DETERMINATION OF LD_{50} FOR *STREPTOCOCCUS AGALACTIAE* AND *STAPHYLOCOCCUS AUREUS* INFECTIONS IN TILAPIA

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SUMMARY

One hundred and sixty fingerlings and 80 adult tilapias were experimentally infected with *Streptococcus agalactiae* and *Stapylococcus aureus* to determine their $LD_{50.}$ Four concentrations of *Streptococcus agalactiae* (10^9, 10^8, 10^7, 10^6 CFU/mL) were used in this experimental infection. This tilapia were divided into 4 groups of 40 fingerlings and 20 adults per group. Groups 1, 2, 3 and 4 of the fingerlings were exposed to 10^9, 10^8, 10^7, 10^6 CFU/mL of *S. agalactiae* by immersion in 2 L inoculum solution for 20 min. Similarly, the adult groups were exposed to the same concentrations of *S. agalactiae* but by intraperitoneal injection at the rate of 1 mL of the inoculum per gram. Similar procedures were repeated using exposure to *Staphylococcus aureus* alone or a combination of *S. agalactiae* and *S. aureus.* All test groups were observed for signs of infections. On Day 7 post-infection (pi), all fish that were still alive were humanely killed. The LD_{50} of the adult tilapia that were exposed to *S. agalactiae*, *S. aureus* or mixed infection was 2.3884×10^7, 2.8151×10^8, and 4.2409×10^5, respectively. For the fingerling groups, the LD_{50} for *S. agalactiae*, *S. aureus*, and mixed infection was 2.9242×10^{20}, 2.8665×10^{17}, and 4.9748×10^{11}, respectively. Experimental infection in adults could be established within 12 h post-injection to 6.3×10^9 CFU per mL and 9.7×10^9 CFU per mL of *S. agalactiae* and *S. aureus*, respectively. For fingerlings, infection could be established within 72 h following bath immersion to 6.3×10^9 CFU per mL and 9.7×10^9 CFU per mL of *S. agalactiae* and *S. aureus*, respectively.

Keywords: *Streptococcus agalactiae, Staphylococcus aureus,* pathogenicity, LD_{50}.

INTRODUCTION

Streptococcal infection in fish was first reported in cultured rainbow trout in Japan in 1957 (Hoshina *et al*., 1958). Since then, numerous species of fish including tilapia have been found to be susceptible to the infection. Ferguson *et al*. (1994) conducted a study using *S. agalactiae,* which showed a virulent infection with 100% mortality rate as compared to other environmental bacteria.

Streptococcus agalactiae was frequently isolated from cases of high mortality of cage-cultured tilapia in Kenyir and Pergau lake in 2002 to 2003 (Siti-Zahrah *et al*., 2004; 2005). This incidence was found to be related with seasonal changes and with poor water quality, which affected the physiological conditions of the fish. Isolation showed the presence of many *Streptococcus* species. including the alpha and beta haemolytic types. Most of the isolates were identified as *S. agalactiae* and no isolation of *S. iniae* as reported in Indonesia or Thailand.

Surveillance of tilapia from ponds, irrigation canals, rivers, reservoirs and ex-mining pools, disclosed that *Staphylococcus aureus* is very common in tilapia. *S. aureus* is the most common species causing infection in man. However, during our screening surveillance in tilapia, the isolates obtained were mainly *Streptococcus* species. From this finding, a study of streptococcosis and staphylococcosis in tilapia was conducted to determine their virulence and disease pattern.

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MATERIALS AND METHODS

Fish

Red tilapia (*Oreochromis* spp.) consisting of fingerlings and adults were obtained from Aquaculture Extension Centre (AEC), Jitra, Kedah, Malaysia. These tilapias were transferred and reared at the National Fish Health Research Centre (NaFisH), Fisheries Research Institute, Department of Fisheries Malaysia, Batu Maung, Penang, Malaysia. Prior to the experiment, all tanks were cleaned and disinfected. The randomly selected fish were then screen for *S. agalactiae* and *S. aureus*. Every fish was weighed and the water was aerated continuously throughout the study. The mean weight was 10 ± 5 g and 100 ± 10 g for the

fingerling and adult, respectively. Once the tilapia were free from both pathogens, 160 fingerlings and 80 adults were randomly assigned to eight 200-L tanks for each experiment. Light cycle was maintained constantly at 12 h light and 12 h dark per day. Water temperature was checked each day and the fish was fed *ad-libitum* with a commercial feed.

