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Transmission and pathology of *Streptococcus inane* in monosex Nile tilapia (*Oreochromis niloticus*) in aquaculture of Bangladesh

Md. Mer Mosharraf Hossain[1] • Amimul Ehsan[2] • Md. Anisur Rahman[1] • Monjurul Haq[1] • Md. Bazlur Rashid Chowdhury[3]

[1] *Department of Fisheries and Marine Bioscience, Jessore University of Science and Technology, Jessore-7408, Bangladesh*
[2] *South-West Area Integrated Water Resources Planning and Management Project, Bangladesh Water Development Board, Bangladesh*
[3] *Department of Aquaculture, Bangladesh Agricultural University, Mymensingh, Bangladesh*

Correspondence: Md. Mer Mosharraf Hossain, Department of Fisheries and Marine Bioscience, Jessore University of Science and Technology; Email: mmiron_bau@yahoo.com

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Abstract

Streptococcus iniae is a major fish pathogen, recently emergent outbreaks were recorded in commercially cultured monosex Nile tilapia (*Oreochromis niloticus*) result in significant losses termed "streptococcosis"- causes unusual appearances with multi-focal pin-point haemorrhages, abscesses, necrosis and ascites in skin, fin, muscle, liver, spleen, kidney, blood, interstitial fluid specially in central nervous system and brain. This disease was more prevalent (>26%) at summer when the water temperature was approximately >25^{o}C, percentage of mortality was higher >41% during the overcrowding and improper water chemistry. Raised levels of glucose and ammonium in blood serum causes reduced number of free blood cells released into the haemolymph to stomach and gut, result in refrain from eating in diseased tilapia. Stocking density (200 fish/decimal; class IV) had significant effect (P<0.01) on the total production (5,000 to 5,500 kg/ha). *S. iniae* in the circulating blood cells, extra-tubular haemal spaces containing blood vessels, fixed phagocytes in the hepatopancreas (gastrointestinal tract), bacteria-like particles in the brain tissue, vacuum and necrosis in hepatocytes revealed with histopathology. In vitro study revealed that cohabitation of dead or infected fish with healthy fish resulted infection (horizontal transmission mechanism) to the healthy fish.

Keywords: Streptococcosis, Monosex tilapia, histopathology, transmission

INTRODUCTION

There is increasing interest in the likely effect of global climate change on diseases in both wild and cultured organisms (Lafferty *et al.* 2004). In particular, aquatic animals are highly sensitive to temperature change; overcrowding and improper water chemistry can reduced oxygen tension in water, higher microbial growth and immunosuppression to fish, resulted higher prevalence of disease in monosex Nile tilapia (*Oreochromis niloticus*) (Le Moullac and Haffner 2000) caused by *Streptococcus iniae* (Shoemaker and Klesius 1997).

S. iniae is the main etiological agent of streptococcosis also caused by *Streptococcus* and *Lactococcus* sp., in a variety of fresh and saltwater fish species in worldwide and results in estimated losses US$150 million in annually (Shoemaker and Klesius 1997). Temperature, dissolved oxygen, nitrite level (Bunch and Bejerano 1997), various stressor factors (Shoemaker and Klesius 1997), high fish density and infectious dose in intensive culture system (Stoffregen *et al.* 1996, Eldar *et al.* 1997) caused high mortality in tilapia with *S. iniae* (Shoemaker *et al.* 2001).

S. iniae was first isolated from a skin lesion of a captive Amazon River fish, *Inia geoffrensis* (Pier and Madin 1976). Since then, the susceptible bacterium has been reported

in many species of fresh, estuarine and marine fish species from 15 countries in 6 continents, including Africa, Asia, Australia, Europe, as well as North and South Africa with ayu (Kitao *et al.* 1981), barramundi (Bromage *et al.* 1999), salmon (Eldar *et al.* 1995), European seabass (Zlotkin *et al.* 1998), grey mullet (Eldar *et al.* 1994), grouper (Kvitt and Colorni 2004), rainbow trout (Eldar *et al.* 1997), red drum (Shen *et al.* 2005), snapper (Ferguson *et al.* 2000), silver bream (Bromage and Owens 2002), channel catfish (Shoemaker *et al.* 2001), tilapia (Abutbul *et al.* 2004), and yellowtail (Kaige *et al.* 1984). Among other, *S. iniae* has been isolated from humans with bacteraemia, cellulitis, meningitis, and osteomyelitis (Facklam *et al.* 2005). The source of human infections has been associated with the preparation of *S. iniae* infected tilapias for cooking (Lehane and Rawlin 2000).

The current study investigates the nature of the causative agent including epidemiology, mode of transmission, seasonal effects on disease prevalence, interaction between the causative agent with host cells or tissues or organs by histopathological analysis and gross pathological observations. The present study also aims to evaluate the growth performance of the all-male Nile tilapia population at various stocking densities and determine an ideal stocking density as well as appropriate water quality under the climatic and ecological conditions prevailing in the cultured systems in southern part of Bangladesh.

METHODOLOGY

Experimental fish and deign: Adult tilapia (*Oreochromis niloticus*) affected by satreptococcal disease were collected from Naoili, Bisali, Kashipur, Chachuri, Kalia, Shingashul in Jessore and Narail districts (arrow indicates the working area in map; Figure 1) from a pilot farm of South-West Area Integrated Water Resources Planning and Management Project (SAIWRPMP) funded by Asian Development Bank (ADB), Ministry of Water Resource and Bangladesh Water Development Board (BWDB) during the period of 2012-2013. Independent experiments were designed *in vitro* at the Department of Fisheries and Marine Bioscience laboratory, Jessore University of Science and Technology and Faculty of Fisheries, Bangladesh Agricultural University, to evaluate the health, dietary needs, density, dose of tilapia (diseased or disease-free) at different replicable stocked such as: 80, 120, 160, 200, 240 and 280 fry/decimal corresponding to density classes (I, II, III, IV, V and VI), bacterial strain, infection trails and histopathology.

