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Streptococcosis in Freshwater Fish in Nong Prajak and Nong Bua Lakes, Udon Thani, Thailand

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Abstract

While streptococcosis in fish is not generally prevalent, wherever it does occur increased mortality rates may significantly affect fish populations. In many cases, however, there may be no apparent signs of infection other than this. Moreover, certain aquatic species in the genus *Streptococcus* may cause zoonosis (disease in humans) under unusual conditions. Trichodinids and flukes are typically found on the gills, skin and fins of infected fish. In order to ascertain whether this bacterial disease is present locally, six species of fish, including *Oreochromis niloticus*, *Barbonymus gonionotus*, *Pangasius hypophthalmus*, *Anabas testudineus*, *Hampala dispar* and *Clarias* sp. were randomly collected from Nong Prajak and Nong Bua lake waters in Udon Thani, Thailand, in the summer. In this survey, clinical signs of streptococcosis infection were observed in some fish, while incipient necropsy was suggested by the presence of haemorrhages, enlarged spleen and pale liver. In a total of 28 colonies of bacteria isolated from the kidney, spleen and liver of necropsied fish, a number of examples of α-haemolysis (2), β-haemolysis (4) and γ-haemolysis (2) were recorded. Biochemical and DNA sequencing assays detected spherical chains of gram-positive bacteria, attributed to *Enterococcus faecalis*, in 3 isolates. Two isolates (P10K2 and B35S1) seem to be pathogenic to fish when injected. When a disease outbreak occurs, access to relevant information and assistance for rapid diagnosis and appropriate therapies are important approaches to minimize losses. However, maintaining optimal water quality, particularly with respect to dissolved oxygen, ammonia and nitrites is also recommended, to reduce the deleterious effects upon the immune system associated with such potential environmental stressors.

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Keywords: streptococci, freshwater fish pathogens, haemolysis, disease outbreaks, environmental stressors

1. Introduction

Streptococci are the most prevalent gram-positive bacterial pathogens in fish. They also infect numerous terrestrial animals, including humans [1, 2]. Fish pathogenic streptococci have been linked with *Streptococcus*

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agalactiae, S. dysgalactiae, S. equi, S. equisimilis, S. pyogenes, S. zooepidemicus, Enterococcus faecalis, E. faecium, Lactococcus lactis and *S. mutans*, which cause diseases similar to streptococcosis in several species [3].

Streptococcal disease in fish is not common; however, where it does occur, it creates increased numbers of potential agents of fish disease, with possible severe losses for aquaculture [4]. The disease was firstly reported in cultured rainbow trout in Japan in 1957 [5]. A variety of marine and fresh water fish species have since been reported as being vulnerable to *Streptococcus* spp. [4]. With the advent of intensive aquaculture, streptococcal infections have become a significant concern for fisheries in many areas of the world [6, 7], with high mortality rates (>50%) which occur over 3 to 7 days [2]. Some outbreaks are chronic infection in nature and mortalities may extend over several weeks, with only a few fish dying each day. Documentary evidence for streptococcal infection as the cause of disease in a population of fish should include reports of abnormal swimming behaviour, often described as spiralling or spinning. While streptococcal infection should be considered as a possible cause of such unusual behaviour, it may not manifest in all infected fish [2].

Clinical identification of streptococcosis is based partly on its haemolytic reactions on blood agar and Lancefield grouping. There are three types of haemolysis reactions: beta, alpha and gamma. Beta-haemolytic streptococci, which tend to cause the most acute streptococcal diseases, completely lyse the red cells surrounding the colony. Alpha-haemolytic streptococci, which cause partial (or greening) haemolysis around the colony, are associated with the reduction of red cell haemoglobin. Gamma-haemolysis is a term sometimes used for non-haemolytic colonies.

The presence of tissue necropsy can also help to determine the cause of morbidity and mortality in fish. However, the importance of historical and environmental factors cannot be overlooked, perhaps more so than for other classes of animal. The vast majority of disease problems in fish are intimately linked to water quality and management issues, factors that do not necessarily translate into directly observable pathologies. This may create a frustrating circumstance for clinicians expecting to determine the cause of disease based solely upon tissue biopsies or necropsy. Complete information on water quality, life support systems, general management practices, the condition of the fish and the nature of the problem is therefore of critical importance. The aims of the study were to isolate and identify bacteria causing streptococcosis in freshwater fish from lakes in central Udon Thani, Thailand. As no evidence of streptococcosis arising from external parasitic infection was found, it is reasonable to suggest that the immune systems of fish within these lakes is suboptimal, leaving the local fish population prone to infection.