Water Quality

The temperature, pH and dissolved oxygen were measured using YSI 556 (YSI, USA). The ammonia, sulphate and nitrites were determined daily using a DR 2800 Portable Spectrophotometer (Hach, USA). The water quality parameters were 4.97 ± 0.3 mg L^{-1} for dissolved oxygen, 32.6 ± 0.8 °C for temperature, 7.47 ± 0.1 for pH, 2.37 mg L^{-1} for ammonia and 0.023 mg L^{-1} for nitrate concentrations.

Bacteria, bacterial culture and inocula

Streptococcus agalactiae and *Staphylococcus aureus* that have been isolated from an outbreak and screening samples were used for the study. Bacteria were cultured on blood agar and further sub-cultured into brain heart infusion broth (BHIB) in 30 °C shaking incubator for 18 h. At this time, the cultures were in the stationary phase of growth. Following incubation the bacterial concentrations were determined using the standard plate count technique (Alcamo, 1997). Approximately 1 mL of the broth was serially diluted 10-fold before 0.1 mL of each dilution was poured and spread onto blood agar and incubated for 24 h at 30 °C. Following incubation, the number of colonies, particularly those plates containing between 25 to 250 colonies, was counted before the concentration was expressed as colony forming unit (CFU). The final concentration of live *S. agalactiae* and *S. aureus* were then recorded. For a lower concentration, the stock solution 10^9 were then diluted tenfold using phosphate buffered saline (PBS) and the inocula were immediately taken. The bacterial cells were harvested by centrifugation at 3500 *g* for 10 min at 4 °C.

LD_{50} challenge and sampling

This study was designed for six days after the exposure. Experiment was divided into three adult groups with duplicates. Group 1 was exposed to only *S. agalactiae*, Group 2 was exposed only to *S. aureus*, and Group 3 was exposed to *S. aureus* then followed by *S. agalactiae* on Day 2. For each group, four different concentration were used; 10^9, 10^8, 10^7, 10^6. All fish were infected by injecting the inoculum at the rate of 1 mL intraperitoneally (i.p.) according to their mean body weight (1 mL ~100 g).

Similar experimental design was assigned to the fingerling group, except the challenge trials were using bath immersion (b.i.) for 20 min. for each concentration before transferred into the tanks. Mortality was recorded every 12 h daily. The dead fish were subjected to bacterial isolation in which samples of the eyes, brain, and kidneys were examined. At the end of the study, the survival fish were humanely killed and necropsied for bacterial isolation. The data on the LD_{50} was analyzed using Curve Expert version 1.34 (USA).

Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 12.4.0.0 (MedCalc Software, Mariakerke, Belgium) and tested at 5% level of significance. The differences in the mortality and other data were analysed using a one-way ANOVA. If significant differences were obtained, a student-Newman-Keuls pairwise comparison post-hoc test was employed to determine the statistical differences between the treatments.

RESULTS

Clinical observations

The clinical signs of the infected fish were erratic swimming, curved body posture (C-shaped), petechial haemorrhages around the eyes, development of corneal opacity and blindness. Another observation in the infected with streptococcosis was that the fish isolated themselves at the corner of the tank before becoming moribund and eventually died the following day.

LD_{50} analysis

For adult tilapia, the LD_{50} for Group 1 being challenged with *S. agalactiae* was 2.3884×10^7 and it was lower than Group 2 being challenged with *S. aureus*, 2.8151×10^8. Group 3 challenged with *S. aureus* and *S. agalactiae* together was 4.2409×10^5 which was lower compared to Group 1 and Group 2 (Table 1). The results showed that a single infection of *S. agalactiae* was more pathogenic compared to a single infection of *S. aureus*. However, combined infection by S.

agalactiae and *S. aureus* together, the infection was worst. In this experiment, *S. aureus* was given first as *S. Aureus'*s virulence depends on its protein A that will bind to the Fc of IgG that protects it from phagocytosis (Easmon *et al*., 1983). Following that *S. agalactiae* was inoculated to the same fish which was in a stress condition due to the primary infection by *S. aureus*.

In fingerlings, the LD_{50} for Group 1 challenged with *S. agalactiae* was 2.9242×10^{20} and this was higher than Group 2 that was challenged with *S. aureus*, 2.8665×10^{17}. This results revealed that *S. aureus* was more pathogenic compared to *S. agalactiae* probably due to the *S. aureus* exotoxins. The fingerlings in Group 3 were challenged with *S. aureus* and *S. agalactiae* and had a LD50 of 4.9748×10^{11} (Table 1).

