Streptococcus suis: an emerging and lethal pathogen in snakeskin gourami, *Trichopodus pectoralis*

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Abstract

The objective of this study was to determine the causative agent of an outbreak with clinical signs similar to those of piscine streptococcosis in farmed snakeskin gourami (*Trichopodus pectoralis*). Initial microscopic examination revealed the predominance of a Gram-positive, cocci bacteria in the brain and kidney of the diseased fish. This bacterium was successfully isolated and identified as *Streptococcus suis* based on nucleotide homology of 16S rDNA and species-specific PCR. Experimental infection was then performed to investigate the pathogenicity of the bacterium and its histopathological manifestation. Naïve juvenile and adult snakeskin gourami were injected intraperitoneally with a low dose (1.2×10^5 CFU/fish) and a high dose (1.2×10^7 CFU/fish) of *S. suis*. Cumulative mortality appeared to be dose- and size-dependent. Experimentally diseased fish exhibited clinical signs consistent with naturally diseased fish. Severe histopathological changes in multiple organs were observed in both juvenile and adult fish, including meningitis, severe congestion in the brain and eyes, thickened stromal layers of the retina, severe hepatic lipidosis and tissue degeneration. Notably, numerous granulomas containing massive bacterial cells in the necrotic core were observed in the infected fish. Relatively pure colonies of *S. suis* were recovered from tissues of experimentally diseased fish. Taken together, this study fulfilled Koch's postulates, indicating that *S. suis* is a newly emerging piscine pathogen. Although this is a case report, public awareness and biosecurity measures should be considered to prevent the spread of the disease. Further surveillance of the pathogen's distribution and research into the underlying causes of fish-host adaptation will provide insights into the genuine impact and appropriate disease control strategies.

1. Introduction

Snakeskin gourami, *Trichopodus pectoralis* (Regan 1910) is one the fishes native to Southeast Asia that are economically important in Cambodia, Laos, Southern Vietnam, Malaysia, and Thailand [1, 2]. It has been domesticated and cultured in Thailand for over 60 years [3, 4] and has become one of the top five freshwater aquaculture species in the country [1, 5]. As fish farming becomes a thriving sector, so does the challenges associated with managing various fish diseases, which are a major obstacle to commercial farming of this species [6].

There are relatively few reports of diseases in the snakeskin gourami [7]. The pathogen fauna of this species is poorly known and may contain pathogens that are not documented in the literature. So far, only a few clinical cases of disease have been reported in snakeskin gourami, such as those caused by parasitic infections (coccidia and metacercaria) [8, 9], virus-associated lymphocystis [10] and *Aphanomyces* fungi associated with epizootic ulcerative syndrome [11]. Most recently, simultaneous infection of a novel *chlamydia*-like organism with a Myxozoa parasite, *Henneguya* sp. was described in this species by Dinh Hung et al. [12]. In general, the most common infectious pathogens found in fish aquaculture are bacteria, viruses, fungi, and parasites [13, 14]. As far as bacterial diseases in fish are concerned, streptococcosis is considered the most devastating disease causing economic losses in the production of freshwater and marine fish worldwide [15, 16]. The well-known symptoms of streptococcosis in fish, although not the only clinical signs, are the "pop eye" (exophthalmos) and erratic swimming (spiralling or spinning) [17, 18]. While several bacterial pathogens have been identified in streptococcosis infections in fish, the Gram-positive, *S. parauberis, S. iniae, S. agalactiae*, and *S. dysgalactiae* are by far the most common agents of these diseases, regardless of geographic region [16, 18–21]. To date, apart from zebrafish, which has already been demonstrated as a model for evaluating the virulence of *S. suis* [22, 23], no association of *S. suis* causing disease in any fish has ever been reported. It is well known that *S. suis* is the major cause of sepsis and meningitis in young pigs, resulting in significant economic losses to the swine industry [24, 25]. *S. suis* is also considered an emerging zoonotic agent, and the number of human cases has increased in recent years [26, 27]. The poor understanding of the epidemiology of *S. suis* in the aquatic environment may underscore the potentially devastating impact of the pathogen in aquaculture.

In this study, we identified for the first time *S. suis* as the bacterial pathogen that caused mortality in farmed snakeskin gourami and then evaluated pathogenicity through experimental challenges to confirm Koch's postulates. Our findings contribute to further epidemiological and pathogenicity studies of an emerging piscine streptococcosis that is lethal to farmed snakeskin gourami and possibly other fish species.

