES FORUM PROCEEDING OF THE SECOND ASIAN FISHERIES FORUM, TOKYO.JAPAN, 17-22. APRIL-1989. PP.697-699.

Aoki, T., Takami, K., Kitao, T.(1990). Drug resistance in a non-hemolytic *Streptococcus* sp. isolated from cultured yellowtail *Seriola quinqueradiata.* DIS AQUAT.ORG. 1990, Vol-8, no.3 pp-171-177.

Aoki, T., Takeshita, S., Kiao, T., (1983) Antibacterial action of chemotherapeutic agents againsts non haemolytic *Streptococcus* sp. isolated from cultured marine fish. Yellowtail *Seriola quinqueradiata.* Bull. Jap.Soc.Sci. FISH. NISSUISHI. 1983. Vol. 49. no.11, pp. 1673-1677 ISSN 0021-5392.

Barham, W.T., Schoonbee, H., Smit. G.L. (1979). The occurrence of Aeromonas and Streptococcus in rainbow trout, *Salmo gairdneri* – Richardson. J.FiSH. BIOL-1979. 15(4), 457-460.

Baya, A.M., Navarro, R.B., Kotopoulis, E. (1996) Streptococcal infection of hybrid striped bass and tilapia. AQUACULTURAL ENGINEERING SOCIETY PROCEEDING II: SUCCESS AND FAILURES IN COMMERCIAL RECIRCUALTING AQUACULTURE. 1996. VOL. 1, PP. 32-40.

Boomker, J., Imes, G.D., Jr. Cameron, C.M., Nude, T.W., Schoonbee, H.J. (1979). Tourt mortalities as a result of Streptococcus infection. Onderstepoort J.VET. RES.,, 46(2), 71-77, 1979.

Bunch, E.C., Bejerano, I., (1997) The effect of environmental factors on the susceptibility of hybrid tilapia *Oreochromis niloticus* X *Orcachromis aureus* to streptococcus. Israeli Journal of Aquaculture/Bamidgeh (ISR, J. AQUACULT./BAMIDGEMENT , VOL.49, NO.2, PP.767-76, JUN 1997.

Collins and Patrica M. Layer (1985) Microbiological methods, Butterworth and Co. Publishers Ltd. pp-450.

Cowan, S.T. (1977) Cowan & Steel's Mannuel for the identification of Medical Bacteria, 2nd Edition, Cambridge University Press, Cambridge, pp-50-55.

Chang, P.H., Plumb, J.H, (1996) Effects of salinity on streptococcus infection of Nile tilapia, *Oreochromis niloicus*. J.APPL.AQUACULT., VOL.6, NO.1, PP. 39-46, 1996.

Chang, P.H., Plumb, J.A. (1996) Histopathology of experimental *Streptococcus* sp. infection in tilapia, *Oreochromis niloticus* (L) and channel catfish, *Ictalurus punctatus* (Ratinesque) J.FISH DIS.VOL.19.NO.3, PP.235-241, 1996.

Crosby, M.D., (1996) Fish health status of aquaculture recirculating systems in Virginia: Three years casework. AQUACULTURE ENGINEERING SOCIETY PROCEEDING II: SUCCESS AND FAILURE IN COMMERCIAL RECIRCULATING AQUACULTURE, 1996-Vol.-2, pp. 611-615.

Eladar, A; Shapiro, O., Bejerano, Y., Bercovier, H. (1995). Vaccination with whole. Cell vaccine and bacterial protein extract protects tilapia against *Streptococcus difficile*. VACCINE, VOL.13, no.9, pp. 867-870. 1995.

Eldar, A., Bejerano, Y., Bercovier, H.(1994). *Streptococcus shiloi* and *Streptococcus difficile*: Two new streptococcal species causing meningoencephalitis in fish. CURR.MICROBIOL., Vol.28, no.3, pp.139-143, 1994.

Eldar, A., Bejerano, Y., Livoff, A; Horovitcz, A; Bercovier, H (1995) Experimental Streptococcal meningo-encephalitis in cultured fish. VET. MICROBIOL, VOL.43, no.1, pp. 33-40, 1995.

Farkas, J; Olah, J.(1984), Occurrence of pasteurella and streptococcus in shellfish (*Silurus glanis* L.) AN ENVIORNMENT IN EUROPEAN POLYCULTURE. Olah. I.ed. 1984 Vol-23 pp 47-54.

Fasanya, OOA., Oladimeji, A.A., Yakubu, U.J. (1988), Bacterial microflora associated with the skin and gills of *Tilapia nitotica* (*Oreochromis niloticus*) NIGER. J. APPL. FISH HYDROBIOL., Vol.3, pp. 49-50 1988.