Bacteria	Adult tilapia (i.p.)	Fingerling tilapia (b.i.)
S. agalactiae	2.3884×10^{7}	2.9242×10^{20}
S. aureus	2.8151×10^{8}	2.8665×10^{17}
S. agalactiae + *S. aureus*	4.2409×10^{5}	4.9748×10^{11}

Table 1: LD50 analysis (CFU/mL)

The results for adults showed that a single infection of *S. agalactiae* was more pathogenic compared to a single infection of *S. aureus*. However, combination of infection by S. *agalactiae* and *S. aureus* together, the infection was more severe. In contrast to fingerlings, the results revealed that *S. aureus* was more pathogenic compared to *S. agalactiae* probably due to the *S. aureus* exotoxins. The combined infection in Group 3 produced less CFU compared to others.

Bacteria	Adult tilapia (i.p.)	Fingerling tilapia (b.i.)
S. agalactiae	6.3×10^{9} (12 h)	6.3×10^{9} (72 h)
S. aureus	9.7×10^{9} (12 h)	9.7×10^{9} (12 h)
S. agalactiae (D1) + *S. aureus* (D2)	6.3×10^{9} (12 h)	6.3×10^{9} (12 h)

Table 2: Pathogenicity (Time)

The results for adults showed all groups can produce the disease within 12 h in i.p. route administration. However for fingerlings, group *S. agalactiae* took a longer time of 72 h to create the disease compared to other groups which took 12 h in b.i. route.

Note: b.i. (bath immersion), i.p. (intraperitoneal), D1 (Day 1), D2 (Day 2), h (hour)

Pathogenicity analysis

In adult tilapia, pathogenicity was evidence from inoculation of *S. agalactiae* 6.3×10^{9} within 12 h by i.p. injection. On the other hand, *S. aureus* with 9.7×10^{9} inoculum was slightly higher. For adults tilapia there was not much difference in the pathogenicity between *S. agalactiae* and *S. Aureus.* As seen in Figure 1, the combined inocula showed the highest mortality followed by *S. agalactiae* and *S. aureus* if given individually.

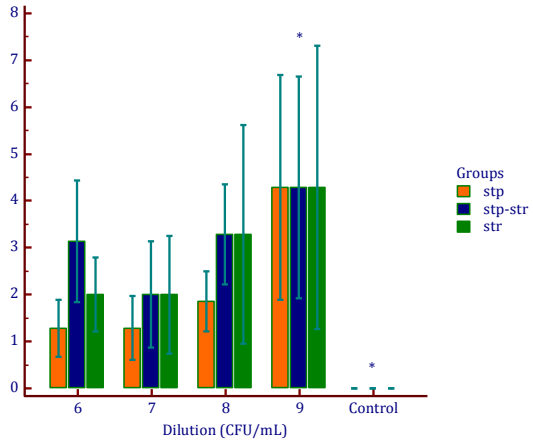

Figure 1: Mortality in adult tilapia with different CFU 6 (10^6), 7 (10^7), 8 (10^8), and 9 (10^9) of *S. agalactiae*, *S. aureus* and combination of S. *aureus* and *S. agalactiae*. Data are presented as SEM. Significance is illustrated between each dilution and each treatment group at $P < 0.05$ as indicated by an asterisk.

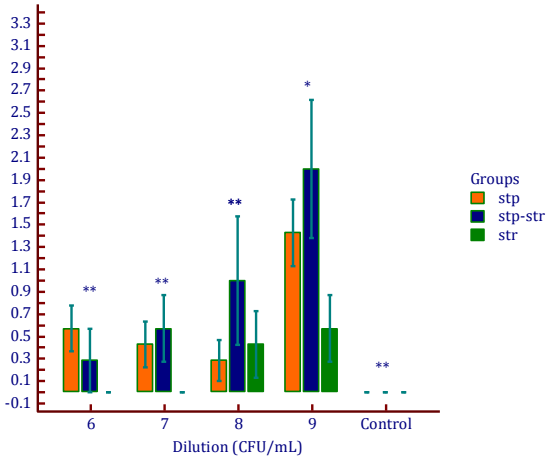

Figure 2: Mortality in fingerling with different CFU 6 (10^6), 7 (10^7), 8 (10^8), and 9 (10^9) of *S. agalactiae*, *S. aureus* and combination of *S. aureus* and *S. agalactiae*. Data are presented as SEM. Significance is illustrated between each dilution and each treatment group at $P < 0.05$ as indicated by different asterisk.