2. Materials And Methods

2.1. Fish necropsy, presumptive diagnosis, and bacteria isolation

In June 2021, we received a set of the snakeskin gourami (*T. pectoralis*) from farms in the central part of Thailand for disease diagnosis. After clinical observation, diseased fish specimens of snakeskin gourami were subjected to aseptic necropsy for presumptive diagnosis by rapid smear staining and subsequent bacterial isolation. Smeared tissues of the brain and head kidney on glass slides were stained with Gram staining reagents (HiMedia, India) and observed under a light microscope (Olympus, Japan). For bacterial isolation, a framed sterile loop was stabbed into the brain and head kidney tissues of the diseased fish prior to streaking on tryptic soy agar (TSA) plates (HiMedia, India). The plates were then incubated for 24–48 hours at 30°C. Based on their morphology, dominant bacterial colonies were selected for re-subculture on TSA plates. Bacterial genomic DNA was extracted from the pure colonies by the boiling method as previously described [28, 29]. DNA purity and concentration were measured using the NanoDrop (Thermo Fisher Scientific, USA) and adjusted to 100 ng/µL with distilled water.

2.2. PCR amplification and DNA sequencing of bacterial 16S rDNA and *S. suis gdh* fragments

A 16S rDNA PCR reaction (25 µl total volume) was prepared consisted of 12.5 µl of 2X Terra PCR Direct Polymerase Mix (Takara Bio, Japan), 0.4 µl of each 10 uM forward and reverse primers (Uni-Bact F: 5'-AGA GTT TGA TCM TGG CTC AG-3', Uni-Bact-R: 5'-ACG GHT ACC TTG TTA CGA CTT-3') [30], 0.5 µl of 1.25 U/µl Terra Taq enzyme (Takara Bio, Japan), 2 µl of bacterial DNA, and distilled water. The cycling conditions were as follows: a denaturation step at 98°C for 5 min, followed by 28 cycles of 98°C for 10s, 55°C for 30s, and 68°C for 80s, with a 5 min extension at 68°C. The expected size of amplified product was ~1.5 kb.

S. suis-specific PCR based on the bacterial glutamate dehydrogenase (*gdh*) gene was performed according to a previous report [31]. A 20 µl PCR reaction consisted of 2 µl of DNA template, 2 µl of 10X PCR buffer (Invitrogen, USA) 0.4 µl of dNTPs, 0.4 µl of 50 mM $MgCl_2$, 1 µl of each 10 µM forward and reverse primers (JP4; 5'-GCA GCG TAT TCT GTC AAA CG-3' and JP5; 5'-CCA TGG ACA GAT AAA GAT GG-3'), 0.2 µl of 5 U/µl Taq DNA polymerase (Invitrogen, USA) and distilled water was prepared. The cycling conditions were as follows: a 5 min denaturation step at 94°C, followed by 35 cycles of 94°C for 10s, 55°C for 15s, and 72°C for 30s, and a 5 min extension at 72°C. The expected size of amplified product was 688 bp.

After agarose gel electrophoresis, amplified products were visualized using gel documentation (Aplegen Omega Fluor™, USA) and subsequently purified using NucleoSpin Gel and PCR clean-up (Takara Bio, Japan). Purified DNA amplicons were subjected to DNA sequencing by a barcode-tagged sequencing technique (U2Bio, Thailand). DNA sequence analysis was performed using Geneious Prime software (Biomatters, Inc., New Zealand). Homology searches were performed using BLAST-n and/or BLAST-p in the GenBank database of the National Center for Biotechnology Information (NCBI). Note that, in addition to employing bacterial DNA as a template, *gdh* PCR was also used to detect *S. suis* from DNA isolated from fish tissues (see below).

2.3. Phylogenetic analysis

To determine taxonomic identification, a BLAST-n query was performed against publicly available nucleotide sequences in the GenBank database (www.ncbi.nlm.nih.gov). The closest known aquatic relatives of *Streptococcus* spp. and numerous sequences of similar species were used for phylogenetic analysis. After multiple alignments against the closely related *Streptococcus* spp. using ClustalW in MEGA X version 10.2.4, the phylogenetic tree was constructed using the neighbour-joining method with 1,000 bootstraps [32]. Sequence 16S rDNA of *Aeromonas veronii* (MG283140.1) was used as outgroup.