Health status assays: Blood and interstitial fluid (haemolymph) was withdrawn from tilapia, *O. niloticus* by puncturing the unsclerotized membrane with a 21 gauge needle and syringe. For total haemocyte (blood cell) counts (THC), the blood was mixed with an equal volume of ice-cold fixative (5% formaldehyde in 3% sodium chloride solution) and cells counted with a Neubauer haemocytometer. For blood chemistry (total protein, ammonium and glucose) blood was allowed to clot for ~60 min on ice, centrifuged (5000 g; 10 min) and the resulting serum stored at -80^{o}C. Total protein was determined using a bicinchromic acid assay kit (Pierce and Warriner) using BSA as a standard, while glucose and ammonium concentrations were determined using methods (Bergmeyer 1984, Bolz and Howel 1978) modifications. Glycogen levels of the hepatopancreas (digestive gland) were determined using the anthrone method (Van Handel 1965) with type II glycogen (Sigma-Aldrich) as the standard. All assays were performed in 96-well microtitre plates and absorbance's read on an Anthos II plate reader.

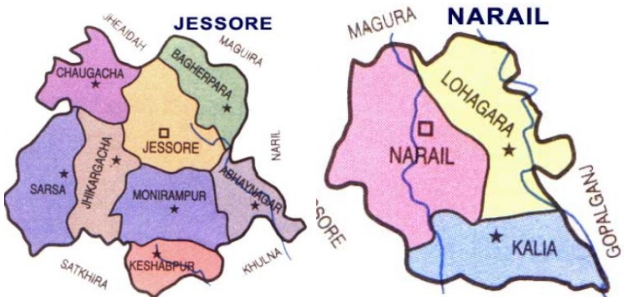


Figure 1: Map of the study areas, Jessore and Narail districts

Culture pond: Fifty newly excavated 0.02 to 0.05 ha freshwater earthen ponds were completely drained, tilled, dried, leveled and limed at the rate of 247 kg/ha during the sunny days after plowing. Ponds limed and one week later water level was increased up to 1.37 m to support natural productivity. Organic fertilizers (cattle manure 1235 kg/ha and compost 1235 kg/ha) and inorganic fertilizers (urea and TSP; 24.7 kg/ha) were applied in time intervals (15 days) during the culture period.

Monosex Nile tilapia production: Three days old mixed sex juveniles of Nile tilapia (mean weight 0.0021±0.005 g; mean length 1.20±0.010 cm) were collected from the hatchery. Fish stocked in each of the culture systems at a density of 50 individuals/m^3 and reared for four months. The fish were fed with 17α-methyltestosterone (17αMT) hormone treated diet (35% protein) with a dose of ≤60 mg MT/kg feed at a rate of 50%, 30% and 20% body weight/day for 21 days (Haylor and Pascual 1991, Popma and Green 1990) and other report showed 10 mg MT/kg feed at a rate of 20% body weight/day for 30 days (Chakraborty *et al.* 2007, Chakraborty and Banerjee 2009) and hormone treated diets were prepared by the 99% ethyl alcohol evaporation technique (Shelton *et al.* 1978). Fish (mean weight 18±2 g) acclimatized and released in

the 50 pilot ponds at six different stocking densities of 19760, 29640, 39520, 49400, 59280 and 69160 fry/ha corresponding to density classes (I, II, III, IV, V and VI). After first month, the fish were fed twice to four times daily with hormone untreated control diet (crude protein content 25 to 30% and total digestibility 2500±300 kcal/kg feed) at a constant rate of 10, 8, 7, 6, 5, 4, 3, 2 and 1% of body weight/day for the next 120 days respectively.

The entire experiment was conducted in three replicating units for statistical validation. Throughout the entire culture period different water quality parameters like temperature, DO, free CO_2, pH, total alkalinity and transparency were monitored daily using standard procedures (Apha 1998). Besides, growth parameters like specific growth rate (SGR), relative growth rate (RGR), daily weight gain (DWG), food conversion ratio (FCR) and protein efficiency ratio (PER) were measured according to standard method (Pechsiri and Yakupitiyage 2005) at the end of the culture period. The fish were harvested by dewatering method using water pump from the pond at 4 month post stocking, fish average weight and total fish production was also calculated.

Bacterial strains and culture of the putative causative agent: The *S. iniae* strain used in this study was *S. iniae* SI01, which was isolated in Nobember 2011 from Nile tilapia, *O. niloticus* suffering from streptococcosis in pilot farm from SAIWRPMP project. Brain heart infusion (BHI) medium (broth and agar) and growth temperature 25°C was used to culture the bacterial strain. The stock organism was kept in culture broth supplemented with 15% (v/v) glycerol at -80°C. Attempts to isolate and culture the causative agent of streptococcal disease were made using various dilutions (0–10000 fold) of infected haemolymph spread on a variety of solid culture media. These included tryptic soy agar, marine agar 2216, Mueller–Hinton agar, brain heart infusion agar, blood agar (5% sheep's blood), fetal calf (5% final concentration) supplemented tryptic soy agar and tryptic soy agar supplemented with filter-sterilized (0.22 mm) fresh tilapia blood (5% final concentration). Where appropriate, 2% sodium chloride (final concentration) was added to adjust the tonicity of the media. Plates were incubated at temperatures ranging from 15 to 25°C for more than 72 hours.

Infection trails: The *S. iniae* strain SI01 and *O. niloticus* identified biochemically (Berridge *et al.* 1998) was used to challenge the healthy tilapia. The isolate of *S. iniae* taken from a blood agar plate was grown in tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) for 24 hours prior to use. Tilapia in the density by dose experiment was infected by immersion exposure to *S. iniae*. Tilapia were added at a density of 25 fish per liter of water with aeration (e.g., 50 fish into 2 l water; 100 fish into 4 l water) with the appropriate amount of broth culture of *S. iniae* added followed by a 15 min timed exposure before release to aquarium. Plate counts from immersion water (triplicate) showed that the dose used was about 3.2x107, 6.1x107 and 2.5x108 colony-forming units (CFU)/ml (Table 3) were the infectious dose was 9.6x107 CFU/ml. Cohabitation was accomplished by placing five moribund or fresh dead *S. iniae* infected tilapia into each tank for 48 hours prior to removal. The dead or moribund tilapia used to infect by cohabitation were previously infected by immersion exposure to 3x107 CFU/ml and showed signs of streptococcal disease prior to stock to the tanks. No attempt was made to quantify the number of bacteria in the cohabitation experiment using dead or moribund fish. After fish were infected, mortalities were monitored twice daily for 28 days. Sample from brain and anterior kidney of dead fish were cultured on blood agar (Remel, Lenexa, KS) to confirm *S. iniae*.

