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RESEARCH ARTICLE

Susceptibility of Soiny Mullet (*Liza Haematocheila*) to *Streptococcus Dysgalactiae* and Physiological Response to Formalin Inactivated *S. Dysgalactiae*

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ABSTRACT

Streptococcosis caused great lost in intensive aquaculture in China every year. The *Streptococcus dysgalactiae*, one of the main pathogen of streptococcosis in cultured fish in China, caused high mortality of Soiny Mullet (*Liza hematocheila*) with typical symptom after intraperitoneal injection. The semi-lethal dose of *S. dysgalactiae* to mullet was 5.4×10^6 CFU. The antigen against *S. dysgalactiae* was prepared by formalin inactivated *S. dysgalactiae*. After intraperitoneal injection of the antigen, the survival rate of fish infected with *S. dysgalactiae* was significantly increased. At the same time, the splenic and hepatic lysozymes of mullet were also significantly increased. The splenic and hepatic ALP were decreased from 1 day to 3 day and back to normal level at 7 days after vaccine injection. All these data strongly suggested that the formalin-inactivated *S. dysgalactiae* is an effective strategy to protect mullet against *S. dysgalactiae*.

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INTRODUCTION

Bacterial diseases and infections are the most cause of economic losses in fish farming. Among them, the streptococcal infections usually cause high mortality (more than 50%) and last a long period of time (Yanong and Francis-Floyd, 2006). Fish streptococcosis is currently considered as one of the main limiting factors in the aquaculture industry (Pourgholam *et al*., 2011). In china, the outbreak and infected area of streptococcosis increased year by year. In June 2006, a streptococcosis outbreak occurred on cage-cultured sturgeon (*Acipenser schrenckii*) with high mortality and continued at least until October (Yang and Li, 2009). In the summer of 2009 and 2010, outbreaks occurred on pond-cultured tilapia (*Oreochromis* spp.) in major cultivation areas of southern China (Ye *et al*., 2011).

To date many strains of the genus *Streptococcus* have been isolated and proved to be fish pathogen, including *S. iniae*, *S. milleri* (Yanong and Francis-Floyd, 2006), *S. parauberis* (Doménech *et al*., 1996), *S. agalactiae* (Evans *et al*., 2002), *S. ictaluri* (Shewmaker *et al*., 2007) and *S. dysgalactiae* (Zhou *et al*., 2007). *S. dysgalactiae* is a Gram-positive cocci and more outbreaks of *S. dysgalactiae* have been reported globally (Netto *et al*., 2011). During outbreaks, *S. dysgalactiae* exerts moderate to high mortality in many cultured fish including amberjack (*Seriola dumerili* Risso), yellowtail *Seriola quinqueradiata* (Nomoto *et al*., 2004), seal (*Phoca vitulina*) (Vossen *et al*., 2004), grass carp (*Ctenopharyngodon idella*) and crucian carp (*Carassius carassius*) (Yang and Li, 2009).

Soiny Mullet is an economically important fish in China, especially in Jiangsu Province. The Dafeng town, one of city in Jiangsu Province, alone produces over 7500 tons of mullet every year. Strong disease resistance is one of the most important characteristics of mullet. However, in recent years with the application of intensive aquaculture for this species, the occurrence of bacterial infection in mullet has increased every year (Ye *et al*., 2008). The present study was to detect the virulence of *S. dysgalactiae* to mullet and evaluate the Physiological response to formalin inactivated *S. dysgalactiae* antigen in mullet.

MATERIALS AND METHODS

Fish: Healthy mullet (Avg. weight 5.0±0.5 g) were obtained from a local commercial fish farm at Dafeng

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town, Jiangsu Province, China. The fish were transported to the Laboratory of Department of Ocean Technology by oxygen supplying car. Fish were kept in 120 L plastic aquaria and supplied oxygen using an electric air pumping compressors. The fish were maintained on a 14-h light/ 10-h dark cycle at 22°C and fed twice daily with a commercial diet. The YSI multiprobe (YSI 556 Multiprobe system) was used to monitor dissolved oxygen (DO), pH, and temperature. The water conditions were controlled as: DO 6.2 mg/L, pH 7.0, total ammonia <0.2 mg/L, and nitrite < 0.02 mg/L. All fish were acclimatized for 2 weeks prior to use.