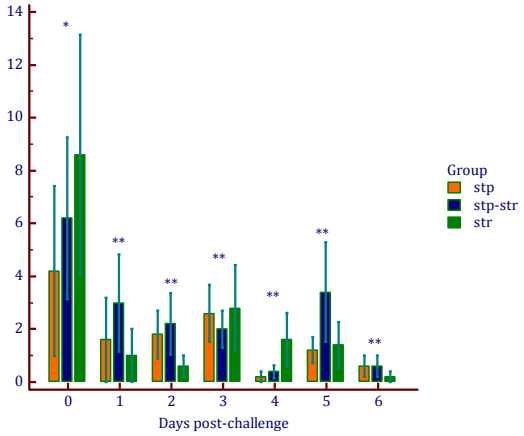

Figure 3: Administration of infection leading highest mortality of adult tilapia occurred in day 0 with the Strep group was the highest compared to other groups. Data are presented as SEM. Significance is illustrated between days post-challenge and each treatment group at *P* < 0.05 as indicated by different asterisk.

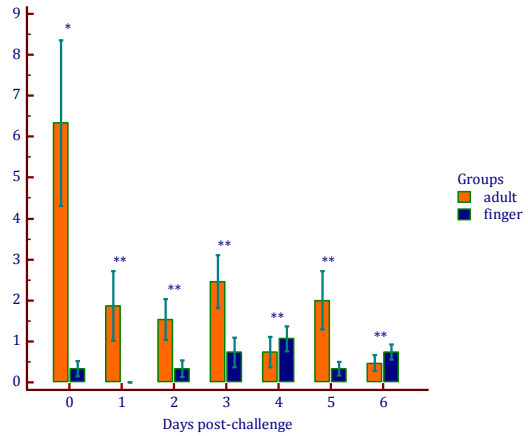

Figure 5: Mortality in the adult and fingerling groups from day 0 to day 6. Data are presented as SEM. Significance is illustrated between days post-challenge and each treatment group at *P* < 0.05 as indicated by different asterisk.

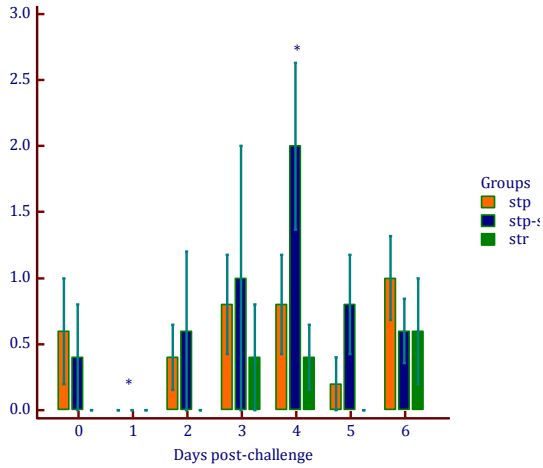

Figure 4: Administration of infection leading highest mortality of fingerling occurred in day 0 with the stp-str group was the highest compared to other groups. Data are presented as SEM. Significance is illustrated between days post-challenge and each treatment group at *P* < 0.05 as indicated by an asterisk.

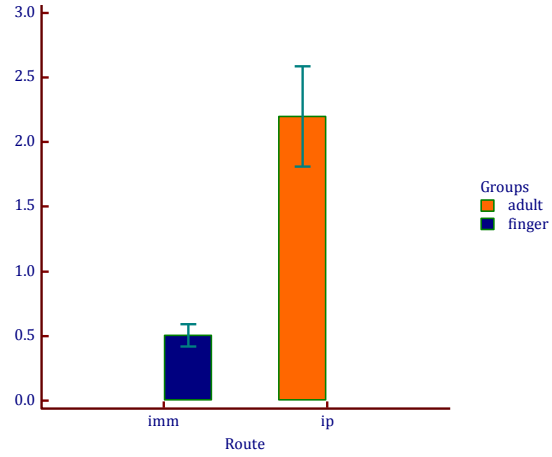

Figure 6: Mortality between the adult and fingerling by comparing the route of infection adminsitration. Data are presented as SEM. Significance is illustrated between route of infection and treatment group at *P* < 0.05 as indicated by different error bar.

