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Effect of Water Temperature on Susceptibility to Streptococcus agalactiae Serotype Ia Infection in Nile Tilapia (Oreochromis niloticus)

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Original Article

Effect of Water Temperature on Susceptibility to *Streptococcus agalactiae* Serotype Ia Infection in Nile Tilapia (*Oreochromis niloticus*)

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Abstract

Streptococcus agalactiae is one of the causative agents associated with warm-water streptococcosis, in which produce massive mortality in aquaculture. Emergence of disease in tilapia farm usually occurs in high temperature season, which suggested for higher susceptibility of tilapia in this particular condition. Thus, the objective of this study is to investigate the association between water temperature and susceptibility of Nile tilapia (*Oreochromis niloticus*) to *S. agalactiae* serotype Ia infection. Nile tilapia were inoculated with 10^8, 10^7 or 10^6 CFU/ml of *S. agalactiae* serotype Ia field strain via water immersion route and maintained in different water temperature at 25, 30 or 33°C for 1 week. Diseased fish showed typical signs of bacterial septicemia including skin hemorrhage, ascites, kidney enlargement and petechial hemorrhage at liver and brain tissue. Accumulated mortality of tilapia was highest in the group maintained at 33°C followed by 30°C, while at 25°C most of the fish survived and clinical signs were not exhibited. The results from this study suggested that Nile tilapia reared in high water temperature condition susceptible to *S. agalactiae* via water exposure route.

Keywords: immersion route, Nile tilapia, *Streptococcus agalactiae*, susceptibility, water temperature

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บทคัดย่อ

ผลของอุณหภูมิน้ำที่มีต่อความไวต่อการติดเชื้อ สเตรปโตคอคคัส อกาแลคติเอ้ ซีโรไทป์ วันเอ ในปลานิล

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โรคติดเชื้อสเตรปโตคอคคัส อกาแลคติเอ้ ถือเป็นโรคที่สำคัญในสัตว์น้ำเนื่องจากก่อให้เกิดอัตราการตายที่ค่อนข้างสูง การเกิดโรคมักพบในฤดูที่มีอากาศร้อนแสดงให้เห็นว่าความรุนแรงของโรคมีความสัมพันธ์กับอุณหภูมิ ในการทดลองครั้งนี้จึงได้ทำการพิสูจน์ความสัมพันธ์ดังกล่าว โดยการวัดอัตราการตายของปลานิล (*Oreochromis niloticus*) ที่ได้รับเชื้อด้วยวิธีแช่น้ำที่มีความเข้มข้นของเชื้อ 10^8, 10^7 และ 10^6 CFU/ml โดยนำไปเลี้ยงไว้ในน้ำที่มีอุณหภูมิแตกต่างกันคือ 33, 30 และ 25 องศาเซลเซียส เป็นเวลา 1 สัปดาห์ หลังจากที่ปลาได้รับเชื้อพบว่าปลาบางส่วนแสดงอาการป่วยและมีรอยโรคดังต่อไปนี้ คือ มีปื้นเลือดออกตามผิวหนัง ท้องมาน ไตบวม มีจุดเลือดออกที่ตับ ไตและสมอง ซึ่งล้วนเป็นอาการที่มักพบในสภาวะที่มีการติดเชื้อในกระแสเลือด สำหรับปลาที่เลี้ยงไว้ที่ระดับอุณหภูมิน้ำ 33 องศาเซลเซียส พบว่ามีอัตราการตายสูงที่สุด ในขณะที่ 25 องศาเซลเซียส นั้นปลาส่วนใหญ่รอดชีวิตโดยที่ไม่แสดงอาการผิดปกติหรือรอยโรคใดๆ จากผลการทดลองดังกล่าวสามารถสรุปได้ว่าปลานิลที่เลี้ยงสภาพอุณหภูมิสูงนั้นมีความไวต่อการเกิดโรคติดเชื้อสเตรปโตคอคคัส อกาแลคติเอ้ ด้วยวิธีการแช่น้ำ

คำสำคัญ: การแช่น้ำ ปลานิล สเตรปโตคอคคัส อกาแลคติเอ้ ความไวต่อการเกิดโรค อุณหภูมิน้ำ

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Introduction

Streptococcus spp. are the cause of streptococcosis in many fish and mammalian species as well. To date, streptococcosis is recognized as a major infectious disease producing significant economic loss in aquaculture worldwide. Various species of the fish were reported as susceptible hosts for streptococcosis including rainbow trout (*Oncorhynchus mykiss*), hybrid striped bass (*Morone saxatilis* x *M. saxatilis*), channel catfish (*Ictalurus punctatus*), wild mullet (*Liza klunzingeri*) and Nile tilapia (*Oreochromis niloticus*) (Eldar and Ghittino, 1999; Shoemaker et al., 2001; Evans et al., 2002; Suanyuk et al., 2008). In Thailand, tilapia is regarded as the highest valuable fresh water aquaculture species (Fishery Statistics Analysis and Research Group, 2010). Two species of streptococcus, *Streptococcus iniae* and *S. agalactiae*, were reported in farmed fish in which the latter specie is in the majority with more than 80% prevalence rate (Maisak et al., 2008; Suanyuk et al., 2010).

