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Research article

Experimental *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*) via different routes

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Abstract

Streptococcus agalactiae is one of the most pathogenic bacteria responsible for causing mortality in Nile tilapia and the routes of infection have not been clear. To determine the mortality, 320 Nile tilapias were assigned to 4 treatments (80 fish/treatment). Each fish was inoculated with 0.1 ml of *S. agalactiae* concentration of $7.25x10^7$ CFU/ml via oral, nares, gills or eyes routes and was then maintained out of water for 30 seconds. Control fish were inoculated with sterile 0.9% normal saline solution using a similar manner. Samples from anterior kidney, spleen, eyes and/or brain from dead fish were sampled by bacteriological technique for confirming *S. agalactiae* infection. The results showed clinical signs of fish inoculated with *S. agalactiae* including lethargy, spiral swimming, corneal opacity and/or darkening of the skin as early as day 1 after inoculation. Cumulative mortality of *S. agalactiae* infection was 31.67%, 18.33%, 0% and 0% via oral, nares, gills and eyes inoculations, respectively. The mortality of fish between oral and nares inoculations was not significant ($P > 0.05$). Death among fish occurred from day 2 to day 8 post-inoculation. The results of this study indicate that *S. agalactiae* could enter through oral and nares routes and consequently led to mortality in Nile tilapia.

Keywords: Infection, Nile tilapia, Route, *Streptococcus agalactiae*

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INTRODUCTION

Streptococcosis is caused by *Streptococcus* spp. and is a serious infectious disease among cultured fish worldwide (Azad et al., 2012; Geng et al., 2012; Ortega-Asencios et al., 2016). Among *Streptococcus* spp., *S. agalactiae* and *S. iniae* are the main species of *Streptococcus* genus affecting a variety of fish species including Rainbow trout (*Oncorhynchus mykiss*) (Eldar et al., 1995), Seabream (*Sparus aurata*) (Doménech et al., 1997), Hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Evans et al., 2000), Red tilapia (*Oreochromis* spp.) (Hernández et al., 2009), Nile tilapia (*Oreochromis niloticus*) (Mian et al., 2009), Queensland grouper (*Epinephelus lanceolatu*) (Bowater et al., 2012), Ya-Fish (*Schizothorax prenanti*) (Geng et al., 2012) and Silver pomfret (*Pampus argenteus* Euphrasen) (Azad et al., 2012). Typical signs of the presence of Streptococcosis in fish could be characterized by erratic swimming, dorsal rigidity, lethargy, corneal opacity, exophthalmoses, splenomegaly, pale and/or haemorrhagic liver as well as some haemorrhage around the eyes, operculum, fin and/or body (Abdullah et al., 2013; Eldar et al., 1995). *S. agalactiae* has become a particularly major Nile tilapia pathogen in the past decade (Li et al., 2014; Mian et al., 2009; Ortega-Asencios et al., 2016).

Generally, *Streptococcus* spp. can infect the fish directly through water. Previous studies have investigated the routes of *S. agalactiae* and *S. iniae* transmissions through the external fish organs. For example, the oral inoculation of *S. agalactiae* in Queensland grouper, Red tilapia and Nile tilapia (Delamare-Debouteville et al., 2015; Iregui et al., 2015; Soto et al., 2016) and of *S. iniae* in Barramundi and Japanese flounder (Bromage and Owens, 2002; Nguyen et al., 2001), the gills inoculation of *S. agalactiae* in Nile tilapia (Mian et al., 2009) and of *S. iniae* in Hybrid striped bass (Mcnulty et al., 2003) as well as the nares and eyes inoculations of *S. iniae* in both Hybrid striped bass and Nile tilapia (Evans et al., 2000), respectively.

However, the study of *S. agalactiae* infection in Nile tilapia via a specifically entry site has been investigated to a lesser extent with, in some cases an absence of reported findings. Thus, this study aims to investigate the mortality of Nile tilapia infected with *S. agalactiae* after oral, nares, gills and eyes inoculations in order to ensure that *S. agalactiae* can enter a specific transmission route.