2.4. Experimental challenge

S. suis bacteria isolate named 3112 obtained from the head kidney of naturally infected fish was used to perform pathogenicity test in two stages of the snakeskin gourami. Briefly, bacteria were streaked on TSA plates and incubated at 30°C for 24 hours. A single pure colony was then cultured in 5 ml TSB for 2 hours. The bacterial suspension (2 ml) was then transferred to 30 ml TSB and incubated at the same temperature for 20 hours with shaking at 250 rpm. The bacterial suspension was pelleted by centrifugation at 3000 rpm for 5 min. The pellet was then washed twice and resuspended with 0.9% saline to achieve an OD_{600} = 0.8, which corresponds to a concentration of approximately 10^8 CFU/ml by conventional plate counting. Apparently healthy juvenile (small fish, mean weight = 6.17 ± 1.76 g, mean total length = 7.67 ± 0.61 cm) and adult (big fish, mean weight = 110.67 ± 17.00 g, mean total length = 19.43 ± 0.89 cm) snakeskin gourami were obtained from a commercial hatchery with no recent record of disease. All fish groups were acclimated and maintained in an indoor aquaculture system at a water temperature of 29 ± 1^{o}C with aeration and fed twice daily with commercial pellets at a feeding rate of 3% body weight. For the small fish, there were two replicate setups, each with 2 different treatment groups (20 fish per group). Fish in each treatment group were challenged by intraperitoneal injection (i.p) of *S. suis* at a low dose (1.2×10^5 CFU/fish) and a high dose (1.2×10^7 CFU/fish). Two negative control groups consisted of two replicates of 20 fish each, were injected with 0.1 ml of 0.9% saline (i.p.). Experiments with big fish were performed in the same manner. The fish were subsequently housed in plastic tanks of 200 liters (small fish) and 500 liters (big fish) with aerated and dechlorinated water. The challenged fish were monitored daily for 21 days for signs of disease and mortality. The experimental protocols and animals use were approved by the Institutional Animal Care and Use Committee of Kasetsart University (approval ID: ACKU64-FIS-013).

2.5. Confirmation of infection by bacteriology and PCR

Bacteria from liver, spleen, and kidney of representative moribund fish were re-isolated on TSA plates and incubated at 30°C for up to 3 days. To detect *S. suis*, *gdh* PCR was carried out using DNA extracted from the re-isolated bacteria. The *gdh* PCR was also performed using DNA extracted from the infected pooled tissues (liver, kidney, brain and spleen) of the experimental fish. Fish DNA was extracted using proteinase K-containing lysis buffer, followed by phenol/chloroform extraction and ethanol precipitation [33]. PCR amplifications were performed as described in section 2.2.

2.6. Histopathological analysis

Samples of liver, spleen, kidney, brain and eye were taken and placed in 10% neutral buffered formalin. After 24 hours, the tissues were transferred to 70% ethanol and then routinely processed for histology. Specimens were embedded in paraffin, sectioned to a thickness of 5 µm, and stained with hematoxylin and eosin (H&E). The slides were examined under a BX51 light microscope (Olympus, Japan). Two control fish (pre- and post-challenge) and three challenged fish were collected from each low and high injection dose fish group.

2.7. Statistic test

Data on cumulative percent mortality (CPM) of fish of different sizes were statistically compared using the SPSS program (SPSS Inc., USA). Significant differences between the observed means of CPM were determined using one-way ANOVA followed by Duncan's new multiple range test (DMRT). A p-value of less than 0.05 was considered significant.

3. Results

3.1. Macroscopic examination and presumptive diagnosis

According to the farmer, fish mortality of unknown cause has occurred in the last two years with various symptoms affecting cultured fish at all stages. Clinical signs of this recent unknown disease resembling streptococcosis were noted, such as marked hemorrhage, corneal opacities, spinning near the water surface, erosion of the caudal fin, and exophthalmos (eye protrusion). The obtained specimens were about 5 months old and had a body length of approximately 13 cm. The diseased fish showed external lesions with intraocular haemorrhages and exophthalmos and internal changes such as necrotic and hemorrhagic liver **(Figure S1)**. Presumptive diagnosis by microscopic examination of Gram stain of stamp-smeared tissues of the brain and head kidney revealed numerous Gram-positive cocci bacteria in both smeared tissues (representative results in brain in **Figure S2A**).