S. agalactiae can infect several mammalian species and fish. The pathogen can produce septicemia and meningoencephalitis in diseased fish, which show various clinical signs such as skin hemorrhage, exopthalmia, ascites and erratic swimming concerned as a typical sign for streptococcosis (Austin and Austin, 2007). Several transmission routes were successful in experimentally infecting the fish with *Streptococcus spp.*, but infection via water exposure route is considered as major key role responsible for pathogen transmission in natural situation (Agnew and Barnes, 2007).

It is well-known that for aquatic animal, environments play an important role equally or more than pathogenic and host factor in the aspect of disease pathogenesis. In the case of warm-water streptococcosis associated with *S. iniae*, recent studies in barramundi (*Lates calcarifer*) and Mozambique tilapia (*Oreochromis mossambicus*) revealed that the mortality of the challenged fish was increased due to inappropriate water temperature (Bromage and Owens, 2009; Mian et al., 2009) and the critical temperature points causing fish susceptible to the disease varied from 17-28^{o}C depending on fish species (Nomoto et al., 2004; Agnew and Barnes, 2007; Bromage and Owens, 2009; Mian et al., 2009). Moreover, a quite recent study of *S. iniae* infection in rainbow trout showed that number of *S. iniae* in fish tissue was associated with bacterial pathogenesis as well (Lahav et al., 2004). Unfortunately, for S. *agalactiae*, a previous epidemiological investigation about streptococcosis occurrence in Nile tilapia farm

in Brazil was the only evidence suggesting that susceptibility to the disease is closely related to dynamic change in water temperature since the disease outbreak is usually found when the water temperature is higher than 26°C (Mian et al., 2009), while other studies of the correlation between *S. agalactiae* virulence and distribution behavior inside fish body are not available yet. With few scientific studies supported, how water temperature affects the *S. agalactiae* pathogenicity in Nile tilapia has not been clearly understood.

In this paper, we studied the distribution of *S. agalactiae* inside infected fish and also the direct effect of water temperature on the susceptibility of Nile tilapia to *S. agalactiae* infection via water exposure route, which will provide helpful information about disease pathogenesis and environment-pathogen relationship in cultured tilapia farms.

Materials and Methods

Fish: Nile tilapia (*O. niloticus*), each weighing one hundred grams (100 g) were maintained in three-ton aerated PVC tanks and acclimatized at least for 2 weeks before trial. A totally of 120 Nile tilapia were used in this study. The fish were sampled for bacterial isolation to ensure that they were streptococcal-free. During experiment, the fish were transferred into a 30 litre aerated acrylic glass tank for each experiment group. The tilapia would fed daily on commercial tilapia feed by 3% of bodyweight.

Preparation of bacterial inoculants: *S. agalactiae* serotype Ia isolated from Nile tilapia during disease outbreak in tilapia farms in central region of Thailand in the year 2008 was used in this study. Bacterial specie was identified using conventional biochemical assay and confirmed by species specific PCR technique (Martinez et al., 2001). Serotype of *S. agalactiae* was identified by using recent published multiplex PCR method (Imperi et al., 2010). Glycerol stocked bacteria was sub-cultured on tryptic soy agar (Difco, USA) containing 5% sheep blood.

Preparation of bacteria for inoculation was performed by dissolving pure colony of *S. agalactiae* growth on plate into 5 ml peptone water. The concentration of mixture was adjusted equivalent to 0.5 McFarland standard then add to 45 ml of tryptic soy broth (TSB) (Difco, USA) and incubated at 37°C overnight in 100 rpm rotary shaker. The concentration of incubated bacterial mixtures determined by standard direct plate count was 1.06×10^9 CFU/ml. This suspension was diluted in 3 litre of phosphate buffer saline (PBS) for equilibrating the final concentration as 10^8, 10^7 or 10^6 CFU/ml. Mixtures were prepared for challenging the fish.

Fish inoculation: One hundred and twenty Nile tilapia (*O. niloticus*) (n=120) were used in 4x3 factorial designed experiment. The animals were divided into 12 experiment groups containing 10 tilapia each. Each group was challenged by 10^8, 10^7 or 10^6 CFU/ml of *S. agalactiae* suspension via water immersion for 15 min followed by washing the fish by dipping in sterile PBS for 15 sec. These bacterial inoculation dose we use in this experiment were decided base on previous information which found that *S. agalactiae* virulence strain successfully infected Nile tilapia by water exposure route at the concentration of 10^6 CFU/ml (Mian et al., 2009).