Histopathology: Both streptococcal disease-free and affected tilapia, *O. niloticus* were killed by the injection of ~5 ml Bouins sea-water fixative, 5% formaldehyde in sea water and Davidson's sea-water fixative. Tissues (brain, gills, muscle, gonad, hepatopancreas) were removed and left for 24 hours in these fixatives, dehydrated and embedded in paraffin wax. Sections (~8 µm thick) were stained with Cole's haematoxylin and eosin, Giemsa, or Pappenheim's stain. Photomicrographs were taken on an Olympus BX50 microscope equipped with a digital camera.

Statistical analyses: Duncan's multiple tests (at 5%) were applied to evaluate the differences among means and due to dose, density and dose by density interactions (Duncan 1955). The data between treatment groups were represented as means±SD and analyzed by one-way analysis of variance (ANOVA) and Tukey's test was used to compare the mean values between individual treatments (Zar 1984) using Stat Plus 2007 professional.

RESULTS

Tilapia culture: All the different density class ponds (Table 1) showed DO (4.5 mg/l to 7.5 mg/l), temperature (25.2°C to 33.7°C), pH 7.2 to 8.0 (max. 8.0±0.1), alkalinity (122.5 mg/l to 145.6 mg/l), transparency (25.2 cm to 39.5 cm), free CO_2 concentration (highest 8.2±0.4 mg/l; lowest (3.1±0.4 mg/l), and average survival rate 73.92%. Stocking density had significant effect (P<0.01) on the survival percentage, maximum (84.5±1.55%) and minimum (62±1.08%) survival were found in the density classes I and VI, respectively (Figure 2). Stocking density (200 fish/decimal; class IV) had significant effect (P<0.01) on the total production (5,000 to 5,500 kg/ha) and average weight 300 to 320 g and also showed the highest weight,

length, RGR, SGR, PER and protein content (Table 2). Among the six different density categories, fishes of ponds of class IV showed maximum yield while the minimum yield was observed in density class I (Figure 2). FCR was maximum for the density class I where stocking density was 19760 fish/ha and minimum for density class VI corresponding to 59280 fish/ha (Table 2). The overall average production of all the density groups was 3195.6±287.8 kg/ha.

Table 1. Weekly values of physicochemical parameters of different density class ponds during monosex tilapia culture

Density classes	Physicochemical parameters					
	Temperature (^{0}C)	DO (mg/l)	Free CO_2 (mg/l)	pH	Transparency (cm)	Alkalinity (mg/l)
I	25.2±0.8	6.1±0.08	5.1±0.6	7.2±0.08	39.5±0.3	123.4±3.3
II	28.6±0.8	7.5±0.08	6.5±0.4	7.4±0.1	35.0±2.2	139.7±2.0
III	31.0±0.7	7.1±0.1	3.1±0.4	8.0±0.1	35.3±0.3	145.6±1.8
IV	30.7±0.7	7.1±0.09	5.4±1.0	7.4±0.08	30.2±1.0	124.9±4.6
V	31.2±0.8	7.4±0.1	6.5±0.4	7.8±0.09	25.2±0.8	122.5±2.5
VI	33.7±1.0	4.1±0.1	8.2±0.4	7.2±0.09	27.8±1.0	132.1±4.3

Table 2: Growth performances of monosex nile tilapia at various stocking densities during pond culture

Density classes	Growth parameters							
	Weight (g)	Length (cm)	DWG (g/day)	RGR (%)	SGR (%)	FCR	PER	Protein (% wet weight)
I	192.7f ±2.3	21.2f ±0.2	1.74f ±0.02	448.43f ±0.6	1.9d ±0.04	2.5a ±0.04	1.3d ±0.03	13.8e ±0.04
II	231.0d ±2.3	22.6d ±0.2	2.19d ±0.08	557.48d ±0.6	2.1c ±0.06	2.3b ±0.02	1.4c ±0.02	14.18d ±0.09
III	291.6b ±2.4	24.7b ±0.1	2.87b ±0.02	729.95b ±0.3	2.3a ±0.05	1.9d ±0.03	1.5b ±0.01	16.03b ±0.1
IV	309.6a ±2.6	25.4a ±0.2	3.13a ±0.03	780.22a ±0.5	2.4a ±0.03	2.1c ±0.03	1.6a ±0.01	16.58a ±0.09
V	261.5c ±1.6	23.5c ±0.1	2.49c ±0.02	644.15c ±0.3	2.2b ±0.02	1.8e ±0.02	1.5b ±0.02	15.73c ±0.09
VI	208.9e ±2.2	21.8e ±0.1	1.93e ±0.02	494.23e ±0.4	1.9d ±0.02	2.4a ±0.03	1.4c ±0.02	14.15e ±0.06

Culture of the putative streptococcal disease agent: Attempts to isolate and grow the potential causative agent on a wide range of agar-based culture media failed despite the use of long incubation periods and a range of dilutions of the infected haemolymph. These procedures were attempted using haemolymph from different streptococcal diseased affected fish. In all cases, the plates were devoid of colonies apart from the occasional contaminant (<0.001% of the total number of bacteria seen in blood by direct microscopy). Such colonies were not found on a routine basis from streptococcal disease affected fish.

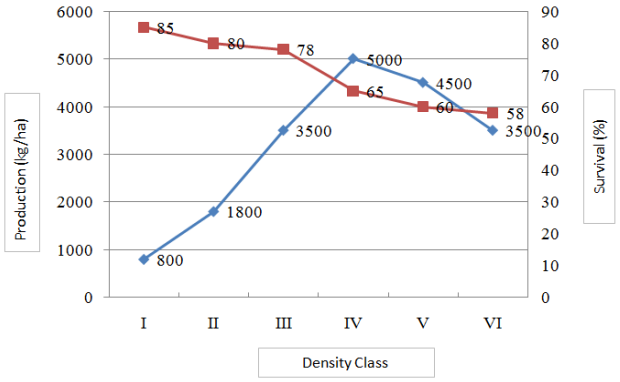


Figure 2: Total production and survival percentage of monosex tilapia under different stocking density classes

Infection: Mortalities in the low, medium and high-density treatments began occurring 2 to 4 days post infection. Typical signs of streptococcal disease *i.e.*, erratic swimming, darkening of the fish, exophthalmia and cloudy eyes were observed (Plumb 1997) in the dead and dying tilapia following infection. *S. iniae* was isolated from both brain and anterior kidney of fresh dead tilapia on blood agar. Density was shown to significantly affect mortality of tilapia infected by immersion exposure to *S. iniae* (Table 3).