3.2. Recovery of *S. suis* from naturally diseased fish

The predominant bacterial colonies on 24 hours incubated TSA plates were small whitish colonies **(Figure S2B)** that were Gram-positive cocci. One representative isolate, namely 3112, was used for further investigation. PCR amplification of 16S rDNA and *gdh* gene using DNA extracted from bacterium 3112 as template yielded amplicon products of approximately 1.5 kb and 0.7 kb, respectively **(Figure S3A)**. DNA sequencing and BLAST analysis identified bacterium 3112 as *Streptococcus suis* based on 99.67-99.87% nucleotide identity of the 16S rDNA sequence and 98.18-98.48% nucleotide identity and 100% amino acid sequence identity of the *gdh* gene to the top 10 BLAST hits of various isolates of *S. suis* complete genomes available in the GenBank database. The partial 688 bp DNA sequence of the *gdh* gene of *S. suis* 3112 is shown in **Figure S3B** while the sequence of the partial 16S rDNA (1.5 kb) was deposited in the GenBank database under accession number OK356401. Phylogenetic analysis based on 16S rDNA sequences revealed that *S. suis* 3112 isolated from the diseased snakeskin gourami belonged to the same cluster as *S. suis* from pigs and cattle (**Figure 1**).

3.3. *S. suis* is highly pathogenic to both juvenile and adult snakeskin gourami

The results of experimental challenge showed that *S. suis* exhibited significant virulence in snakeskin gourami and the mortality rate depended on the size and dose **(Figure 2)**. The high dose of *S. suis*, which was rapidly lethal without clear clinical signs, caused 67.5% mortality in small fish within 3 days post-infection (dpi) and slower mortality in big fish with the same percentage after 5 dpi. The low dose of *S. suis* was also highly virulent, but the disease developed quite slowly, with mortality of 42.5 and 60% in small and big fish, respectively, at one week after injection. Of note, mortality in small fish at high and low doses ceased at 8 and 10 dpi, respectively, whereas mortality in big fish persisted up to 15 dpi at both high and low doses. At the end of the experiment, the mean cumulative mortality and standard error of the mean (SEM) of small and big fish at low and high doses were 50% (±5.0), 75% (±0.00), 80% (±0.00) and 92.5% (±2.5), respectively. Statistical analysis of differences between treatments showed that mortality was significantly higher in big fish than in small fish when the same low dose was administered ($p<0.05$, **Table 1**).

Starting at 3 dpi, after acute death, fish showed lethargic behavior and erratic swimming, swirling or loss of balance, 'C'- or 'S' - shaped posture **(Figure S4)**. The clinical symptoms that developed after injection with *S. suis* are shown in **Figure 2** and **Table 1**. The clinical signs in experimentally infected fish in all groups were lethargy, swimming disorders, loss of appetite, corneal opacity/exophthalmos, and skin haemorrhages/erosions, and internal signs were enlarged liver and splenomegaly **(Figure 3)**. Although clinical signs were similar, small fish died acutely, but mortality, macroscopic lesions, and histopathology were less variable than in big fish. *S. suis* was successfully recovered as pure colonies from the head kidney, liver, and spleen of moribund and dead fish after challenge. The *gdh* PCR results of DNA extracted from the isolated bacteria and DNA extracted from the internal organs of the experimental fish are also shown in **Figure S5** and **Table 1**. No morbidity or mortality was observed in the control small fish group until 21 dpi, while two out of 40 individuals in the control big fish group (5%) died at 3 dpi without major clinical signs but with some injuries on their bodies. This could be because the fish were aggressive and fighting each other, which led to the death of some fish. *S. suis* was not recovered from the internal organs of these fish as well as *gdh* PCR results that were negative and histological findings revealed no abnormal signs (data not shown).

3.4. Histopathology of snakeskin gourami experimentally infected with *S. suis*

Similar histological changes were observed in multiple organs of both small and big fish. The detailed changes in individual organ are described below.