MATERIAL AND METHODS

Fish

Healthy Nile tilapia (*Oreochromis niloticus*) fingerlings with an average weight of 8.17 g were obtained from a commercial production farm in Chiang Mai Province. The animals were acclimated in 80-L aquaria supplied in aeration with an air stone and were fed to apparent satiation with a commercial feed (CP, Thailand) twice a day as well as allowed to acclimate for at least 2 weeks prior to challenge. A subsample of fish was verified by monitoring the

typical signs of Streptococcosis and conducting some bacteriological investigation in order to ensure that no *S. agalactiae* was found in the population taking part in this experiment. The animals in this study were approved and handled in accordance with the guidelines of the Faculty of Veterinary Medicine, Chiang Mai University Animal Care and Use Committee (FVM-ACUC), approval no. S20/2017.

Bacteria

Streptococcus agalactiae III, which originally isolated Tilapia (*Oreochromis* sp.) in Chiang Mai Province was used to infect in fish (Wongsathein et al., 2018). The isolate was identified as *S. agalactiae* by its white colony, coccus morphology, translucent and slightly opaque, round, convex and 1.5-2.0 mm in diameter, Gram-positive, negative catalase, negative oxidase, β-haemolytic activity in sheep's blood agar, non-motility, ability to enhance β-lysin produced by *Staphylococcus aureus* (CAMP test), positive reaction for Voges Proskauer, hippurate hydrolysis, alkaline phosphatase, leucine aminopeptidase, arginine dihydrolase, ribose and negative reaction occurred for β-glucosidase, pyrrolidonyl arylamidase, α-galactosidase, β-glucuronidase, β-galactosidase, arabinose, mannitol, sorbitol, lactose, inulin, raffinose, amidon and glycogen using API 20 STREP kit (BioMerieux, France), reacted with group B antiserum tested by Lancefield serogrouping kit (Oxiod, England) as well as was confirmed by GBS-specific PCR targeting 16S rRNA (Meiri-Bendek et al., 2002). The bacteria were cultured into BHI broth (Merck, USA) supplemented with 15% glycerol and then stored at -80 °C until use.

The infectious bacteria were prepared by culturing on Trypticase Soy Agar (TSA) (Merck, USA) media supplemented with 5% sheep blood and incubated at 28 °C for 24 h. The purified colony on agar media was inoculated in 3 ml of Trypticase Soy Broth (TSB) (Merck, USA) media and incubated at 28 °C for 24 h. After incubation, the cultural media was transferred into 30 ml of new TSB media (1:10) and incubated at 28 °C for 7 h (mid-log phase). The cultural media was adjusted spectophotometically to an optical density of 10^8 CFU/ml. The dose of bacteria used in this study was then prepared by dilution in sterile 0.9% normal saline solution (NSS) and the CFU was later confirmed by plate count technique (United States Department of Agriculture Center for Veterinary Biologics, 2015). The bacteria used in this study were approved by and complied with the guidelines of the Chiang Mai University Institutional Biosafety Committee (CMUIBC), approval no. CMUIBC A-0560004.

Experimental challenge

Prior to the experimental challenge, the bacterial isolation was passaged through the fish with 0.1 ml of 10^8 CFU/ml by intraperitoneal (IP) injection twice to enhance their virulence post storage. The bacteria were recovered from the anterior kidney, spleen, eyes and/or brain of freshly dead fish and were cultured on TSA media with 5% sheep blood. The colonies grown on the culture media were identified by the conventional methods as described above and then used for the challenge.