A significant difference (P<0.05) was seen in the mortality when compared low (6.4%) and medium (30.6%) and low and high (26.4%) density treatments. No significant difference was observed when compared medium and high-density treatments. Two-way analysis of variance demonstrated density had a significant effect on *S. iniae* mortality (P=0.0001). Dose utilized had little effect on mortality. High density by dose however, showed a significant interaction (P=0.001), which is evident when looked at the mortality of each dose in the high-density trial (Table 3). Mortalities in the cohabitation treatment began to occur between day 5 and day 7 after addition of the dead moribund fish.

Dead fish were seen earlier (day 2) in the immersion treatments. Signs of streptococcal disease were observed in both cohabitation and immersion treatments and *S. iniae* was isolated from fresh dead fish on blood agar. No mortality occurred in the noninfected tilapia for the 28 day trial. No significant differences (P>0.05) in mortality was observed between replicates 1 and 2 of the immersion exposed treatments (41.3% and 37.3%, respectively) and of the cohabitation treatment (26.6%, Table 4). All the infected treatments exhibited significantly higher (P=0.0008) mortality when compared to the control treatment where no fish died.

Gross pathology and disease prevalence: Infected tilapia initially refrained from eating, the stomach and gut becomes empty or nearly empty, in most cases fibrinous material seen in the peritoneal cavity; seriously weakened tilapia ultimately died (within 7 days of initial sampling). Diseased fish had significantly higher levels of ammonium in the blood than non-diseased individuals sampled at the same times (18.3±1.8 µg ml-1 blood in diseased fish compared with 3.4±0.4 µg ml-1 blood in non-diseased fish; mean values±SEM, n=20–81, $P<0.001$, Student's t-test).

During routine sampling employed as a method of identifying affected individuals showed eye lesions, haemorrhages (endophthalmia or exophthalmia) and also lead to unilateral (one eye) or bilateral (both eyes) opacification of the eye (Figure 3A and 3B). Streptococcosis developed abscesses on the inferior jaw lead to burst and turn into haemorrhagic ulcers (Figure 3B and 3C). This disease affected the central nervous system of tilapia and infected fish engaged in various forms of abnormal behavior, such as lethargy, disorientation, swirling, bending of the body and ascites was accompanied by a protruding anus (Figure 3A-3D). Skin was found to exhibit the characteristic multi-focal pin-point haemorrhage, normally located near the mouth, anus, genital papilla and the base of the fins (Figure 3C and 3D).

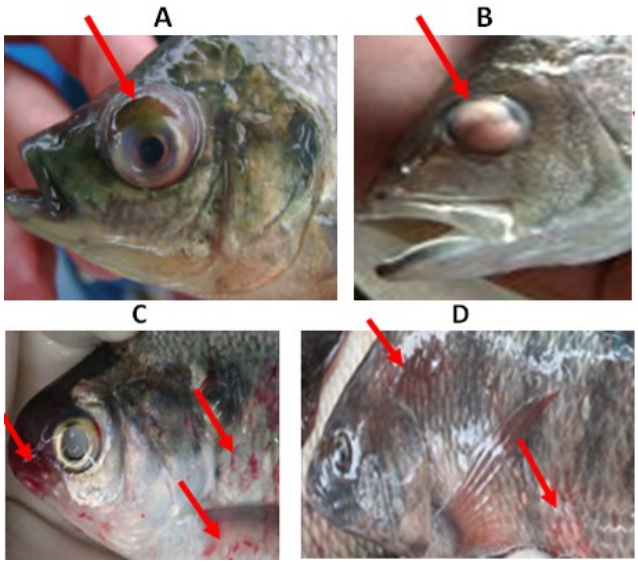


Figure 3: Gross signs of disease in an infected tilapia with *Streptococcus iniae*

Tilapia collected from the wild at all times of the year showed a very low (<1%) prevalence of disease. A long-term survey of tilapia from the pilot tilapia farm (SAIWRPMP) showed a high prevalence of streptococcal disease in tilapia during late summer when water temperatures were high (Figure 4).

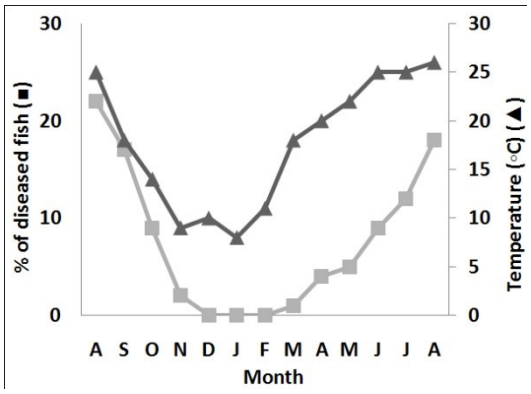
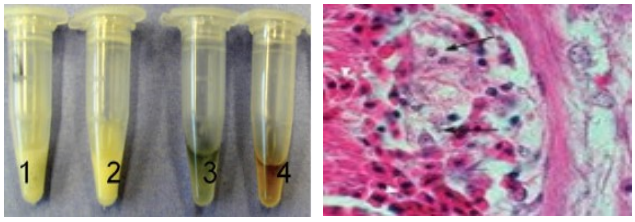


Figure 4: Effect of season and water temperature on the prevalence of streptococcal disease in a pilot farm

During routine sampling, blood was found from infected tilapia to exhibit the characteristic bacterial appearance for this disease (Figure 5A). Examination of blood by phase-contrast microscopy showed the existence of large numbers of bacteria-like particles ~1 mm long together with circulating blood cells (haemocytes) in the brain lead to septicaemia, haemorrhages and inflammation around the nervous ganglion on the external muscular layer of the stomach of a tilapia (Figure 5B) and followed by eye, spleen, kidney, liver and heart.



(A) *(B)*

Figure 5: (A) Appearance of haemolymph from streptococcal disease affected tilapia (1, 2) and non-diseased tilapia (3, 4). (B) Phase-contrast micrograph of blood from a streptococcal disease affected tilapia

Effects of disease on blood cells, serum protein, glucose and ammonium, and hepatopancreatic glycogen: Blood from the streptococcal disease affected tilapia showed THC of apparently healthy fish decreased significantly upon development of streptococcal disease (Figure 6A). Similarly, the amount of total protein in serum also significantly declined before the initial diagnosis of disease (Figure 6A), while in the case of glucose and ammonium (Figure 6B), a significant increase in these factors was found at the time of diagnosis. Levels of glycogen in the hepatopancreas (digestive gland) of affected and non-affected fish were not found to differ significantly.