Liver: Unlike the normal liver, which is made up of densely packed hepatocytes with polyhedral shapes **(Figure 4A)**, the infected liver showed congestion in hepatoportal and sinusoidal blood vessels, severe hepatic lipidosis with large number of lipid vacuoles in both small **(Figure 4B)** and big fish **(Figure 4C).** Affected hepatocytes exhibited atrophy, lipid storage loss and chromatin margination **(Figure 4B1)**. Notably, all affected big fish

had multiple coalescing granulomas in the liver that occupied a large portion of the tissue parenchyma **(Figure 4C)**, which was explicitly not presented in small fish. Granulomas, which may have necrotic cores, typically progress through characteristic stages with overall progressive fibrosis. The main part of granulomas contained large bacterial colonies in their necrotic centers, tissue death (necrosis), accumulation of pigmented macrophages, and were surrounded by fibroblast-like cells **(Figure 4C1)**.

Kidney: The most notable changes were severely diminished hemopoietic tissue accompanied by interstitial haemorrhage and congestion of the intertubular blood vessels **(Figure 4E and F)**. Obvious necrosis of tubular epithelial cells was observed **(Figure 4E1)**. Glomerular necrosis with dilatation of Bowman's space was observed in most cases (data not shown). Typical granulomas and increased melanoma macrophage centres (MMc) were observed only in the kidneys of the big fish **(Figure 4F)**, but not in the kidneys of the small fish **(Figure 4E)**.

Spleen: The splenic lesions showed a marked reduction in the amount of red pulp and red blood cells **(Figure 4H and I).** Pigmented macrophage aggregations were also noted in certain areas **(Figure 4H)**. The hematopoietic components were degenerated and necrotized **(Figure 4H1)**. An increasing number of MMc and their multifocal areas of necrosis as well as typical granulomas were observed only in the spleen of the big fish **(Figure 4I and I1)**.

Brain: The examined sections showed that the affected brain had severe meningeal congestion **(Figure 5B and D)**. The brain parenchyma was degenerated and meningitis was characterized by infiltration with mononuclear cells resembling lymphocytes around the area of congestion **(Figure 5B1)**. It is noteworthy that severe blood vessel congestion was observed in the cerebellum compared to other parts of the brain **(Figure 5C)**.

Eye: The eyes of many fish examined showed marked pathology, with varying degrees of severity of blood congestion in the lens cortex **(Figure 6B)**. The retina was ulcerated with thickened stromal layers, including advanced lesions with disruption and separation of retinal fibres and vacuolization in the stromal layers **(Figure 6B1)**.

4. Discussion

4.1. *Streptococcus suis* is new to fish

Since piscine streptococcosis was first identified in Japanese rainbow trout in 1957 [34], the disease has become a serious problem for the aquaculture industry worldwide [15]. There are several streptococcal species that are pathogenic to fish, including *S. iniae, S. agalactiae, S. dysgalactiae*, and *S. parauberis* [16–21]. To date, there is no documented record of *S. suis* being associated with disease outbreaks in fish. This is the first time that *S. suis*, a major bacterial pathogen of swine [24, 27], has been identified as a new pathogen causing a disease outbreak in farmed snakeskin gourami.

Identification of the causative agent of streptococcosis in aquaculture at the species level becomes more complicated because different streptococcal and lactococcal species cause similar gross signs and pathology [35, 36]. According to farmers, similar clinical signs of streptococcal infection (i.e. abnormal swimming behavior, hemorrhage, and pop eyes) have been observed in snakeskin gourami farms over the last two years but no proper diagnosis has been made. This implies that streptococcosis in snakeskin gourami may associate with a complex of *Streptococcus* species, including an unstudied *S. suis*. Therefore, extensive surveillance is necessary to gain insights on their prevalence in snakeskin gourami and other piscine hosts.

4.2. *S. suis* proven to be pathogenic to snakeskin gourami in artificial infection