2. Materials and methods

2.1 Sample collection

Bacterial infections grew very fast during the summer months when the water was warm. A total of 20 fresh water fish were randomly collected from Nong Prajak and Nong Bua lakes in central Udon Thani, Thailand, in May 2012, during summer. The samples were measured (in cm), weighed (in g) and examined externally and internally for clinical signs or other abnormalities.

2.2 External parasites analysis

Skin and fin smears were prepared for examination immediately after sample collection by gently scraping these surfaces and carefully examining the tissue. The gill arches and filaments were examined under a dissecting microscope. The external parasites were analysed using the wet mount preparation and microscopic inspection method at 40-400X magnification. Photographs were obtained with an Olympus camera. The parasites detected were classified according to their typical morphology [8].

2.3 Bacteriological analysis

Three fish organs (kidney, spleen and liver) were collected aseptically. The streak plate technique was used for microbial isolation. Each sample was streaked on a Tryptic Soy Agar (TSA) plate, then incubated at 35°C for 48 hours. The incubated plates were examined for morphological characteristics of the cultures representing distinct colonies. Colonies were randomly selected and subcultured to obtain pure isolates on fresh plates containing TSA with 5% human blood, then incubated at 35°C for 48 hours. Stock cultures were obtained and carefully labeled, then used for conventional identification using Gram's staining, catalase, oxidase and bile esculin tests, haemolytic reaction and carbohydrate fermentation.

2.4 In vivo study on the virulence of spherical chains of gram-positive bacteria

To confirm whether the isolated colonies were pathogenic to fish, cells (P10K2, B35S1 and *S. agalactiae* standard) were cultured in brain heart infusion (BHI) broth and incubated at 37°C for 48 hrs. Nile tilapia were infected with *E. faecalis* by intraperitoneal injection of 500 µl of bacterial suspension (3.5 x 10^3 CFU/fish). Mortality, appearance and behaviour were observed daily for 7 days. Bacteria isolated from dead and surviving fish were identified as described earlier.

2.5 PCR amplification and DNA sequencing analysis

The amplification and sequencing of three isolates (P2S2, P10K2, P10L1 and B35S1) were performed at the National Center for Genetic Engineering and Biotechnology (BIOTEC). DNA templates were prepared using a Genomic DNA mini kit (Geneaid Biotech Ltd., Taiwan). A PCR product for sequencing 16S rDNA regions was prepared using the following two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'). One hundred µl of a reaction mixture contained 15-20 mg of DNA template, 2.0 µmoles of each primer, 2.5 U of *Taq* polymerase, 2.0 mM $MgCl_2$, 0.2 mM dNTP and 1.0 µl of 10x*Taq* buffer (pH 8.8). The PCR amplification was programmed to carry out an initial denaturation step at 94°C for 3 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min, followed by a final amplification step at 72°C for 3 min. The PCR product was analysed by 0.8% (w/v) agarose gel electrophoresis and purified. Direct sequencing of the single-banded and purified PCR products (ca. 1500 bp) on 16S rDNA was performed in accordance with the *E. coli* numbering system. The nucleotide sequences were analysed using the BioEdit (Biological sequence alignment editor) Program http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Identification of phylogenetic neighbours was initially carried out by the BLAST and megaBLAST programs against the database of strain types and published valid prokaryotic nomenclature.

3. Results

3.1 Sampling

In May 2012 twenty fish representing six species were collected from Nong Prajak and Nong Bua lakes in central Udon Thani. The sample comprised of *Oreochromis niloticus*, *Barbonymus gonionotus*, *Pangasius hypophthalmus*, *Anabas testudineus*, *Hampala dispar* and *Clarias* sp. The clinical signs of abnormality and necrosy were shown in Table 1.

3.2 External parasites analysis

One ciliated protozoan parasite (*Trichodina* sp.) and one monogenetic fluke parasite (*Dactylogyrus* sp.) were observed in wet mounts of mucus from skin, fin and gills of *O. niloticus* and *B. gonionotus* during external parasite screening. Clinical signs and evidence of necropsy were typically confirmed the presence of streptococcosis infection.