In the experimental challenge, 320 fish were conducted into 4 treat-

ments. In each treatment, 80 fish were divided into four groups in triplicate (20 fish/group) with average density of 8.17 g/L. One group was designed as the control group. Each fish was inoculated with 0.1 ml of bacterial concentration of $7.25x10^7$ CFU/ml and was maintained out of water for 30 seconds. All fish were anaesthetized in 2 L of water containing 50 mg of clove oil/L and were placed on moist towels in preparation for the *S. agalactiae* inoculation. Treatment 1, the fish were orally inoculated with 0.1 ml of the bacterial suspension using a 1-ml syringe connected to 24-gauges plastic catheter (BD medical system, USA). The tube was slowly inserted along the mouth of the fish through the esophagus until it reached the stomach. Then, the fish were maintained out of water for 30 seconds and were then returned to their respective tank. Treatment 2, the fish were carefully inserted with 0.05 ml of the same bacterial concentration into the incurrent nares using a plastic tube catheter and the fish were then maintained out of water for 15 seconds. These procedures were repeated to inoculate and maintained out of the water to the other nares of each fish. Treatment 3, 0.05 ml of the same bacterial concentration was slowly dropped onto the gills surface of the fish using a micropipette connected to a tip (>50 µl volume) and the fish were then maintained out of water for 15 seconds. These procedures were repeated to inoculate and maintained out of the water to the other gills of each fish. Treatment 4, 0.05 ml of the same bacterial concentration was slowly dropped onto the eyes surface of the fish using a micropipette connected to a microtip and the fish were then maintained out of water for 15 seconds. These procedures were repeated to inoculate and maintained out of the water to the other eyes of each fish. The control group fish in each treatment were inoculated with a sterile 0.9% normal saline solution (NSS) in a similar manner. The clinical signs, lesions and mortality of fish after inoculation was monitored and was recorded within 14 days.

Sample collection and evaluation

Following *S. agalactiae* infection, the mortality in each treatment was recorded daily within 14 days. Samples from anterior kidney, spleen, eyes and/or brain of dead fish used in the experiments were cultured on TSA media and incubated at 28°C for 48 h. The presence of colonies on culture media was identified by Gram staining, catalase test, oxidase test, CAMP test and Lancefield serogrouping test so as to be confirmed as being *S. agalactiae*.

Water quality evaluation

The temperature using a glass thermometer (SK SATO, Japan) and the dissolved oxygen (DO) using a DO meter version YSI 550A (YSI, USA) were measured daily. The pH using a pH meter version 500 pH (Eutech Instruments, Singapore) and the ammonia and the nitrite following the Ammonia and Nitrite-measurement's protocols as described by Clesceri et al. (1998) were measured every 3 days throughout the experiment.

Statistical analysis

The difference in mortality data between the treatments was analyzed with the independent t-test using R program for windows, version 3.5.0 (https://www.rstudio.com/ products/rstudio/) and tested at 5% level of significance.

RESULTS

Clinical signs and lesions of Nile tilapia infected with *S. agalactiae*

Following *S. agalactiae* infection in Nile tilapia, the fish showed spiral swimming, corneal opacity and darkening of the skin on day 1 and 2 after oral and nares inoculations, respectively. The fish that were challenged by the gills route showed little clinical signs such as lethargy and stayed on the surface of the water after the challenge. Besides, the fish that were challenged by the eyes route showed neither clinical signs nor lesions after challenge.

Mortality of *S. agalactiae* infection in Nile tilapia

The mortality of fish infected with *S. agalactiae* was observed on day 2 to 8 (Figure 1) and the cumulative percentage of mortality reached 31.67%, 18.33%, 0% and 0% after challenges via oral, nares, gills and eyes, respectively (Table 1). However, there were no significant differences in the mortality rates between oral and nares challenges ($P > 0.05$).