Fujioka, R., Ueno, A., Narikawa, O. (1984) Recovery of false presumptive fecal streptococcus colonies from marine waters using KF Agar ABSTRACTS. OF THE ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY – 1984. 1989, P.209.

Getchell, R., (1998) *S. iniae* causes tilapia infection FISH.FARM NEWS., Vol.6, P.16, Apr. 1998.

Hasimoto, H., (1982) Classification and pathogenicity of the genus streptococcus. FISH PAHOL. TOKYO. 1982 Vol. 17 no-1 pp. 1-10.

Hobbert, R.M., Shilo, M., (1989) Bacterial diseases in warmwater aquaculture. FISH CULTURE IN WARM WATER SYSTEMS: PROBLEMS AND TRENDS. CRC PRESS, BOCA RATON, FL (USA), 1989, PP.179-194.

Ibiwoye, TII., Okaeme, AN., Okojie, PUA., Agbontable, JA (1989) Bacterial fish disease investigation and pathology of the integument (Skin and fins) of Clarias and Tilapia species in the NIFFR hatcheries. ANNU. REP.NATL.INST. FRESHWAT. FISH. (NIGERIA) 1989. PP. 32-35. 1990.

Karaseva, T.A., Serdyuk, A.V., Longinova G.A., (1991) Experience in treatment of *Streptococcus* sp. infection in Atlantic salmon *Salmo solar*. COPENHAGEN-DENMARK ICES 1991 11PP

Kitan, T; Aoki, T., Iwata, K; (1979) Epidermiological study on Streptococcosis of cultured yellow tail (*Seriola quinqueradiata*) 1. Distribution of *Streptococcus* sp. in sea water and muds around yellowtail farms. BULL.JAP. SOC.SCI.FISH. NISSUISHI 1979. Vol-45, no.5, pp. 567-572.

Kitao, T. (1982). The methods for detection of Streptococcus sp. causative bacteria of Streptococcal disease of cultural yellowtail *(Seriola quinqueradiata)*-especially their cultural, biochemical and serological properties. FISH. PATHOL., TOKYO, 1982. Vol-17, no.1 pp. 17-26.

Kitao, T; Aoki, T; Sakoh, R. (1981). Epizootic caused by beta-Haemolytic *Streptococcus* species in Cultured freshwater fish. FISH PATHOL., TOKYO., VOL-15, no- 34 pp. 301-307, 1981.

Kott. Y., (1981) Faecal Streptococcus as an indicator in disinfected water and waste water. ANTONIE VAN LEEUNINHOEK, 1982. Vol-48, no.6 pp. 639-641.

Kusuda, R. Kawai, K., (1982b) Characteristics of *Streptococcus* sp. pathogenic to yellowtail. FISH. PATHOL. TOKYO 1982. Vol. 17, no.1, PP. 11-16. ISSN 0388-788.

Kusuda, R., Kawai, K; Shirakawa, T. (1982a) Serological study of *Streptococcus* sp. Pathogenic to cultured yellowtail. BULL.JAP.SOC.SCI.FISH. 1982. Vol. 48, no.12, pp. 1731-1738.

Kusuda, R., Kimura, H; (1978) Studies on the pathogenesis of streptococcal infection in cultured yellowtail *Seriola* spp. the fate of *Streptococcus* sp. after inoculation. J. FISH. DIS. 1978 1(1), 109-114.

Kusuda, R., Komatsu, I., Kawai, K; (1978a). *Streptococcus* sp. isolated from an epizootic of cultured eels. BULL. JAP. SOC. SCI. FISH 1978 44(3). 295.

Kusuda, R., Komatsu, L. (1978b). A comparative study of fish pathogenic Streptococcus isolated from saltwater and freshwater fishes. BULL. JAP. SOC. SCI. FISH. 1978 44 (10) 1073-1078.

Liu, C.I., Huang, J.L., Hung, H.H; Pen, C.F. (1990). The experimental infection of Streptococcus and or Aeromonas to induce bacterial septicaemia in cultured tilapia. COA fisheries series. Taipei (COA FISH. SER. T, no.24, PP.40-45, 1990.

Minami, T. (1979) *Streptococcus* sp. Pathogenic to cultured yellowtail isolated from fishes for diet. FISH PATHS. 14: 15-19.

Muniz, I., Jimenez, L., Teranzes, G.A., Hazen, T.C., (1989). Survival and activity of *Streptococcus faecalis* and *Escherichia coli* in tropical freshwatrer. MICROBIAL. ECP;. 1989. Vol. 18, no.2. pp-125.

Ohnishi, K., Jo, Y. (1981) Studies on streptococcal infection in pond-cultured fishes-1 characteristics of a β-Haemolytic Streptococcus isolated from Ayu and

Amago in 1977-1978. FISH. PATHOL., TOKYO 1981. Vol.16, no.2 pp. 63-67.