Table 1. Clinical abnormalities and evidence of necropsy in collected fish

Water lakes	Fish number	Clinical signs	Necropsy finding
Nong Prajak	P2	corneal opacity	-
	P10	dropsy	gastrointestinal, enlarged spleen and pale liver
Nong Bua	B32	darkening	-
	B34	dropsy	ascites (dropsy/bloating) and gastrointestinal
	B35	dropsy	ascites (dropsy/bloating) and gastrointestinal
	B40	hemorrhage	-

3.3 Bacteriological analysis

A total of 28 colonies of bacteria was isolated from the kidney, spleen and liver of necropsied fish. Biochemical assays detected spherical chains of gram-positive bacteria in 8 isolates (Table 2). A number of examples of α-haemolysis (2), β-haemolysis (4) and γ-haemolysis (2) was found.

Table 2. Gram positive bacteria isolated from target organs (liver, spleen and kidney)

Fish number	Fish species	No. of isolates			
		Liver	Spleen	Kidney	Total
B35	*Pangasius hypophthalmus*	0	2	0	2
P2	*Oreochromis niloticus*	0	1	0	1
P5	*Oreochromis niloticus*	1	0	0	1
P10	*Barbonymus gonionotus*	0	2	2	4

Table 3. Apperance of infected fish after bacterial injection

Time (hrs)	Appearance		
	P10K2	B35S1	*S. agalactiae* Std.
0	no clinical sign	no clinical sign	no clinical sign
24	exophthalmia and death	no clinical sign	ulceration of the operculum
48	-	disorientation	decrease in feeding
72	-	circling listlessly	ulceration of the tail
96	-	still alive	corneal opacity, ascites and death
120	-	still alive	-
144	-	still alive	-
168	-	still alive	-

3.4 In vivo study of virulence of spherical chains of gram-positive bacteria

Nile tilapia began to show clinical signs of morbidity within 24 hrs after injection with the two isolates (P10K2 and B35S1) and the *S. agalactiae* standard (which was isolated from a patient in Udon Thani hospital). There was evidence of both acute and chronic streptococcosis in infected fish, as shown in Table 3 and Fig 1.

Biochemical tests of the colonies isolated from dead fish and fish injected with B35S1 showed the same results as P10K2, B35S1 and the *S. agalactiae* standard before injection into the fish (Fig 2).

3.5 PCR amplification and DNA sequencing analysis

The 16S rDNA amplicons were cloned and verified using information retrieved from GenBank databases. Three isolated colonies (P10K2, P10L1 and B35S1) were matched to *Enterococcus faecalis* with similar scores of 99.80, 100.00 and 100.00% respectively (Table 4). The phylogenetic relationships also showed results very close to the other isolates, and also to a related species, *Enterococcus faecalis* (Fig 3).

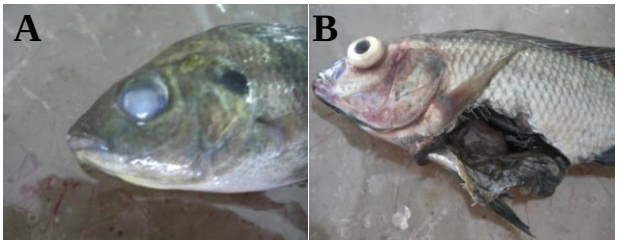

Fig. 1. Appearance of streptococcosis in infected tilapia after injection with (A) B35S1 and (B) P10K2 isolated bacteria

