INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 14, No. 1, p. 476-483, 2019

RESEARCH PAPER OPEN ACCESS

Immunological responses of vaccinated proactive and reactive Nile tilapia (*Oreochromis niloticus*) subjected to handling stress

Jemuel S. Doctolero*, Emmanuel M. Vera Cruz

Department of Aquaculture, College of Fisheries, Central Luzon State University
Science City of Muñoz, Nueva Ecija, Philippines

Key words: Proactive, reactive, immune response, agglutination, formalin-killed vaccine, *Aeromonas hydrophila*.

http://dx.doi.org/10.12692/ijb/14.1.476-483 Article published on January 31, 2019

Abstract

The study was conducted to determine the effect of handling stress application on the immune responses of Nile tilapia vaccinated with formalin-killed *Aeromonas hydrophila* and to determine which stress-coping styles had better responses. Proactive individuals resemble the bold ones and respond to challenges with high general activity and high levels of aggression while reactive individuals respond to the challenges with immobility and lack of initiative, which resembles the response pattern of the shy. FaST strain of Nile tilapia were screened according to stress-coping style whether proactive and reactive using eye color pattern (ECP) values. Post-vaccination of formalin-killed *Aeromonas hydrophila* and application of handling stress was administered prior to the commencement of the study. Immune response through hemaaglutination test revealed that proactive individuals had better immune response than reactive individuals as significantly higher agglutination titer was notable among proactive fish. The study concluded that proactive Nile tilapia had better immune stimulation against *A. hydrophila* upon utilization of formalin-killed vaccine even with the influence of handling stress.

* **Corresponding Author:** Jemuel S. Doctolero ✉ jemsado09@yahoo.com.ph

Introduction

Fish disease, especially bacterial infections, is a major problem facing the fish farming industry (FAO, 2004). Nile tilapia (*Oreochromis niloticus*) is an important species for freshwater aquaculture, and improvements of its culture and disease-resistance is a major challenge facing fish culturists (Abdel-Tawwab *et al.*, 2008). The major causes of economic losses in tilapia culture are bacterial diseases, mostly caused by the genera *Aeromonas* (Lim and Webster, 2006) and consequently stress is often considered to be a contributing factor in disease outbreaks caused by these bacteria (Yin *et al.*, 2009).

There are many potential stressors in fish culture that may affect the immune system (Iwama *et al.*, 1986). One of the common stressors encountered by fish is handling stress (Li *et al.,* 1998). The relations between stress and disease resistance in fish have been extensively examined, and correlations of increased susceptibility to disease during stress have been shown (Mock and Peters, 1990 and Fevolden *et al.*, 1992); however, the process are poorly understood, and few studies have described the progression of fish diseases from a physiological perspective (Ackerman and Iwama, 2001).

When an animal is subjected to a challenge that has a negative effect on its fitness this will cause the animal to respond with a combination of behavioral, neuroendocrine and autonomic changes that aim to reduce the adverse effect of that challenge (Schjolden *et al.*, 2005). Individual animals differ in their behavioral responses to a variety of challenges or situations, such as feeding, mating and aggression. A tendency to respond in a certain manner has been referred to as a coping style (Korte *et al.*, 2005; Koolhaas *et al.*, 2007). Proactive individuals resemble the bold ones and are those that respond to challenges with high general activity and high levels of aggression and physiologically, they predominantly display a sympathetic activation (the fight/flight response) (Bohus *et al.*, 1987; Koolhaas *et al.*, 1999; 2001). On the other hand, animals with a reactive coping style respond to the same challenge with immobility and lack of initiative, which resembles the response pattern of the shy individuals and physiologically, the reactive animals predominantly show a parasympathetic/hypothalamic activation (the conservation/withdrawal response) (Bohus *et al.*, 1987; Koolhaas *et al.*, 1999; 2001). Within recent years considerable progress has been made in understanding the immune mechanism of' fish (Smith *et al.*, 1966). The need for further information on the factors influencing immunity stimulation of fish have recently been emphasized (Klontz, 1972).

Attainment of additional information in terms of immune responses of fish to bacterial diseases will provide a great tool in developing mechanisms and methodologies to further provide desirable culture condition among reared species relating to disease prevention. The study was conducted to determine the effect of vaccination using formalin-killed *Aeromonas hydrophila* on the immune response of experimental fish when handling stress were administered. In addition, result of the study addressed the potential of screening and selection of stress-coping style as well as the sex of individual fish as a way of improving the immune stimulation of Nile tilapia against *Aeromonas hydrophila* and handling stress resistance of fish.

Materials and methods

Exprimental Fish and Compartments

The study used FAC Selected Strain of Tilapia (FaST) which were obtained from Freshwater Aquaculture Center (FAC) ranging from 70 - 80 grams. Total of 96 Fish were individually stock in a 10 x 10 x 10 aquaria. The three sides of each aquarium were covered with paper to eliminate stress associated with social and visual interaction. Aeration were provided in each unit of aquarium. Feeding and maintenance of good water quality was administered during the whole duration of conduct.