In Figure 3, mortality started at Day 0 in all group but the *S. agalactiae* group recorded the highest mortality on the Day 0 itself. Mortality continued to occur until the final day of experiments.

In fingerling tilapia, the pathogenicity was different as seen in *S. agalactiae* it was 6.3×10^9 within 72 h whilst *S. aureus*, it was 9.7×10^9 within 12 h. Fingerlings infected with *S. aureus* showed the earliest clinical signs of staphylococcosis. Pathogenicity for *S. agalactiae* with *S. aureus* as stress factor was 6.3×10^9 within 12 h (Table 1). As seen in Fig. 2, the combined group showed the highest mortality followed by *S. aureus* and *S. agalactiae* individually. The mortality was also significant with the concentration accordingly and the *S. agalactiae* group was the lowest mortalities among the groups.

In fingerling tilapia, *S. aureus* group recorded the highest mortality on Day 0 as seen in Figure 4 but on Day 4, the combined group recorded the highest mortality. Simialar to the adult tilapia, mortality continued to occur till the end of experiment.

Figure 5 showed that mortality of both sizes of experimental fish started to occur from the first day of infection. It was observed that the adult group recorded the higher mortality compared to the fingerling group and that the i.p. inoculation caused systemic infection while the bath immersion caused mucosal infection.

DISCUSSION

Streptococcus agalactiae infection can be established in tilapia without any symptoms, where the bacteria can be isolated from the fish's brain (Amal *et al*., 2008). However, during stress the disease may progress to evoke signs of abnormalities as well as causing an acute infection leading to death. This disease is related to any stress that may be faced by the fish in any condition such as transportation from farm to farm, incline and decline water temperature, low dissolved oxygen and other factors that contribute to the disease development by *S. agalactiae* including *S. iniae* (Klesius *et al*., 2008). An outbreak of *S. agalactiae* infection occurred at water temperature of 27 °C and the many fish were observed in floating cage systems in Brazil (Mian *et al*., 2008).

Farm must take stringent measures to overcome the infection by having good practice in handling water quality and if possible to conduct vaccination program if it is appropriate. The disease can be controlled or prevented by vaccination given by intra-peritoneal injection, immersion or other techniques. Here, we managed to produce the disease within 12 h post-injection by i.p. (Figures 1 & 3). The disease was less if by the immersion route (Figures 2 & 4). Due to the importance of stress in disease development we used *S. aureus* either by i.p. injection or bath immersion to lower the immune response of tilapia before exposure to *S. agalactiae* (Figs. 1, 2, 3, & 4). It appears that the disease developed faster and with lower CFU of bacteria to initiate the infection in the fish (Figures 1 & 2). Acute infection by *S. agalactiae* can be established using i.p. injection. However, in this study we found that single i.p. injection need a high dose of bacteria compared to a combined inoculum which requires a lower dose of bacteria.

We found that i.p. route of infection of *S. agalactiae* in tilapia successfully established the infection as early 12 h post-inoculation ($P < 0.05$) compared to immersion with high mortality rate (Figures 5 & 6). It was observed that high doses of bacteria, route of infection and age of tilapia either fingerling or adult together could easily cause infection leading to death. But, in low CFU dose, tilapia showed slow development of the disease with appearance of exophthalmia, haemorrhagic eyes, spiralling and erratic swimming, curved body posture (C-shaped) or position themselves from the group in the corner of the tank with torn dorsal fins. In the end, the infected fish succumbed to the infection. Other workers have produced fish streptococcosis experimentally by immersion, injection and cohabitation (Robinson & Meyer 1966) and by skin injury followed by immersion (Rasheed & Plumb 1984). Oral administration of the bacteria did not produce mortality nor did crowding and low oxygen concentrations following by immersion of the fish (Rasheed & Plumb, 1984).

It is shown that tilapia is more susceptible by i.p. route rather than immersion (Figures 5 & 6). The factors that play a role in infection were not only the route of infection, but also the dose of bacteria and their virulence. To increase the virulence of the *S. agalactiae* and *S. aureus* prior to infection, the pathogens were inoculated into the tilapia and re-isolated from them before inoculating the organisms into the tilapia via i.p. The experimental results concluded that *S. agalactiae* is more pathogenic compared to *S. aureus.*

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