After inoculation, each group of tilapia was maintained in particular tank that water temperature were regulated as 25, 30 and 33°C using thermostat. For negative control, three groups of tilapia were immersed in sterile PBS and raised in different water temperature as well. Clinical signs and mortality of experimental and control fish were recorded for 7 days. Dead fish were rapidly removed from the tank and the gross lesions also recorded. Internal organs (spleen, kidney and brain) were collected for determination of histopathological change using standard H&E stain. Bacterial species were isolated from those organs and cultured on TSA containing 5% sheep blood. Additionally, the kidney, liver and brain from 5 freshly dead fish were also aseptically collected and pooled prior to being ground with tissue mortar. Homogenate tissue were diluted in sterile PBS and spread on streptococcus specific media (TSA + 10 mg/l of colistin sulfate (Sigma, USA) + 5 mg/l of oxolinic acid (Sigma, USA) to enumerate *S. aglactiae* sustained in homogenate fish tissue (Nguyen and Kanai, 1999).

Statistical analysis: Data analysis was performed using Statistical Package for the Social Sciences (SPSS) version 11.5 for Windows (SPSS Inc.). For tilapias that were challenged with the same concentration of bacterial suspension, the mortality of the fish kept in different water temperature conditions was compared by Pearson's chi-square test.

Results

Number of Dead tilapia challenged with 10^6, 10^7 and 10^8 CFU/ml of *S. agalactiae* serotype Ia were 3, 8 and 10, respectively. Mortalities first occurred at 2 day post inoculation (dpi) and increased until 7 dpi. Most of the external lesions showed on diseased and dead tilapia were skin hemorrhage, ascites and anal swelling. For internal lesions, severe peritonitis, splenomegaly, renomegaly, petechial hemorrhage at liver and brain tissue were most frequently found among infected fish (Fig 1). Histopathological changes were found in numerous internal organs. The head and trunk kidney showed haemolysis and tubular degeneration respectively (Fig 2A & 2C). Granuloma-like lesion with hemosiderosis was observed at multiple site of splenic tissue (Fig 2B). Leukocytes were infiltrated in surrounded area of brain tissue indicating meningitis condition (Fig 2D).

Five (5) freshly dead tilapia at 2-4 dpi were randomly selected and enumerated *S. agalactiae* sustained in visceral organs of the fish. Results showed that *S. agalactiae* serotype Ia was highly distributed to brain and kidney of infected fish (Table 1).

Figure 1 Lesions of Nile tilapia challenges with *S. agalactiae* via water immersion route. A: Hemorrhage at operculum and base of the fin, B: Anal swelling (arrow), C: Kidney enlargement (arrow) and petechial hemorrhage at liver (arrow head), D: Hemorrhage at brain tissue.

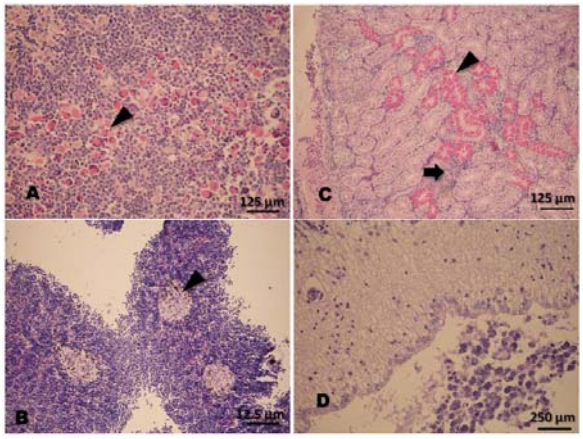

Figure 2 Histopathological lesions of internal organs of Nile tilapia infected with *S. agalactiae* showed A: hemolysis at head kidney (arrow head), B: Granuloma-like lesion at spleen (arrow head), C: tubular degeneration (arrow head) with leukocytic infiltration (arrow) at trunk kidney, D: Leukocytic infiltration in surrounding area of brain tissue.

Table 1 Average number of *S. agalactiae* sustained in brain, kidney and liver tissue from randomly selected 5 dead tilapia at 2-4 day post inoculation (dpi) with bacterial pathogen.

	Brain	Kidney	Liver
Number of *S. agalactiae* (log CFU/g of fish tissue ±SD)	7.70±0.7	7.58±0.5	6.79±0.4

Accumulated mortality at 7 dpi of the fish challenged with 10^8 CFU/ml bacterial cells are highest in the group maintained at 33 ^oC ($p<0.05$). For fish that were challenged with 10^7 or 10^6 CFU/ml, the mortality showed no significant difference ($p>0.05$) among experimental groups kept in various temperature conditions (Fig 3). *S. agalactiae* serotype Ia could be re-isolated from every dead fish confirming streptococcal septicemia.