The piscine isolate *S. suis* 3112 in this study reproduced the disease with clinical and pathological signs similar to natural infection and those found in other streptococcal infections [17, 18]. Apart from general gross signs, neurological-related symptoms (i.e., lethargy, swimming disorders) occur during the course of the disease, suggesting that the brain (central nervous system) may be the important target organ for *S. suis*. Pathological changes associated with the brain, such as meningitis, congestion of blood vessels caused by *S. suis* in snakeskin gourami, are consistent with the typical pathological lesions of streptococcal infections in other fish species [37–39]. The severe congestion of blood vessels observed in the cerebellum compared with other parts of the brain corresponds strongly with the gross aspects of erratic swimming, as the cerebellum is an area responsible for regulating locomotion and balance stimuli in fish [40, 41]. Similar to previous studies with *S. agalactiae* and *Tilapia tilapinevirus* in tilapia, damages to the cerebellum may lead to swimming disorders [38, 42]. Although the LD_{50} (median lethal dose) has not been determined, both small and big fish experienced mortality in excess of 50% even at a low dose (1.2×10^5 CFU/fish). This provided sufficient evidence of the high virulence of *S. suis* in snakeskin gourami. Previous studies have reported LD_{50} values for other piscine *Streptococcus* species ranging from 1.8×10^6 to 3.6×10^7 CFU/fish in tilapia *Oreochromis niloticus* infected with *S. agalactiae* [43, 44]; from 2.45×10^8 to 5.54×10^8 CFU/fish in crucian carp *Carassius carassius* infected with *S. dysgalactiae* [45]; from 5.1×10^5 to 6.4×10^5 CFU/fish in Siberian sturgeon *Acipenser baerii* [46] and 3.2×10^4 to 2.5×10^5 CFU/fish in Asian sea bass *Lates calcarifer* [47] infected with *S. iniae*. In fact, virulence and pathogenesis of streptococci differs greatly, upon different fish stage and species, bacterial species, infection routes, isolates, and environmental parameters [17, 44, 48].

Histopathological findings in experimental fish in this study revealed that *S. suis* infections in snakeskin gourami were systemic that caused degeneration and focal necrosis in multiple organs, similar to what have been described in Siberian sturgeon [46], tilapia [49], Asian sea bass [37]

infected with *S. iniae* and Asian sea bass [39] infected with *S. agalactiae.* As suggested by Miyazaki et al. [50] and Olufemi & Roberts [51], a hematogenous pathway appears to be the major route for the spread of streptococcal infections in fish. It is noteworthy that the occurrence of granulomas and increased MMc was observed only in big fish. Indeed, the differential immunity of fish stages may lead to differences in susceptibility and histopathological manifestation during bacterial infections [52]. A granuloma is an accumulation of granulation tissue that forms in response to microbial infection or foreign material and serves as a tactic of the immune system to confine microbes or foreign materials [53]. Granulomas are a notable histologic feature of several bacterial infections, particularly those with intracellular lifestyles, including *Streptococcus* spp. [49, 54, 55], *Edwardsiella* spp. [56], *Nocardia* spp. [57, 58], *Francisella* spp. [59, 60], and *Mycobacteria* spp. [61, 62]. Similarlity, MMc are thought to cooperate with lymphocytes to scavenge antigens, which then elicit a specific immune response [63, 64]. The majority of MMc are melanoma macrophages, so activation of these cells during streptococcal infection may be associated with MMc formation and proliferation in lymphoid tissue. Increasing MMc numbers in lymphoid tissues such as kidney and spleen and rarely in the liver of infected fish in this study is consistent with previous publications showing an increase in MMc as a result of a cellular immune response in fish [37, 63]

4.3. 3. Early warning of an emerging piscine Streptococcus

Despite the fact that this is a case report of *S. suis* in snakeskin gourami, it serves as an early warning of a newly emerging virulent strain of *S. suis* infecting fish. The finding also underscores the need for comprehensive monitoring of this pathogen in aquatic hosts to gain better insight into its spread and adverse effects on farmed fish. In addition, how the snakeskin gourami became naturally infected with *S. suis* remains an open question; however, a possible source of infection for *S. suis* could be contaminated water. The affected farm consists of earthen ponds that received water from rainfall, village sewage, and agricultural run-off, so it is possible that water contaminated by animal excrement may be a potential source of *S. suis*. Therefore, follow-up investigations on the origin, host range, prevalence, risk factors for disease, and mode of transmission will provide better insight into this emerging pathogen. This will lead to a better understanding of the pathogenesis and epidemiology of the disease so that effective control measures can be developed.

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Tables

TABLE 1 The percentage of small (S) and big (B) snakeskin gourami that developed clinical signs after injection experiments with *Streptococcus suis* at low dose (Low; 1.2×10^5 CFU/fish) and high doses (High; 1.2×10^7 CFU/fish) as well as the results of bacteriology, PCR and histopathology.