Experimental Treatment

The study utilized factors of stress-coping style whether proactive (P) or reactive individuals (R), sex of fish whether male (M) or female (F) and application of stressor whether with (c) or without handling stress (hs). The treatment was named PMc, PMhs, PFc, PFhs, RMc, RMhs, RFc and RFhs

Experimental Methods

Identification of Proactive and Reactive Individuals

A number of previously reared and Nile tilapia were subjected for isolation and were introduced in a novel environment for 7 days. The eye color pattern (ECP) of each fish were recorded daily during the isolation period. Eye color change was observed to be fractional changes of the color of the iris and sclera around the pupil which was transformed into scores ranging from 0 (no darkening) to 8 (total darkening). Proactive and reactive individual were determined through ECP scores. ECP values of less than 4 after the isolation period of 7 days were classified as proactive individual while those with ECP values of more than 4 were classified as reactive individual.

Preparation of Formalin-killed A. hydrophila Vaccine

A. hydrophila was mass produced on Tryptic Soy Broth (TSB) at 30°C for 48h. Bacterial cells were collected by centrifugation at 6500 rpm for 15 minutes and washed three times with sodium phosphate saline. The *A. hydrophila* was re-suspended in PBS at 10^{10} cells mL-1. Formalin (37% w/v) was used and added to the bacterial cell suspension at a final concentration of 0.4% (V/V). The final concentration (0.4% formalin) was derived by diluting 10 mL of 37% formalin with 90 mL of distilled water to produce 3.7% formalin.

The suspension was incubated for 3 hours at room temperature and then at 4°C overnight. The dilutions were adjusted to a turbidity score at 1 x 10^8 cells/mL following McFarland scale. The bacteria were tested for their sterility (free from the living cells) by streaking them onto newly prepared nutrient agar. Non-appearance of white colony means no bacterial growth.

Vaccination and Implementation of handling stress

Post-vaccination was conducted two weeks before initial gathering of data. A 300µL- formalin-killed *A. hydrophila* vaccine was injected intramuscularly. Implementation of handling stress as a stressor were conducted among experimental treatments by keeping them out of the water for one minute using scooping net.

Blood Collection and Microtiter Plate Hemagglutination Test

Cardiac puncture was done for blood collection. Blood was dispensed into sterile test tubes and was allowed to stand at room temperature or preferably at 4°C overnight to let the serum retract from clot. The first lane of the 96-well microtiter plate was filled with 100µL of fish serum and 50µL of physiological saline was added to the second lane up to the last lane of a 96-well round bottom microtiter plate. Mixing of solution was done before carrying out two-fold serial dilution by transferring 50µL solution starting from the first lane down to the last lane. As a result, the serum dilutions were 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048 and 1:4096, 1:8192, 1:16384 and 1:32768. Thereafter, 50µL of live *A. hydrophila* (5.8 × 10^{10} CFU/mL) suspension was added to each well. Then, microplates were mixed by gently tapping on the side of the microtiter tray, covered and then left incubated for 16-18 h overnight at 37°C for agglutination reaction to proceed. The agglutination end point was established as the last serum dilution where agglutination was visible. Agglutination antibody titers were expressed as log 2 (x+1) of the reciprocal of the highest serum dilution showing visible agglutination as compared to the control. Presence of visible spot or sharp button (i.e, accumulated bacteria at the bottom of the wells) indicated negative agglutination, while positive agglutination was viewed with visible mat or absence of visible spot at the bottom. Hemagglutination test was done after the stressor was applied followed by an interval of five days until on the 15th day.

Statistical Analyses

The relationship of the eye color pattern, ventilation rate and dilution level of agglutination were determined using Pearson correlation coefficient and Linear Regression. Statistical analyses was done using factorial, one-way ANOVA, T-test and means were compared using Duncan Multiple Range Test.

Results

Microplate Hemagglutination Test

The effect of the interaction among factors of the study was analyzed in terms of the agglutination level occurrences of each experimental treatment.

It was notable that no interaction took placed on the combination of factors except for the main effect of stress-coping style classification (p=0.014). This indicated that stress-coping style classification of experimental fish whether proactive or reactive individuals influenced the result obtained in hemagglutination test. As shown in Table 1, all treatments were not significantly different from each other among all sampling periods except for the treatment PMc of the final sampling which was revealed to be significantly different with the rest of the treatments.

Furthermore based on mean agglutination titers at different sampling times, significant differences among treatments were observed between PMc and RMhs, PMc and RFc and PMc and RFhs. Treatments PMhs, PFc, PFhs and RMc were not significantly different to the rest of experimental treatments.

Table 1. Mean (± S.D.) agglutination titer per treatments in different sampling period.