Preparation of formalin-inactivated *S. dysgalactiae* antigen: The *S. dysgalactiae* was kindly provided by Pro. Li from the State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, the Chinese Academy of Sciences. This bacterial was isolated from ten diseased Amur sturgeons and was confirmed as *S. dysgalactiae* by using physiological, biochemical properties and molecular analysis (Yang and Li, 2009). The *S. dysgalactiae* was inoculated in 100 mL flask having Todd Hewitt Broth (THB) broth and kept on an orbital shaker at 100 rpm for 24 hours under 24°C. After that the cells were collected by centrifugation at 10000 *g* for 10 min and washed three times with sterile phosphate buffer solution (PBS, 0.1 M, pH 7.0). Then 1.0% formalin was added to kill the *S. dysgalactiae*. After the formalized solution was kept for 24 h, the cells were harvested by centrifugation at 10000 *g* for 10 min and washed twice with sterile PBS. The cells were re-suspended in sterile PBS and the concentration of cells was adjusted to 1.0×10^8/ mL. The preparation was stored at 4°C until use.

Experimental infection: Firstly, sixty healthy mullet (5.0±0.5 g) were divided into six groups, including one control group and five bacterial challenge groups. There were 10 fish in each group. The fish in the challenge groups were injected intraperitoneally with *S. dysgalactiae* at the dosage ranging from 2×10^5 to 2×10^9 CFU per fish while the fish in the control group with equal amount of 0.1 M PBS. These fish were maintained at 22°C in six 120-L aquaria with artificial oxygen-supplement respectively. The trial lasted for 1 week. The mortality was recorded daily.

Secondly, a total of twenty healthy mullet (5.0±0.5 g), divided randomly into 2 groups (vaccinated group and control group) containing 10 fish each group, were utilized for assessing the effect of vaccine on the mortality after *S. dysgalactiae* infection. The fish in vaccinated group were intraperitoneally injected with 0.1 mL of the inoculum containing 1.0×10^8/mL of formalin-inactivated *S. dysgalactiae*, while the fish in control group were injected with equal amount of 0.1 M PBS. After 24 h of antigen injection, the fish in both groups were injected intraperitoneally with 0.1 mL 2×10^9 CFU *S. dysgalactiae*. After injection, the fish were monitored daily for 7 days and the mortality was recorded each day.

Physiological response in mullet: For this analysis, 24 healthy mullets were randomly divided into 2 groups (formalin inactivated *S. dysgalactiae* antigen group and control group). Each group contained twelve fish. For the antigen group, the fish were injected intraperitoneally with 0.1 mL of the inoculum containing 1.0×10^8/ mL of formalin inactivated *S. dysgalactiae* antigen while the fish in control group were injected with equal amount of PBS. After injection for 1 d, 3 d, 5 d and 7 d, three fish from each group were sacrificed and their livers and spleens were collected for the enzyme assays, respectively. The fish in control group were also sampled at the same time. The liver (avg. weight 0.133±0.051 g) and spleen (avg. weight 0.037±0.011 g) samples were homogenized and the supernatants were diluted using 0.5 mL PBS for enzyme assay. The protein concentration was measured by the Braford method.

The level of lysozyme in samples was determined by turbidimetric assay kit (Lie *et al*., 1989) from Jiancheng Bioengineering Institute (Nanjing, China) with slight modifications. Briefly, 2 mL *Micrococcus lysodeikticus* solution was mixed with 0.2 mL of each sample and the transmittance was measured after 5 and 125 seconds by UV-2100 spectrophotometer (Unic Co., Ltd., China) at 530 nm wavelength. PBS was used as the blank and the pure lysozyme (provided by Jiancheng) as positive control.

The samples used for alkaline phosphatase (ALP) activity were prepared as mentioned above. The activity of ALP was determined using the commercial kit from Jiancheng Bioengineering Institute (Nanjing, China).

Statistical analysis: Data were statistically analyzed using one way analysis of variance (ANOVA) in SPSS statistical software. Significant differences were set as $P<0.05$.