Histopathology: Figure 7A shows variety of epithelial cells, extra-tubular haemal spaces containing blood vessels, fixed phagocytes, and freely circulating

haemocytes in gastrointestinal tract. A gram stained, impression print of *S. iniae* infected brain tissue illustrating the gram-positive chains of cocci (Figure 7B). Occasionally, haemocyte accumulations, termed nodules, were found in the haemal space; some of these were seen to contain debris and bacteria-like particles in brain tissue of diseased tilapia (Figure 7C). All other tissues examined (gonad, alimentary canal and muscle) were structurally normal (not shown). The degeneration in hepatocytes, vaculation, congestion and necrosis in hepatocytes *O. niloticus* injected with *S. iniae* (Figure 7D).

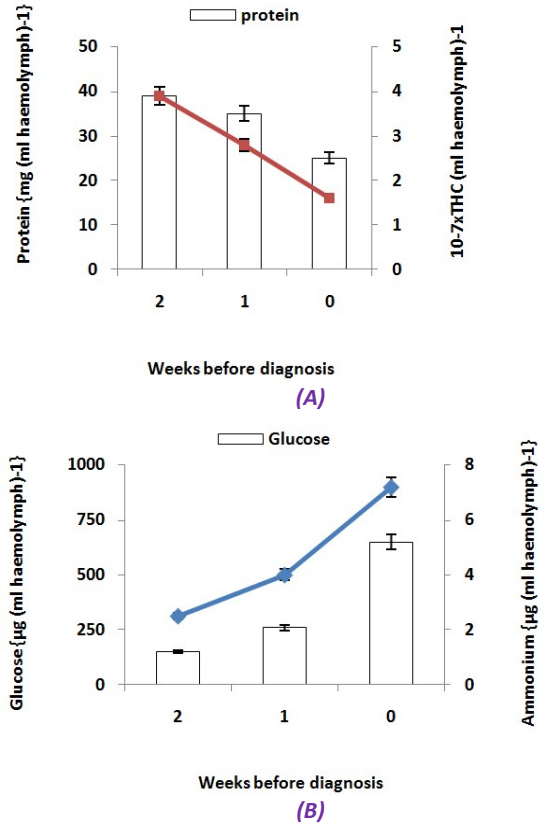


Figure 6: (A) Changes in total haemocyte counts and total serum protein, and (B) serum glucose and serum ammonium levels prior to the initial identification of milky disease in crabs. Values shown are means±SD, n=12–17. **P*<0.05, ***P*<0.01 and ****P*<0.001 compared with time 0

Transmission of disease and temperature stress experiments: Asymptomatic carriers have been found and horizontal transmission has been reported during outbreaks of streptococcal disease. This disease transmitted horizontally and major routes of transmission apparently via infected fish to disease-free tilapia with fed skin, organs and trash fish or via cannibalism, injuries, etc., and also from the contaminated water to the fish after 6 to 8 weeks post-challenge (Table 3 and 4). Although the disease caused mortality in fish as small as 10 g, however, bigger fish (from 100 g to market size) were more susceptible to the disease. The greatest losses occurred in large fish close to the market size (300 to 400 g). Outbreaks occurred when fish exposed to stress with suboptimal oxygen levels (<2ppm) in the water and overcrowding (50 fish/m^3) for a long period of time (Table 3 and 4). Fish also showed acute symptoms with peaks of mortality lasting 2-3 weeks during the high water temperature (>25^{o}C), it was chronic, when water temperature was low (<10^{o}C) and showed persistent level of mortality (Table 3 and 4). Furthermore, during this time, the THC of these individuals was differing from that affected fish to non disease affected animals (data not shown). Intrahaemocoelic injection of apparently healthy tilapia with blood (100-300 ml containing ~108 bacteria) from disease affected individuals resulted in large mortalities (100%) within 24 hours of injection. Similarly, injection of fish with the same volume of cell-free haemolymph (*i.e.* with neither haemocytes nor bacteria) from disease affected fish resulted in 100% mortality at 24 hours post-challenge. In the case of fish injected with the same volume of whole blood from uninfected fish, no mortalities were observed 24-48 hours post-challenge.

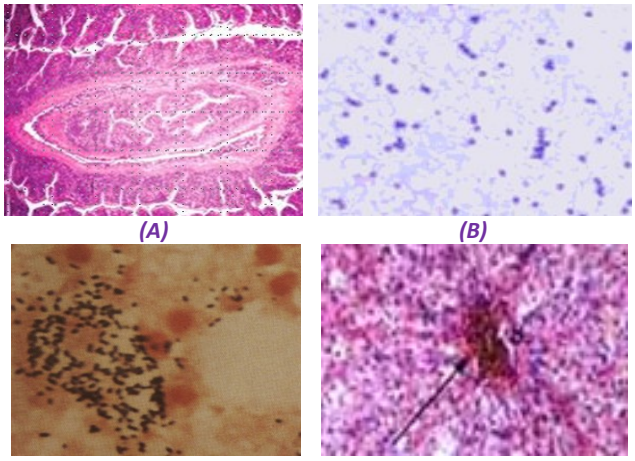


Figure 7: Histopathological changes in *Oreochromis niloticus* injected with *Streptococcus iniae* (stained with H & E), A; extra-tubular haemal spaces containing blood vessels in gastrointestinal tract. B; brain tissue with gram-positive chains of cocci. C; brain tissue contain debris and bacteria-like particles. D; liver with degeneration and congestion

DISCUSSION

The current study has revealed the epidemiology and pathology of streptococcal disease in tilapia cultured farms during summer months when high water temperatures prevail. It is well established that tilapia, like all aquatic animals, are highly sensitive to environmental degradation such as elevated or depressed temperature, reduced oxygen tension, overcrowding and changes in the concentrations of xenobiotics or waste products in the water (Le Moullac and Haffner 2000). Temperature affected on the immune systems of tilapia

resulting in immune suppression and also the antibacterial activity of the haemocytes of tilapia which enhanced disease prevalence and severity (Chang and Plumb 1996) which was similarly observed in the farm and in vitro conditions during the summer months in the present study. In the present study THC was found rapid decline in early stages of streptococcal disease and significant elevation in the amounts of glucose and ammonia in the blood of affected tilapia due to environmental stress (Yoganandhan *et al.* 2003). Intrahaemocoelic injection of healthy tilapia with blood from streptococcal disease affected tilapia resulted in their rapid (>24 hours) mortality but without any of the key symptoms (septicaemia-like infection) of the disease as seen in natural infections in the farm and *in vitro* conditions.