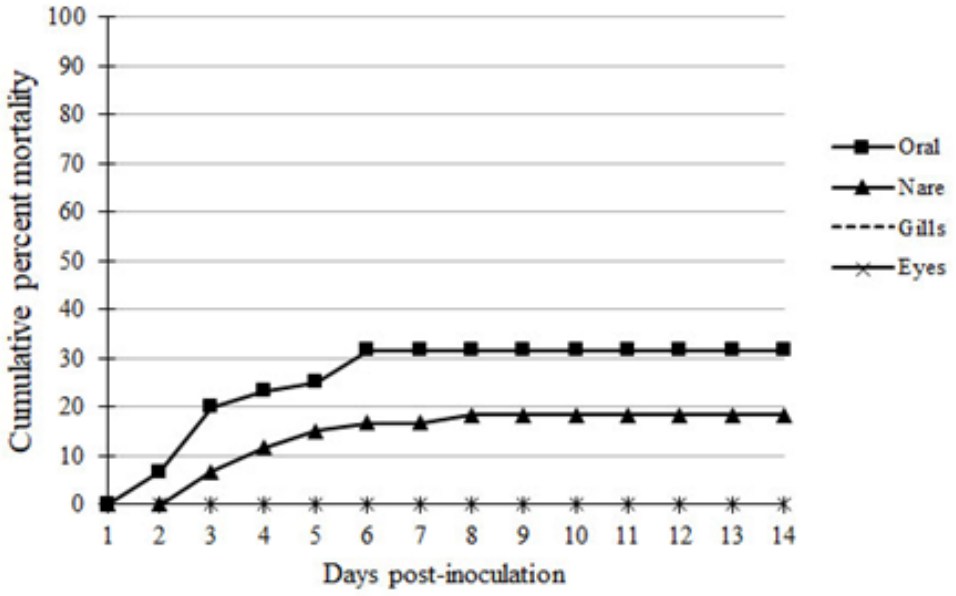

Figure 1 Cumulative mortality of *S. agalactiae* infection in Nile tilapia via oral, nares, gills and eyes inoculations within 14 days post-inoculation.

Table 1 Mortality of *S. agalactiae* infection in Nile tilapia via different routes.

Treatments	Number of fish	Number of culture positive fish with *S. agalactiae*	Mortality of dead fish with *S. agalactiae (%)*
oral inoculation			
group 1	20	2	10
group 2	20	6	30
group 3	20	11	55
Total	60	19	95 (31.67)
control group	20	0	0
nares inoculation			
group 1	20	4	20
group 2	20	5	25
group 3	20	2	10
Total	60	11	55 (18.33)
control group	20	0	0
gills inoculation			
group 1	20	0	0
group 2	20	0	0
group 3	20	0	0
Total	60	0	0
control group	20	0	0
eyes inoculation			
group 1	20	0	0
group 2	20	0	0
group 3	20	0	0
Total	60	0	0
control group	20	0	0

Recovering *S. agalactiae* from experimentally infected Nile tilapia

The isolated bacteria from samples of anterior kidney, spleen, eyes and/or brain showed a white color, translucent and slightly opaque, round, convex and 0.5-2.0 mm in diameter on culture media. The bacterial cells showed the Gram-positive cocci in pairs or chains, catalase and oxidase negative, CAMP test positive and reacted serologically with group B antiserum. The primary identification was characteristically presumptive of *S. agalactiae*.

Water quality

The water quality of all treatments during the experiment was closely similar. The temperature was 26.5±0.39°C, DO was 7.25±0.29 mg/l, pH was 7.6±0.21, ammonia was 0.013±0.01 mg/l and nitrite was 0.064±0.07 mg/l.

DISCUSSION

S. agalactiae is one of the most important pathogens and causes high mortality in Nile tilapia. The occurrence of mortality in this study has led to the understanding of a specific entry site of *S. agalactiae* into Nile tilapia. After challenge, the results indicate that the challenge with *S. agalactiae* can cause clinical signs and lesions in Nile tilapia, when the bacteria were delivered orally or by nares inoculation, rapid clinical signs and lesions were observed within 48 h. Clinical signs and lesions observed in this study were similar to those observed in Seabream (*Sparus aurata*) (Evans et al., 2002), Queensland grouper (*Epinephelus lanceolatu*) (Delamare-Debouteville et al., 2015), Yafish (*Schizothorax prenanti*) (Geng et al., 2012), Red tilapia (*Oreochromis* spp.) (Hernández et al., 2009) and Nile tilapia (*Oreochromis niloticus*) (Abdullah et al., 2013) experimentally or naturally infected with *S. agalactiae*. Although *S. agalactiae* can infect in various fish species, the presence of clinical signs and lesions of fish infected the bacteria were similar. In addition, the distinct sign was when the fish swam in a spiralling fashion, this particular behavior had previously been attributed to a central nervous system (CNS) damage by *Streptococcus* spp. (Eldar et al., 1994).