ıments	Mean cumulative percent mortality at 21 dpi. (n=20/replicate)	Observed clinical signs as a mean percentage (n=20/replicate)					Bacteriology	PCR organs	Histology (Presenting granulomas)
		Lethargy	Appetite loss	Swimming errors	Skin haemorrhages/ erosion	Cornea opacity/ exophthalmos	Number of affected fish/ examined fish		
ıtrol	0^a	0	0	0	0	0	ND	0/4	0/2
ıtrol	5^a (±0.0)	0	0	0	0	0	ND	0/4	0/2
w	50^b (±5.0)	40	60	20	10	20	2/2	1/4	0/3
w	75^c (±0.0)	70	80	50	40	30	2/2	3/4	3/3
gh	80^d (±0.0)	60	80	30	20	30	2/2	2/4	0/3
gh	92.5^d (±2.5)	90	100	60	60	40	2/2	2/4	3/3

Results were calculated as the mean of two repeated experimental groups (±SEM, standard error of the mean). Significant differences between treatments are indicated by superscript alphabets (P <0.05). See text for additional details. ND, Not determined.

Declarations

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Kasetsart University (Approval ID: ACKU64-FIS-013).

AUTHOR CONTRIBUTIONS

HTD, SC, and SS involved in conceptualization. ND-H, CS and ST underwent investigation. ND-H, HTD, SC, and SS performed formal analysis and wrote the original draft. ND-H, HTD, SS, CR and SC reviewed and edited. All authors have read and agreed to the current version of the manuscript.

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Figures

Figure 1

The phylogenetic tree was constructed based on the partial 16S rDNA sequence (1.5 kb) of *S. suis* isolated from the snakeskin gourami *(Trichopodus pectoralis)* from this study (OK356401) and closely related species. Accession numbers and the origin of organisms are indicated in the parentheses. *Aeromonas veronii* was selected as an outgroup. The tree was constructed using the neighbour-joining method. The scale bar represents 0.05 - nucleotide substitution per site, while the number at the node of the tree indicates the bootstrap value in percent.