Table 3. Density and dose effect on mortality following *S. iniae* immersion challenge in tilapia held for 28 days

Dose CFU/ml plate count	*No. fish per tank five tanks for each dose*	*No. dead/No. total**	*% Mortality (SEM)***
$3.2x10^7$	25	8/125	6.4 (±1.5)[a]
$6.1x10^7$	25	6/125	4.8(±0.8)[a]
$2.5x10^8$	25	10/125	8.0(±1.6)[a]
	Low density***	Mean % mortality (SEM)[¶]	6.4(±0.8)[a]
$2.5x10^7$	50	93/250	37.2(±2.1)[a]
$3.8x10^7$	50	64/250	25.6(±2.6)[b]
$7.1x10^7$	50	73/250	29.2(±3.7)[a,b]
	Medium density	Mean % mortality (SEM)	30.6(±2.0)[B]
$2.7x10^7$	100	83/500	16.6(±1.9)[a]
$4.9x10^7$	100	137/500	27.4(±5.1)[a,b]
$1.9x10^8$	100	176/500	35.2(±4.6)[b]
	High density[≠]	Mean % mortality (SEM)	26.4(±3.0)[B]

* *Fresh dead fish were cultured on blood agar and S. iniae was isolated from the brain and kidney.*

** *Lower case superscripts denote differences in the means on each group of mortality data in low, medium and high treatments (i.e., dose) by Duncan's Multiple Range Test.*

*** *Low density = 5.6 g/l*

¶ *Upper case superscripts denote differences in the mean percent mortality as compared by two-way analysis of variance using Duncan's multiple range test to compare means. Significance level was equal to P<0.05.*

ǂ *Medium density = 11.2 g/l*

≠ *High density = 22.4 g/l*

Lethargy, loss of appetite, sluggish movement, dark discoloration of the skin and respiratory manifestations were found as common clinical sings and histopathological observations revealed lesions, necrosis, haemorrgahe and ulcer in all vital organs of necropsied fish with accumulation of fluids in abdomen, congested gills and dark liver in this study. Similar clinical sings and histopathological hepatic lesions, severe congestion, vacuolar degeneration of hepatocytes, focal coagulative necrosis, focal replacements of the hepatic parenchyma with extravagated blood, hemorrhage, activation of melanomacrophages were reported of aflatoxicated Nile tilapia (Mehrim *et al.* 2006).

Table 4: Density effect on route of infection with *Streptococcus iniae* (100 fish/tank; 3 tanks/treatment)

Treatment	*No. dead/no. total**	*% Mortality (SEM)***
Non infected***	0/300	0.0 (±0.0)[A]
$9.6x10^7$ (re 1)[¶]	124/300	41.3(±7.3)[B]
$9.6x10^7$ (rep 2)	112/300	37.3(±2.8)[B]
Cohabitation[≠]	80/300	26.6(±2.5)[B]

* *Fresh dead fish were cultured on blood agar and S. iniae was isolated from the brain and kidney.*

** *Significant differences in the means are noted by different letters (P=0.0008). The standard error of the mean is presented in parentheses.*

*** *Non infected tilapia (controls) which were immersed in tryptic soy broth only.*

¶ *Tilapia infected with S. iniae (CFU/ml) as determined from triplicate plate counts of immersion water on blood agar plates.*

≠ *Cohabitation infection was accomplished by adding five moribund or fresh dead tilapia to each tank for 48 h before removal.*

For tilapia culture generally considered the optimum temperature 20-30°C or above; DO: 4-5 mg/l; free CO_2: 20 mg/l; pH: 6.5 to 11.0; transparency: 25-35 cm and stocking density: 200-250 fish/decimal (Tran-Duy *et al.* 2008), similar results were observed more or less stable during the experimental period. The cannibalism had found a main cause of tilapia fry mortality at high stocking densities (El-Sayed 2002) this study also indicates an inverse relationship between the stocking density and growth potential of the fish when it is stocked at a very high density. But interestingly, the maximum growth and well survival percentage of the fish is obtained at an intermediate stocking level. Monosex Nile tilapia stocked in ponds at a low density showed better growth than at a higher density, due to higher density showed intense antagonistic behavioral interaction, competition for food and living space, environmental stress, social dominance, lower individual growth rates, deterioration in water quality, rise plasma cortisol concentrations (Diana *et al.* 2004). The present study established an optimum stocking density level of tilapia for maximum utilization of food and space with minimum stress and energy expenditure resulting in higher growth potential of the fish, on other hand at the low stocking densities showed decreased feed utilization efficiency, stunted growth, feed wastage and increase production cost.

In our study, fish were housed in aquaria that allowed for one-half water exchanges per hour. But in closed culture systems, tilapia held at high densities, little or no water is exchanged, which leads the significant increases in mortality 70% or more (Craig *et al.* 2000). Dose of *S. iniae*

applied to infect the tilapia via immersion exposure with highest density treatment, showed significantly higher mortality with the greater number of *S. iniae* and low-density treatments resulted in little mortality. Increased density enhances direct contact with a greater concentration of *S. iniae* in water from dead diseased fish that favors transmission of *S. iniae* and subsequent inoculation via abrasion of the infected fish skin, resulted in large mortality (Perera *et al.* 1997). We were successful in inducing mortality in tilapia following cohabitation by placing previously infected tilapia in the same tank for 48 hours exposure before removal (Robinson and Meyer 1966). While the dead or moribund fish were in the tanks, we observed cannibalism of eyes and viscera. This suggests an oral and/or olfactory mode of infection; able to infect tilapia using direct delivery of *S. iniae* to the gut which was responsible for infection by waterborne *S. iniae*.

Many studies have been carried out to reveal the transmissions of *Streptococcus* sp. from the newly introduced fish into the farm (Nguyen *et al.* 2002), bacteria on excreted feces of infected fishes, survive in the water, be infectious to other healthy fish (Nguyen *et al.* 2002), infected thrashed fish as fish feed also responsible for the outbreaks of streptococcosis in farm (Kim *et al.* 2007). The present study revealed the horizontal transmission of the pathogens between fish in cohabitation experiment, similarly this mechanism usually involved in fish that were cultured in high densities, same as different species of wild and cultured fish (Evans *et al.* 2002), infection have been occurred through wounds and abrasions of the skin (Xu *et al.* 2007). The fish cohabiting barramundi pens had the same *S. iniae* strains as the barramundi, in addition, the transmission among the species of reef fish to fish via contact and abrasion were also observed (Bromage and Owens 2002).