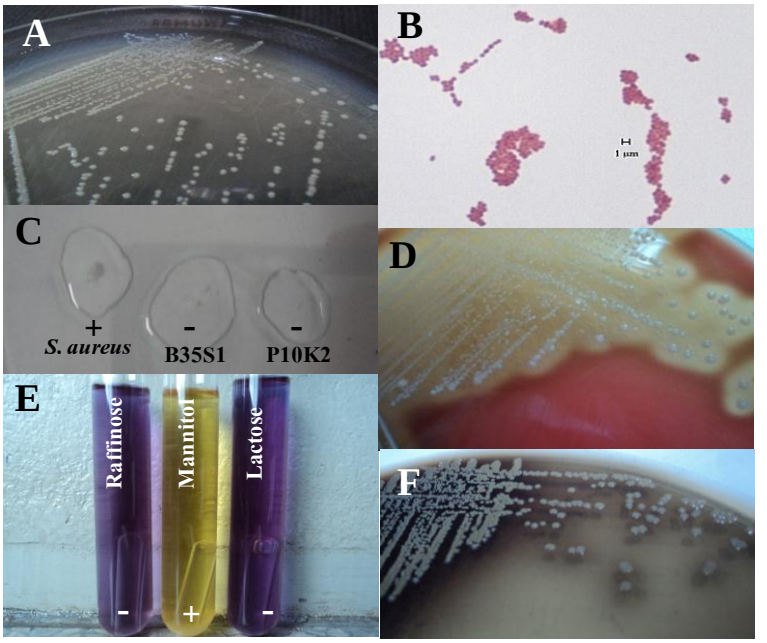

Fig. 2. Biochemical tests of the 2 isolates (P10K2 and B53S1) showing (A) morphology of colony, (B) cocci shape, (C) non-catalase compared to *Staphyococcus aureus*, (D) β-haemolysis, (E) acid produced from fermented manitol and (F) esculin hydrolysis

Table 4. %Similarity of 16S rDNA compare with closely related species

Sample number	Strain	Authors	Accession	Pairwise Similarity (%)
P10K2	*Enterococcus faecalis*	V583	AE016830	99.80
P10L1	*Enterococcus faecalis*	JCM 5803(T)	AB012212	100.00
B35S1	*Enterococcus faecalis*	JCM 5803(T)	AB012212	100.00