RESULTS AND DISCUSSION

S. dysgalactiae has been proved to be virulent and epidemic in several fish. Our observations showed that this bacterium also shared high virulence to mullet. After intraperitoneally infected with live *S. dysgalactiae*, the mullet first became quiet and stay on the tank bottom. Then the morbid mullet exhibited anorexia, eccentric swimming behavior such as spiraling or spinning and finally died. Compared with healthy mullet (Fig. 1a), necropsy showed that morbid fish developed hepatomegaly, splenomegaly and gall bladder enlargement. The spleen was highly congested. Additionally, there was much bloody and yellowish fluid in the abdominal cavity (Fig. 1b). Yang and Li (2009) found that healthy Amur sturgeon died after being challenged with the dosage of 4×10^8 CFU *S. dysgalactiae*. Meanwhile, this isolate could also cause death of carps and the LD_{50} for crucian carps was 2.45×10^8 -5.54×10^8 CFU. Pan *et al.* (2009) also found *S. dysgalactiae* was lethal to Siberian sturgeon (*Acipenser baerii*) at a dosage of 3×10^8 CFU. In present work, we found the LD_{50} for *S. dysgalactiae* to mullet was 5.4×10^6 CFU and the virulence of *S. dysgalactiae* to mullet was dose dependent. At the first day after challenge, there was no mortality of mullet for each dose of *S. dysgalactiae*; then there were different numbers of fish dying in *S. dysgalactiae* injected groups. The mortality reached 30, 50, 60 , 70 and 90% after 7 days injection for 2×10^5 CFU, 2×10^6 CFU, 2×10^7 CFU, 2×10^8 CFU and 2×10^9 CFU *S. dysgalactiae* concentrations, respectively (Fig. 2). Our results were

coincidence with the observation of Yang and Li (2009). This condition was also similar to the natural status of *S. dysgalactiae* infection. Those results further confirmed that the *S. dysgalactiae* was epidemic in different fish species and this might one of reasons for the difficult prevention of streptococcosis in aquaculture.

Fig. 1: Anatomic feature of healthy mullet (a) and mullet infected with live *S. dysgalactiae* (b). Arrows showed the splenomegaly and gall bladder enlargement in diseased mullet.

Vaccines that developed by formalin killed bacteria have been found to be effectively for fish to defend streptococcosis. The formalin-killed *S. agalactiae* vaccine could significantly increase the survival rate of tilapia (*Orechromis niloticus*) after *S. agalactiae* challenged (Evans *et al*., 2004). Eldar *et al.* (1997) also found that formalin killed *S. iniae* vaccine could decrease the mortality of rainbow trout (*Oncorhynchus mykiss*) after *S.iniae* infection. The using of formalin inactivated vaccine in Sea bass and sea bream against pasteurellosis and turbot against streptococcosis had been successful carried out (Håstein *et al*., 2005). Those all confirmed that the formalin inactivated bacteria might be a good choice for vaccine preparation. In present study, we found that the formalin inactivated *S. dysgalactiae* antigen could significantly increase the survival rate of mullet that infected with *S. dysgalactiae*. After antigen injected for 7 days, the fish were challenged with live 2×10^8 CFU *S. dysgalactiae*. The mortality was then calculated and the result was shown in Fig. 2. The vaccine could significantly decrease the mortality that caused by live *S. dysgalactiae*. For the vaccine group, the mortality at 7 days after challenge was only 30%, which was significantly lower than that of control group (70%) ($P<0.05$). The reason for the mortality decreasing might be the fish innate immunity was activated by formalin-killed bacteria. Of course, the exact reason needed to be studied in the future work. At least, our results further confirmed the formalin-killed bacterial was helpful for the fish to prevent the occurrence and development of bacterial disease.

Many researches focused on the physiological response of animal to formalin inactivated pathogen antigen. Ispir *et al.* (2009) found the formalin inactivated *Yersinia ruckeri* vaccine could increase the immune indicators of rainbow trout. Liu *et al.* (2008) also found formalin-inactivated *Flavobacterium columnare* could up-regulate the immune related genes, e.g. C-reactive protein, and tumor necrosis factor α. Our observations showed that the levels of hepatic and splenic lysozyme were increased after antigen injection and exerted time-dependent pattern. The level of hepatic lysozyme was 0.59±0.06 U/mL for the control group. After 3 days of injection the hepatic lysozyme was 3.0 U/mL, which was significantly higher than that of control group ($P<0.05$). At 7 days after injection, the hepatic lysozyme was 7.50 U/ml, which was 12.7-fold of hepatic lysozyme in control group (Fig. 3a). For the splenic lysozyme, the level in control group was 0.32 U/ ml. After 1 days of injection the splenic lysozyme increased to be 4.95 U/ml and reached to be 11.82 U/ml at 7 day after injection (Fig. 3a).