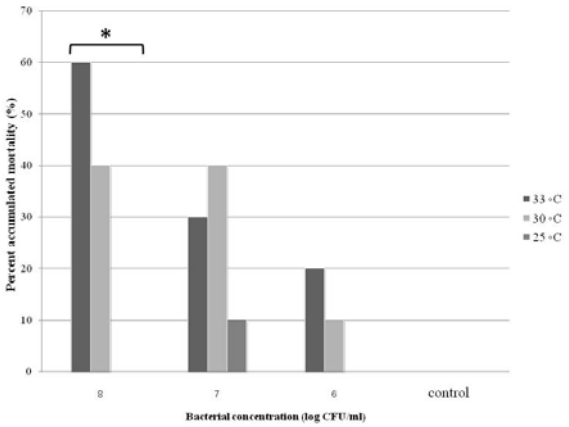

Figure 3 Percent accumulated mortalities at 7 day post inoculation (dpi). The data is shown mortalities of Nile tilapia that exposed with various concentration of *S. agalactiae* contained in PBS for 15 min, for control group the fish were challenged with sterile PBS instead. Each experimental group was kept in separated tank, which water temperature was set at 25, 30 or 33^oC. An asterisk is indicated for significantly different ($p<0.05$) of accumulated mortality among experimental groups.

Discussion

In this study, Nile tilapia challenged with *S. agalactiae* serotype Ia exhibited various signs and lesions resembling with streptococcosis such as skin or visceral organs hemorrhage, peritonitis, ascites, and anal protrusion while histopathological changes were observed in several organs including brain tissue, which suggested that septicemia condition occurred (Austin and Austin, 2007). These gross and histopathological lesions indicated the successful of experimental infection of *S. agalactiae* via water immersion route. Enumeration of streptococcus contaminated in visceral organs of fish (Table 1) also confirmed the bacteremia condition and suggested that brain and kidney might be target organs specified to this pathogen since large amount of bacteria was found. The result was similar to the previous study about warm-water streptococcosis associated with *S. inae* in rainbow trout which showed that *S. iniae* mostly distributed into brain tissue and the number of sustained bacteria was higher than 10^8 CFU/g of fish tissue (Lahav et al., 2004).

For inoculation experiment, water temperatures tended to associate with accumulated mortality only in the fish challenged with 10^8 CFU/ml, while another showed no significant importance. Only one tilapia kept at 25^oC died after bacterial challenge. It may suggest that Nile tilapia tolerate *S. agalactiae* in this circumstance. On the other hand, susceptibility to infection was higher when the temperature was ≥ 30^oC. Our results conform to study from Mian et al. (2009) which found that streptococcosis associated with *S. agalactiae* in Nile tilapia farms emerged only when water temperature was above 26^oC. Additionally, the challenging model we chose was not injectable challenging since it unlikely to natural route of transmission because adhesion and invasion step of infection are passed

over. Hence, to mimic the natural infection route, the water immersion route was selected instead and the results showed that the susceptibility to infection still depended on water temperature.

According to previous studies, non-specific and specific immune response of teleost are significantly decreased either when the water temperature is lower or higher than normal physiological range of the fish (Le Morvan et al., 1998; Langston et al., 2002). In the case of tilapia, the optimum temperature for growing is about 29 to 31°C (Yanong and Francis-Floyd, 2002). A study in Mozambique tilapia (*O. mossambicus*) demonstrated that at 19 and 35°C, fish challenged with *S. iniae* via injection route exhibited the highest mortality rate due to the lower non-specific immune response of the fish (Ndong et al., 2007). According to our study, the highest mortalities in the fish reared at 33°C might be affected by the reduction of host immunogenic activity. However, the decrease in host immunity might not be the only factor responsible for fish susceptibility. *S. agalactiae* growth activity and pathogenicity were also influenced by fluctuation of surrounded temperature. A study of human S. agalactiae revealed that the transcription of some importance virulence factors, including hemolysin, was increased at 40°C compared with 30°C (Mereghetti et al., 2008). Up to this date, there still has not been any scientific evidence showing whether the change of virulence of fish *S. agalactiae* corresponds with water temperature.

Conclusion

In conclusion, this study documented that Nile tilapia reared at high water temperature was susceptible to streptococcosis associated with water transmission of *S. agalactiae* serotype Ia. For a tropical country like Thailand, susceptibility to streptococcus infection due to high temperature might be significantly important especially in the summer period. Therefore, the study of the relationship among fish, *S. agalactiae* and environmental conditions, especially water temperature, should be further investigated.

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