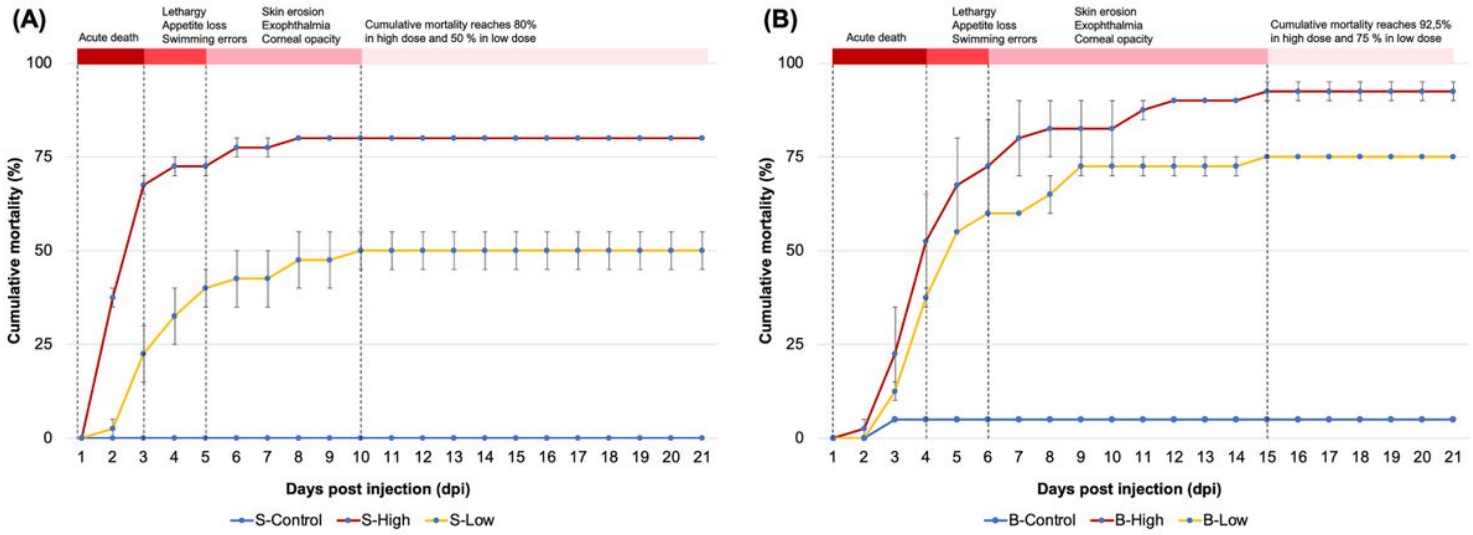

Figure 2

Experimental challenge of snakeskin gourami (*Trichopodus pectoralis*) infected with *S. suis*. Time to onset of clinical symptoms and cumulative mortality of snakeskin gourami after injection (i.p.) of *S. suis* with a low dose (1.2×10^5CFU/fish), a high dose (1.2×10^7 CFU/fish) and controls were injected with 0.1 ml of 0.9% saline (i.p.) in the small fish group (A) and the big fish group (B). Results were calculated as the mean of two replicate experimental groups (±SEM, standard error of the mean). S, small fish; B, big fish.

Figure 3

External and internal lesions in snakeskin gourami (*Trichopodus pectoralis*) infected by *S. suis*. (A) Experimentally infected small fish show eye with exophthalmos (ocular protrusion) and hepatosplenomegaly (arrowheads). (B) Experimentally infected big fish show ulceration and marked haemorrhages on the skin (arrows), eye with intra-ocular haemorrhages and exophthalmos, and hepatosplenomegaly (arrowheads). Scale bars are shown in the picture.

Figure 4

Histological lesions in liver, kidney, and spleen of snakeskin gourami *(Trichopodus pectoralis)* infected with *S. suis*. Representative histopathology of liver (A), kidney (D) and spleen (G and G1) of normal fish. In infected fish, liver shows severe hepatic lipidosis with large lipid vacuoles (arrowhead in B and C), atrophic and degenerated hepatocytes (arrow in B1), congestion of hepatoportal blood vessels (asterisk in B). Kidney shows severely decreased hematopoietic tissue (E and F), necrosis in tubular epithelial cells (arrowheads in E1), interstitial haemorrhage and congestion of intertubular blood vessels (asterisk in E1, and F). Spleen shows marked depletion of red pulp and red blood cells (H and I), pigmented macrophages aggregation (arrowhead in H), degeneration and necrosis of hematopoietic elements (asterisk in H1). Multiple, coalescing granulomas (Gr) in the liver, kidney, and spleen, occupying a large portion of the tissue parenchyma, was observed only in big fish (C, F, and I). At high magnification, the granulomas showed large bacterial colonies in their necrotic centers (asterisk in C1 and F1), dead tissue (arrowhead in C1) and pigmented macrophages (arrowhead in F1), surrounded by fibroblast-like cells. Melanoma macrophage centres (MMc) were necrotic (I1) compared with normal MMc (G1). B, big fish; S, small fish; C, control; L, low dose; H, high dose; the number after the abbreviation represents the date of sampling (dpi). Scale bars are shown in the picture.

Figure 5

Histological lesions in the brain of the snakeskin gourami *(Trichopodus pectoralis)* infected with *S. suis*. The non-infected brain (A) shows that the primitive meninges (PM) (A1, between arrows) consist of the superficial and loose connective tissue layers of the primitive meninges (arrowhead in A1). In the infected fish, the meninges were severely congested (arrowheads in B and D), and the parenchyma was degenerated, and meningitis characterized by infiltration with mononuclear cells resembling lymphocytes around the area of congestion (asterisk in B1). Severe congestion of blood vessels was observed in the cerebellum (arrowheads in C) compared with other parts of the brain; cerebellum without abnormalities in control fish is shown in the box. B, big fish; S, small fish; C, control; H, high dose; the number after the abbreviation represents the date of sampling (dpi). Scale bars are shown in the picture.

Figure 6

Histological lesions in the eye of the snakeskin gourami *(Trichopodus pectoralis)* infected with *S. suis*. The non-infected retina (A) is well structured and composed of the various layers (A1). The eyes of many infected fish examined showed marked pathology, with varying degrees of severity of blood congestion in the lens cortex (white arrows in B and B1). The retina is ulcerated with thickened stromal layers (B1, note that Figures A1 and B1 are the same scale). Advanced lesions include disruption and separation of retinal fibres and vacuolization in the stromal layers (arrowheads in B1). B, big fish; S, small fish; C, control; L, lose dose; the number after the abbreviation represents the date of sampling (dpi). Scale bars are shown in the picture.

Supplementary Files

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