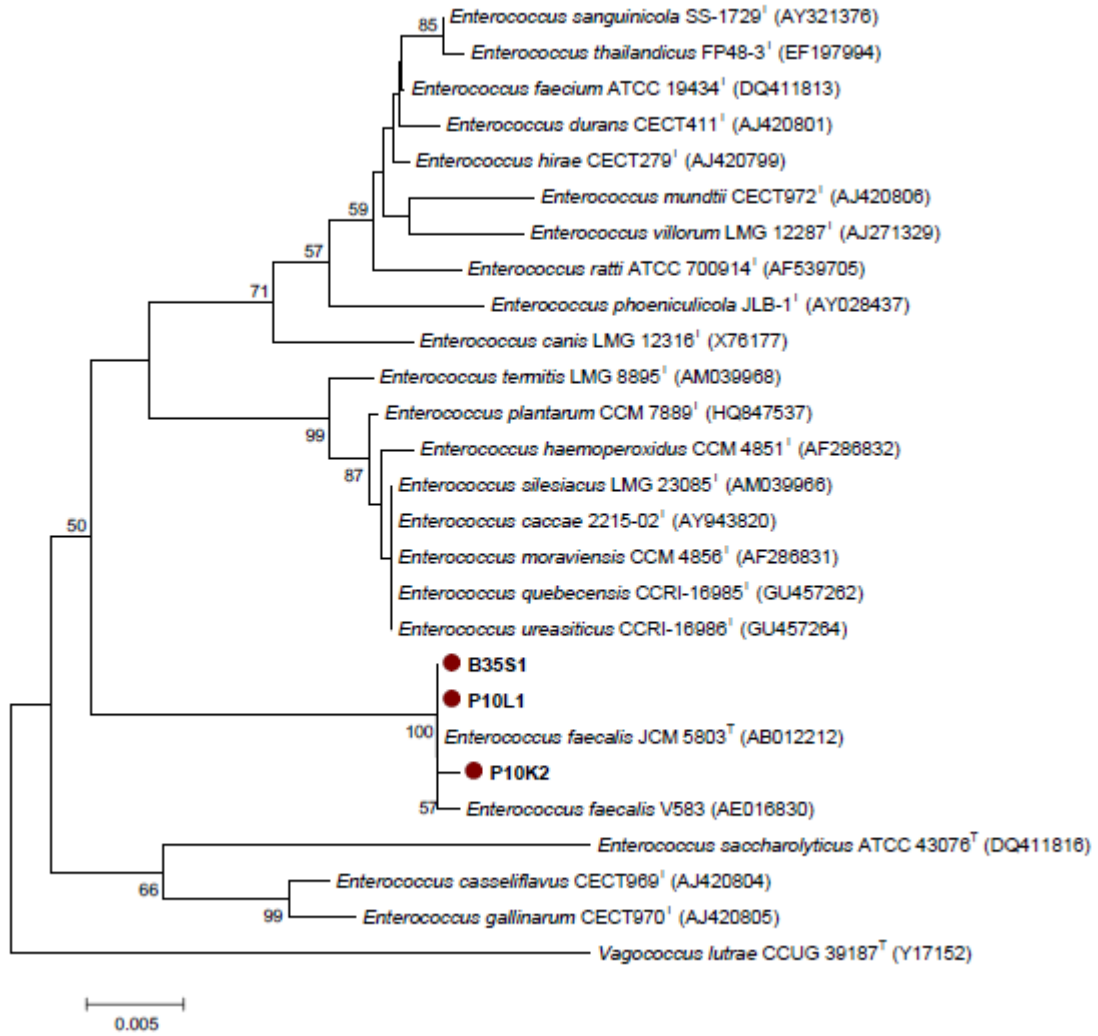

Fig. 3. Phylogenetic relationships between isolates and closely related species, based on partial lengths of 16S rDNA of P10K2, P10L1 and B35S1. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches

4. Discussion

Fish are susceptible to a wide variety of potentially pathogenic bacteria [9]. Many of these are considered to be saprophytic in nature, only becoming pathogenic when fish are physiologically unbalanced, nutritionally

deficient, or subjected to the influence of environmental stressors such as poor water quality and overstocking. Such conditions enable opportunistic bacterial infections to proliferate and may lead to considerable economic losses in aquaculture throughout the world as a result of high mortality rates in both cultured and wild fish populations [4, 10].

Compared to other external protozoans infesting fish, Trichodinid protozoa are relatively weak pathogens. However, many of these organisms are serious pathogens, causing high mortality rates, especially in hatchery-cultured fish species. *Trichodina* and *Epistylis* (and other stalked ciliates) can serve as useful indicators of water quality conditions, since both are commonly found in waters with highly organic content [11]. The genus *Trichodina* is found parasitising freshwater and marine fish species worldwide, but *Dactylogyrus*, also found worldwide, parasitises mostly freshwater cyprinids. The monogenes are flatworms with a direct life cycle that can cause significant damage to skin, fins and gills of fish. There are no known human health concerns associated with either *Gyrodactylus* or *Dactylogyrus* [12].

Virulence assays showed streptococcosis symptoms in tilapia which were injected with *Enterococcus faecalis* (P10K2 and B35S1) and gave similar results to the *Streptococcus agalactiae* (isolated from a patient in Udon Thani hospital). The result shows that although streptococcal bacteria can be a fish pathogen, healthy humans are at minimal risk of zoonosis (contracting this disease) from infected fish [2].

E. faecalis is a Gram-positive organism that is part of the normal gut flora of many humans, but which is also a serious pathogen that can cause meningitis, endocarditis and urinary tract infections. *E. faecalis* is one of the most common bacterial pathogens in aquacultural species such as rainbow trout, catfish and brown bullhead [13]. *S. agalactiae, L. garvieae* and *E. faecalis* are closely related groups of bacteria that can cause diseases similar to streptococcosis and lactococcosis [13, 14]. Although less common as other bacterial diseases, streptoccocosis does occur in fish, and can result in significant financial losses to the fish farmer. The disease frequently infects the brain, with the frequent appearance of abnormal behaviours, such as spinning. Other signs include lethargy, darkening, exophthalmia ("pop-eye"), haemorrhages, ascites (dropsy/bloating), and ulcerations, although these are not necessarily specific to streptococcosis. However, in many cases, there may be no obvious clinical signs other than rapid onset of mortality, with *E. faecalis* expressing cytolysin, an effector that can lyse host cells [15].

Clinical observations of live fish before the onset of necropsy can help identify clinically diseased animals and the exact location of lesions. However, knowledge of which species are susceptible to streptococcosis and establishing effective means of obtaining assistance for rapid diagnosis and proper therapy if a disease outbreak should occur is important ways to decrease losses.

5. Conclusion

Streptococcosis infections have increased prevalence and severity during summer months [16]. Some of these problems may manifest in gill, skin or fin and water quality is very probably implicated, at the very least being a significant contributing factor [2]. In Nong Prajak and Nong Bua lakes during summer, water quality is generally poor, with low DO (dissolved oxygen) values and high levels of BOD (biochemical oxygen demand) and phosphates [17]. Infection by bacteria and parasites is often secondary to management problems. Approaches such as external examination, microscopic evaluation, and obtaining an accurate history, including assessment of existing management practices, are often sufficient to determine an appropriate course of action where necessary [18]. However, maintaining optimum water quality, particularly with respect to dissolved oxygen, ammonia and nitrites is also highly recommended, in order to reduce the deleterious effects upon the immune system associated with these environmental stressors.

Acknowledgements

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