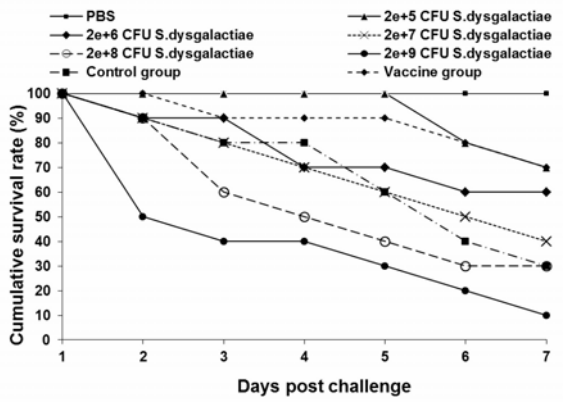

Fig. 2: Cumulative survival rate for mullet in live *S. dysgalactiae* challenge experiment and vaccinated group. For the challenge experiment, the fish were injected intraperitoneally with different dose of *S. dysgalactiae* while the fish in the control group with equal amount of 0.1 M PBS. For the vaccine protected experiment, the vaccinated group was injected with 0.1 mL formalin-inactivated *S. dysgalactiae* (1.0×10^8/mL); control group injected with equal amount of 0.1 M PBS. Then the fish was challenged with 0.1 mL 2×10^9 CFU *S. dysgalactiae* by intraperitoneally injection. The survival rate of both experiments was monitored daily and the mortality was recorded each day. n=10.

Lysozyme is an important defense molecule of fish innate immune system that is important in mediating protection against microbial invasion (Saurabh and Sahoo, 2008). Although the observing time was shorter, our studies found that the formalin inactivated *S. dysgalactiae* could significantly increase the activity of splenic and hepatic lysozyme of mullet. The results indicated that during the early stage after antigen injection, the non-specific immune response was activated to prevent *S. dysgalactiae* infection. We also observed that the levels of hepatic and splenic ALP were significantly decreased from 1 day to 5 day after antigen injection ($P<0.05$), and return the normal level at 7 days after vaccine injection. The hepatic ALP in the control group was 60.6 U/g of total protein, while decreased to be 22.2, 40.6 and 31.74 U/g after 1, 3 and 5 days of vaccine injection, respectively (Fig. 3b). At 7 days after injection, the hepatic ALP was 62.4 U/g. The same case was also observed in splenic ALP. The splenic ALP after 1, 3 and 5 days of vaccine injection were 2.82, 3.63 and 3.35 U/g respectively, which were significantly lower than that of control group (5.11 U/g) ($P<0.05$). At 7 days after injection, the splenic ALP was 4.63 U/g (Fig. 3b). These results suggested that some parenchymal damages in liver and spleen (Ahmad *et al*., 1995). At 7 days after injection, the ALP in liver and spleen were back to normal level indicating the damages

caused by the antigen were reversible. Our study confirmed that the *S. dysgalactiae* antigen could booster the immune response of mullet and could be an initial step for the preparation of successful streptococcosis vaccine.

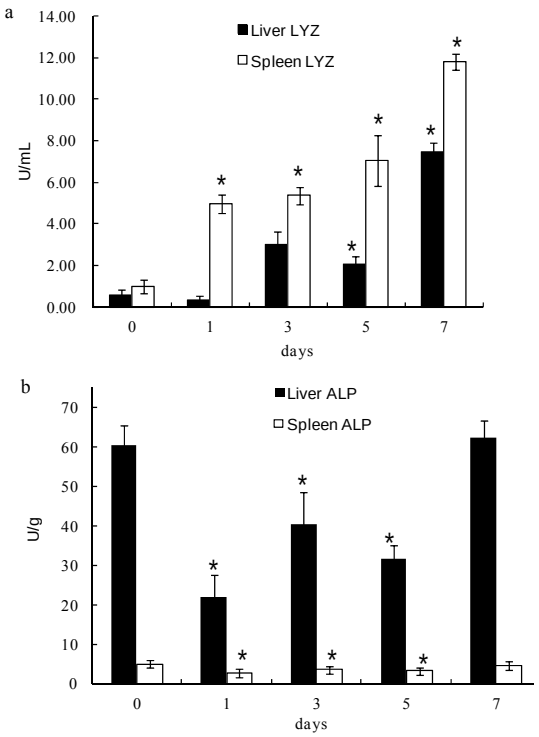

Fig. 3: Level of mullet lysozyme (a) and ALP (b) in liver and spleen after vaccine injection. The fish in vaccinated group were injected intraperitoneally with 0.1 mL of formalin inactivated *S. dysgalactiae* antigen (1.0×10^8/ mL); fish in control group with equal amount of PBS. The lysozyme and ALP activity in liver and spleen of fish were detected after injection for 1 d, 3 d, 5 d and 7 d. Values were shown as the mean ± SD. n=3. *P<0.05.

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