CONCLUSION

The number of *S. iniae* or dose used in the cohabitation experiment (cannibalism) and seasonal effect was properly quantified for streptococcal outbreaks. Stocking density (49400 fish/ha; class IV) had significant effect (P<0.01) on the total production (5,000 to 5,500 kg/ha) and average weight 300 to 320 g and also showed the highest weight, length, RGR, SGR, PER and protein content. High density (100 fish/4 l tank) effect on route of infection *i.e.*, percentage of mortality was 41.3(±7.3), low density (25 fish/4 l tank) showed the percentage of mortality was 4.8(±0.8). Reducing fish density may be another management strategy to limit transmission of *S. iniae* in intensively farmed tilapia. This disease was more prevalent (>26%) at summer when the water temperature was approximately >25^{o}C, percentage of mortality was higher >41% during the overcrowding and improper water chemistry. Raised levels of glucose and ammonium in blood serum causes reduced number of free blood cells released into the haemolymph to stomach and gut, result in refrain from eating in diseased tilapia. The transmissions routes of infection, reduced free blood cell numbers and total serum protein, raised levels of glucose and ammonium in blood and histopathological observation suggested adequate control measures and a best management strategy should be employed to quickly remove dead and moribund fish from intensive culture operations. Reducing fish density, lowering water temperature, stopping overfeeding and water exchange also suggested a new approach for management strategy to limit transmission of *S. iniae* in intensively farmed tilapia.

REFERENCES

Abutbul S, Golan-Goldhirsh A, Barazani O and Zilberg D (2004) Use of Rosmarinus officinalis as a treatment against *Streptococcus iniae* in tilapia (*Oreochromis* sp.). Aquaculture 238(1-4): 97-105. DOI: 10.1016/j.aquaculture.2004.05.016

Apha (1998) Standard Methods for the Examination of Water and Wastewater. 20th edn. APHA (American Public Health Association), Washington, USA.

Bergmeyer HV (1984) Methods of Enzymatic Analysis. Metabolites I: Carbohydrates, 3rd edition. Weinheim: Verlag Chemie.

Berridge B, Fuller JD, de Azavedo J, Low DE, Bercovier H and Frelier PE (1998) Development of specific nested oligonucleotide PCR primers for the *Streptococcus iniae* 16S-23S ribosomal DNA intergenic spacer. Journal of Clinical Microbiology 36(9): 2778-2781.

Bolz DF and Howel JA (1978) Colorimetric Determination of Non-Metals. London: Wiley.

Bromage ES and Owens L (2002) Infection of barramundi *Lates calcarifer* with *Streptococcus iniae*: Effect of different routes of exposure. Diseases of Aquatic Organisms 52(3): 199-205.

Bromage ES, Thomas A and Owens L (1999) *Streptococcus iniae*, a bacterial infection in barramundi *Lates calcarifer*. Diseases of Aquatic Organisms 36(3): 177-181.

Bunch EC and Bejerano I (1997) The effect of environmental factors on the susceptibility of hybrid tilapia *Oreochromis niloticus*; *Oreochromis aureus* to streptococcosis. The Israeli Journal of Aquaculture-Bamidgeh 49: 67-76.

Chakraborty SB and Banerjee S (2009) Culture of monosex Nile tilapia under different traditional and non-traditional methods in India. World Journal of Fish and Marine Sciences 1(3): 212-217.

Chakraborty SB, Sarbajna A, Mazumdar D and Banerjee S (2007) Effects of differential dose and duration of 17-methyltestosterone treatment on sex reversal of Nile tilapia, *Oreochromis niloticus* at different age groups under Indian perspective. Asian Journal of Microbiology, Biotechnology & Environmental Sciences 9(3): 705-710.

Chang PH and Plumb JA (1996) Histopathology of experimental *Streptococcus* sp. infection in tilapia, *O. niloticus* and channel catfish, *Ictalarus punctatus*. Journal of Fish Diseases 19: 235-241.

Diana JS, Yi Y and Lin CK (2004) Stocking densities and fertilization regimes for Nile tilapia (*Oreochromis niloticus*) production in ponds with supplemental feeding, In: Proceedings of the sixth international symposium on tilapia in aquaculture, R. Bolivar, G. Mair and K. Fitzsimmons, Eds. Manila, Philippines, BFAR, Philippines, pp. 487-499.

Duncan DB (1955) Multiple range and multiple F tests. Biometrics 11: 1-42.

El-Sayed AM (2002) Effect of stocking density and feeding levels on growth and feed efficiency of Nile tilapia (*Oreochromis niloticus* L.) fry. Aquaculture Research 33(8): 621-626. DOI: 10.1046/j.1365-2109.2002.00700.x

Eldar A, Horovitcz A and Bercovier H (1997) Development and efficacy of a vaccine against *Streptococcus iniae* infection in farmed rainbow trout. Veterinary Immunology and Immunopathology 56(1-2): 175-183.

Eldar A, Bejerano Y and Bercovier H (1994) *Streptococcus shiloi* and *Streptococcus difficile*, two new streptococcal species causing a meningoecephalitis in fish. Current Microbiology 28(3): 139-143. DOI: 10.1007/BF01571054

Eldar A, Bejerano Y, Livoff A, Horovitez A and Bercovier H (1995) Experimental streptococcal meningo-encephalitis in cultured fish. Veterinary Microbiology 43(1): 33-40.

Evans JJ, Klesius PH, Gilbert PM, Shoemaker CA, Al-Sarawi MA, Landsberg J, Durendez R, Al-Marzouk A and Al-Zenki S (2002) Characterization of β-hemolytic group B *Streptococcus agalactiae* in cultured seabream, *Sparus auratus* and and mullet, *Liza klunzingeri*, in Kuwait. Journal of Fish Diseases 25: 505-513.

Facklam R, Elloitt J, Shewmaker L and Reingold A (2005) Identification and characterization of sporadic isolates of *Streptococcus iniae* from human. Journal of clinical microbiology 43(2): 933-937.

Ferguson HW, St Johns VS, Roach CJ, Willoughby S, Parker C and Ryan R (2000) Caribbean reef fish mortality associated with *Streptococcus iniae*. The Veterinary Record 147(23): 662-664.

Haylor GS and Pascual AB (1991) Effect of using ram testes in a fry diet for *Oreochromis niloticus* (L.) on growth, survival and resultant phenotypic sex ratio. Aquatic Fisheries Management 22: 265-268.