Based on our results, the occurrence of mortality in Nile tilapia infected with *S. agalactiae* was observed on day 2 to day 3 in the oral and nares challenges, respectively. With the oral challenge, although mortality did occur in Nile tilapia, only a few mortalities were observed on day 11 after the challenge with 0.1 ml of bacterial concentration of 10^2 CFU/ml (Soto et al., 2016). The infectivity of the bacteria may depend on the concentration of *S. agalactiae*. Additionally, Kaewngernsong et al., (2019) demonstrated that the high concentration (5.92 x 10^8 CFU/ml) of *S. agalactiae* with 0.3 ml could disseminate into different Nile tilapia organs within 30 min after oral inoculation. Besides, Iregui et al. (2015) found that *S. agalactiae* resulted in septicaemia in Red tilapia after intragastric inoculation and they suggested that the gastrointestinal tract is the main entry site of *S. agalactiae* infection. Similar results were found with the nares route, mortality did also occur in Nile tilapia at 0-20% and

Hybrid striped bass at 13.3-66.7% after the challenge with 0.01 ml of S. iniae concentration of 10^3-10^5 CFU/ml for 5 min by nares inoculation (Evans et al., 2000). This study suggests that the nares tissue could induce *Streptococcus* spp. leading to infection in fish, in high concentration dosage.

Additionally, the results in this study showed that no mortality was observed in Nile tilapia following the eyes and gills challenges. Mortality with the eyes challenge was not either observed in Nile tilapia and Hybrid striped bass after inoculation with 0.01 ml of *S. iniae* concentration of 10^5 CFU/ml and exposed for 5 min (Evans et al., 2000). Moreover, Evans et al. (2000) suggested that the infectivity of fish may be more related to the length of exposure of the eyes to the bacteria, bacterial concentration or other factors such as the anatomy, the physiology and the appropriate mucosal receptors in the eyes rather than the very inoculation protocols because they found that Hybrid striped bass produced some anti-streptococcal antibodies after eyes inoculation. On the contrary, no mortality of Nile tilapia were occurred after *S. agalactiae* inoculation by gills route, whereas Mian et al. (2009) found an approximate 100% of mortality in Nile tilapia following an infection by gills challenge with 0.1 ml of *S. agalactiae* concentration of 10^6 CFU/ml and Mcnulty et al. (2003) found similar results with 13-100% mortality rate in Hybrid striped bass after inoculation with 0.5 ml of *S. iniae* concentration of 10^5-10^8 CFU/ml and 2 min exposure. This study assumes that *S. agalactiae* may still remain in the fish body throughout the experiment, because similar study from Delamare-Debouteville et al. (2015) found that *S. agalactiae* in Queensland grouper (*Epinephelus lanceolatus*) after 15 days of challenge by PCR technique. Moreover, Mian et al. (2009) suggested that the gills tissue is an important site of *S. agalactiae* in Nile tilapia.

Previously, several studies have demonstrated the susceptibility of Nile tilapia to virulent *S. agalactiae* isolates (Abdullah et al., 2013; Mian et al., 2009; Soto et al., 2016). Some studies found the presence of *S. agalactiae* in dead fish or surviving fish following several detection techniques. For example, *S. agalactiae* was detected using PCR technique from Queensland grouper (*Epinephelus lanceolatu*) (Delamare-Debouteville et al., 2015) or immunohistochemistry (IHC) technique from naturally or experimentally infected Red tilapia (*Oreochromis* spp.) (Hernández et al., 2009; Iregui et al., 2015). Furthermore, those studies concluded that those techniques had a higher sensitivity and specificity than bacteriology. Based on those results, this study suggests that the technique used for the investigation of *S. agalactiae* in experimental Nile tilapia should be used along with the other techniques, which have higher sensitivity or specificity than bacteriology techniques for finding the bacteria, which cannot be cultured.

From the results in this study, the detection of *S. agalactiae* among all surviving Nile tilapia after the experimental challenge is required to confirm *S. agalactiae* infection in fish. Additionally, bacterial distribution data is necessary to fulfill the information of *S. agalactiae* infection in Nile tilapia in order to understand the early phases of infection.

CONCLUSION

The present study demonstrated that *S. agalactiae* could enter into Nile tilapia and produced mortality via oral and nares routes.

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