Oragui, J.I., Mara, D.D., (1984) A note on a modified membrance bovis agar for the enumeration of *Streptococcus bovis* by membrance filtration. J. APPL. BOCTER 1984. Vol-56, no.1 pp. 179-181.

Pappalardo, R., Boemare, N., (1982) An intracellular Streptococcus causative agent of a slowly developing disease in the Mediterranean crab. *Carcinus mediterraneus* AQUACULTURE. 1982. Vol. 28. no. 3-4 pp.282-292.

Park, S.W., Sim, D.S., Yoo, Y.C.(1987), Pathogenic *Streptococcus* sp. isolated from on epizootic of cultured yellowtail. *Seriola quinqueradiata.* BULL.NAT.FISH.RES.DEV. ASGENCY-KOREA 1987 no.40, pp- 75-79.

Perera, R.P., Johnson, S.K., Lewis, D.H. (1997) Epizootiological aspects of *Streptococcus iniae* affecting Tilapia in Texas. AQUACULTURE, VOL.192, NO.1-4, PP.25-33, Jun. 1997.

Perera, R.P., Johnson, S.K., Collins, M.D., Lewis, D.H. (1994) *Streptococcus iniae* associated with mortality of *Tilapia nilotica* X.*T.aureus* hybrids. J.AQUAT.ANIMOL HEALTH, Vol-6, no.4, pp. 335-340, 1994.

Pouncher, A.M., Devriese, L.A., Hernandez, J.F., Deltattre, J.M., (1991).Infection in Atlantis salmon *Salmo solar.* COPENHAGEN-DENMARK ICES 1991 11PP

Pouncher, A.M., Devriese, L.A., Hernandez, J.F., Deltattre, J.M., (1991). Enumeration by a miniaturized method of *Escherichia coli, Streptococcus bovis* and enterococci as indicates of the origin of faecal pollution of waters. J.APPL. BACTERIOL. 1991. VOL. 70, NO.6, PP. 525-530.

Rasheed, V; Plumb, J.A., (1984) Pathogenicity of a non-haemolytic group B *Streptococcus* sp. in gulf killifish *Fundulus grandis* Baird and Girard AQUACULTURE 1984, Vol. 37, no.2, pp. 97-105.

Ridina, (1972) Aquatic Microbiology. Edited by R.R Colwell and M.S. Zambruski, Univ. Park Press, Baltimore.

Shiomitsu, K., (1982) Isolation of *Streptococcus* sp. from the brain of cultured yellowtail. FISH, PATHOL, TOKYO., 1982 Vol-17, no.1 pp. 27-31. ISSN 0388-788

Shiomitsu K, Kusuda, R., Osaga and Mimekiyo, M. (1980). Studies on Chemotherapy of fish disease with erythromycin-11 FISH PATHO. 15. 17-23.

Sugita, H., Kawasaki, J., Deguchi, Y. (1997) Production of amylase by the intestinal microflora in cultured freshwater fish. LETT. APPL. MICROBIOL. VOL. 24. NO.2, P. 105-108, Feb. 1997.

Taniguchi, M., (1983) Progress of Streptococcus in preoral inoculation. BULL. JAPAN. SOC. FISH. 49. 1171-1174.

Tung, Ming-chen., Chen, Shin-chu., Tasi, Shinn-Shyang (1987) General septicaemia of streptococcal infection in case. Culture tilapia, *Tilapia mossambica*, in southern Taiwan. THE MEMOIR OF BACTERIOLOGY AND IMMUNOLOGY IN FISH DISEASE. I., 1987, PP. 187-197, COA Fisheries Series, Taipei (COA, FISH SER.), no.10 ISSN 1018-9637.

Ugajin.M., (1981) Studies on *Streptococcus* sp. as a causative agent of an epizootic among the cultured Ayu *(Plecoglossus altivelis)* in Tochigi prefecture, Japan 1980. FISH. PATHOL, TOKYO., 1981. Vol-16 no-3 pp. 119-127.

Weinstein, M.R., Litt, M; Kertesz, D.A., Wyper, P., Rose, D; Coulter, M; Mcgeer, A; Facklam, R; Ostach, C; Willey, B.M., Berczyk, A., Low, D.E. (1997). Invasive infections due to a fish pathogen. *Streptococcus iniae*. New England Journal of Medicine EN.ENGL.J.MED.I. VOL. 337. NO.9. PP. 589-59 Aug. 1997.

Yasunaga, N., (1982) Occurrence of *Streptococcus* sp., a pathogen of cultured yellowtail, in muscle of sardine for diets. FISH. PATHOL., TOKYO. 1982. Vol. 17, no. 3, pp. 195-198.

Zobell, C.E. (1946) Marine Microbiology Chronica Botanica Waltham Mass USA.