Kaige N, Miyazaki T and Kubta SS (1984) The pathogen and histophatology of vertebral deformity in cultured yellowtail. Fish Pathology 19: 173-179.

Kim JH, Gomez DK, Choresca CH and Park SC (2007) Detection of major bacterial and viral pathogens in trash fish used to feed cultured flounder in Korea. Aquaculture 272(1-4): 105-110. DOI: 10.1016/j.aquaculture.2007.09.008

Kitao T, Aoki T and Sakoh R (1981) Epizootic caused by β-haemolytic *Streptococcu*s species in cultured freshwater fish. Fish Pathology 15: 301-307.

Kvitt K and Colorni A (2004) Strain variation and geographical endemism in *Streptococcus iniae*. Diseases of Aquatic Organisms 61(1-2): 67-73.

Lafferty KD, Porter JW and Ford SE (2004) Are diseases increasing in the ocean? Annual Review of Ecology, Evolution, and Systematic 35: 31-54. DOI: 10.1146/annurev.ecolsys.35.021103.105704

Le Moullac G and Haffner P (2000) Environmental factors affecting immune responses in Crustacea. Aquaculture 191(1-3): 121–131. DOI: 10.1016/S0044-8486(00)00422-1

Lehane L and Rawlin GT (2000) Topically acquired bacterial zoonoses from fish: a review. Journal of the Australian Medical Association 173(5): 256-259.

Mehrim AI, Abdelhamid AM, Abu Shousha AAM, Salem MFI and El-Sharawy MAMM (2006) Nutritious attempts to detoxify aflatoxic diets of tilapia fish: 2-Clinical, biochemical and histological parameters. Journal of Arabic and Aquaculture Society 1: 69-90.

Nguyen HT, Kanai K and Yoshikoshi K (2002) Ecological investigation of *Streptococcus iniae* in cultured Japanese flounder (*Paralichthys olivaceus*) using selective isolation procedures. Aquaculture 205(1-2): 7-17. DOI: 10.1016/S0044-8486(01)00667-6

Pechsiri J and Yakupitiyage A (2005) A comparative study of growth and feed utilization efficiency of sex-

reversed diploid and triploid Nile tilapia, *Oreochromis niloticus* L. Aquaculture Research 36(1): 45-51. DOI: 10.1111/j.1365-2109.2004.01182.x

Perera RP, Johnson SK and Lewis DH (1997) Epizootiological aspects of *Streptococcus iniae* affecting tilapia in Texas. Aquaculture 152(1-4): 25-33. DOI: 10.1016/S0044-8486(96)01450-0

Pier GB and Madin SH (1976) *Streptococcus iniae*, a beta-hemolytic *Streptococcus* isolated from Amazon freshwater dolphin, *Inia geoffrensis.* International Journal of Systematic Bacteriology 26: 545-553.

Plumb JA (1997) Infectious diseases of tilapia. In: Costa-Pierce, B.A., Rakocy, J.E. (Eds.), Tilapia Aquaculture in the Americas vol. 1 World Aquaculture Society, Baton Rouge, LA, USA, pp. 212-218.

Popma TJ and Green BW (1990) Sex reversal of tilapia in earthen ponds. Research and Development Series No. 35. International Center for Aquaculture, Alabama Agricultural Experiment Station, Auburn University, AL, USA.

Robinson JA and Meyer FP (1966) Streptococcal fish pathogen. Journal of Bacteriology 92(2): 512.

Shelton WL, Hopkins KD and Jensen GL (1978) Use of hormones to produce monosex tilapia for aquaculture. In The Second International Symposium on Tilapia in Aquaculture. ICLARM Conference Proceedings 15, Eds., Pullin, R.S.V., T. Bhukaswan, K. Tonguthai and J.L. Maclean. Department of Fisheries, Bangkock, Thailand and International Center for Living Aquatic Resources Management, Manila, Philippines, pp: 10-33.

Shen ZH, Qian D, Xiu WJ, Gu JH and Shao JZ (2005) Isolation, identification and pathogenicity of *Streptococcus iniae* isolated from red drum *Sciaenops ocellatus*. Acta Hydrobiologica Scadanavika 29: 678-683.

Shoemaker C and Klesius P (1997) Streptococcal disease problems and control: a review. In: Fitzsimmons, K. (Ed)., Tilapia Aquaculture vol. 2 Northeast Regional Agricultural Engineering Service-106, Ithaca, NY, USA, pp. 671-680.

Shoemaker CA, Evans JJ and Klesius PH (2000) Density and dose: factors affecting mortality of Streptococcus iniae infected tilapia (*Oreochromis niloticus*). Aquaculture 188: 229-235.

Shoemaker CA, Klesius PH and Evans JJ (2001) Prevalence of Streptococcus iniae in tilapia, hybrid striped bass, and channel catfish on commercial fish farms in the United States. American Journal of Veterinary Research 62(2): 174-177

Stoffregen DA, Backman SC, Perham RE, Bowser PR and Babish JG (1996) Initial disease report of *Streptococcus iniae* infection in hybrid striped (sunshine) bass and successful therapeutic intervention with fluoroquinolone antibacterial enerofloxacin. Journal of the World Aquaculture Society 27: 420-436.

Tran-Duy A, Schrama JW, Van Dam AA, Verreth JAJ (2008) Effects of oxygen concentration and body weight on maximum feed intake, growth and hematological parameters of Nile tilapia, *Oreochromis niloticus*. Aquaculture 275(1-4):152-162. DOI: 10.1016/j.aquaculture.2007.12.024

Van Handel E (1965) Microseparation of glycogen, sugars and lipids. Analytical Biochemistry 11(2): 266-271. DOI: 10.1016/0003-2697(65)90014-X

Xu DH, Shoemaker CA and Klesius PH (2007) Evaluation of the link between grodactylosis and *Streptococcus* of Nile tilapia (*O. niloticus*). Fish Diseases 30: 230-238.

Yoganandhan K, Thirupathi S and Sahul Hameed AS (2003) Biochemical, physiological and haematological changes in white spot syndrome virus-infected shrimp, *Penaeus indicus*. Aquaculture 221(1-4): 1-11. DOI: 10.1016/S0044-8486(02)00220-X

Zar JH (1984) Biostatistical analysis. Prentice-Hall Inc., Englewood Cliffs, New Jersey, USA.

Zlotkin A, Hershko H and Eldar A (1998) Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. Applied and Environmental Microbiology 64(10): 4065-4067.