SAMPLING TIME	PMc	PMhs	PFc	PFhs	RMc	RMhs	RFc	RFhs
Initial sampling	66.67 [a] (± 60.040	26.67 [a] (± 9.23)	93.33 [a] (± 140.93)	26.67 [a] (± 33.31)	1.33 [a] (± 2.31)	13.33 [a] (± 16.65)	25.33 [a] (± 33.55)	21.33 [a] (± 36.95)
2nd sampling (5th day)	178.67 [a] (± 288.70)	176.00 [a] (± 290.98)	192.00 [a] (± 278.10)	429.33 [a] (± 529.71)	106.67 [a] (± 133.23)	48.00 [a] (± 27.71)	9.33 [a] (± 6.11)	45.33 [a] (± 71.70)
3rd sampling (10th day)	106.67 [a] (± 36.95)	74.67 [a] (± 48.88)	48.00 [a] (± 69.28)	66.67 [a] (± 60.04)	74.67 [a] (± 48.88)	32.00 [a] (± 32.00)	53.33 [a] (± 66.61)	24.00 [a] (± 34.87)
Final sampling (15th day)	853.33 [b] (± 295.60)	181.33 [a] (± 129.33)	261.33 [a] (± 248.04)	109.33 [a] (± 130.07)	36.67 [a] (± 25.32)	8.00 [a] (± 0.00)	53.33 [a] (± 66.61)	16.00 [a] (± 16.00)
MEAN (±S.D.)	301.33 [b] (± 370.91)	114.67 [ab] (± 76.49)	148.67 [ab] (± 96.21)	158 [ab] (± 184.01)	54.83 [ab] (± 45.73)	25.33 [a] (± 18.28)	35.33 [a] (± 21.79)	26.67 [a] (± 16.00)

Means in rows having similar superscript are not significantly different at 5 % level of significance.

Table 2 shows the mean agglutination titer of proactive and reactive treatments. Statistical analysis revealed that proactive fish (200.33 ± 142.69) had significantly higher (p = 0.040) agglutination titer than reactive fish (34.21 ± 14.13).

On the analysis of agglutination titer of the factor sex of fish (Table 3), the result revealed that agglutination titer of male (150.79 ± 178.03) was not significantly different (p = 0.163) from that of female (83.75 ± 65.31).

Table 2. Mean agglutination titer per treatment of the factor stress-coping style group.

Stress-Coping Style Group			
Proactive		Reactive	
Treatment	Agglutination Titer	Treatment	Agglutination Titer
PMc	412.67	RMc	54.50
PMhs	110.00	RMhs	26.00
PFc	125.33	RFc	33.00
PFhs	153.33	RFhs	23.33
Mean (± SD)	200.33 (± 142.69) [a]		34.21 (±14.13) [b]

Means in rows having similar superscript are not significantly different at 5 % level of significance.

Table 3. Mean agglutination titer per treatment of the factor sex of fish.

Sex of Fish			
Male		Female	
Treatment	Agglutination Titer	Treatment	Agglutination Titer
PMc	412.67	PFc	125.33
PMhs	110.00	PFhs	153.33
RMc	54.50	RFc	33.00
RMhs	26.00	RFhs	23.33
Mean (± SD)	150.79 (± 178.03) [a]		83.75 (± 65.31) [a]

Means in rows having similar superscript are not significantly different at 5 % level of significance.

Tables 4, 5, 6 and 7 show the mean agglutination dilution level of experimental treatments in different sampling period of serum (initial, 2nd, 3rd and final sampling).

It was notable that all experimental treatments in different sampling period appeared to have positive agglutination in various agglutination dilution levels.

This indicated that immune stimulation had occurred among experimental fish even handling stress was administered. Highest mean agglutination was observed in the final sampling on the treatment PMc at titer 1:1024 while lowest was obtained in initial sampling on the treatment RMc at titer 1:2. Analysis on the correlation of agglutination level as time progressed revealed to have positive correlation (r = 0.264, P < 0.144) which signified that immune stimulation was notable through time.

Table 4. Mean dilution level of agglutination test of experimental treatments conducted on initial sampling

Treatment	Dilution Level of Agglutination											
	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	1 2048	1 409
PMc	+	+	+	+	+	-	-	-	-	-	-	-
PMhs	+	+	+	+	+	-	-	-	-	-	-	-
PFc	+	+	+	+	+	-	-	-	-	-	-	-
PFhs	+	+	+	-	-	-	-	-	-	-	-	-
RMc	+	-	-	-	-	-	-	-	-	-	-	-
RMhs	+	+	+	-	-	-	-	-	-	-	-	-
RFc	+	+	+	+	-	-	-	-	-	-	-	-
RFhs	+	+	-	-	-	-	-	-	-	-	-	-

*Note: (+) positive agglutination; (-) no agglutination

Table 5. Mean dilution level of agglutination test of experimental treatments conducted on 2nd sampling.

Treatment	Dilution Level of Agglutination											
	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	1 2048	1 409
PMc	+	+	+	+	+	-	-	-	-	-	-	-
PMhs	+	+	+	+	+	-	-	-	-	-	-	-
PFc	+	+	+	+	+	-	-	-	-	-	-	-
PFhs	+	+	+	+	+	+	+	-	-	-	-	-
RMc	+	+	+	+	+	-	-	-	-	-	-	-
RMhs	+	+	+	+	+	-	-	-	-	-	-	-
RFc	+	+	+	-	-	-	-	-	-	-	-	-
RFhs	+	+	+	-	-	-	-	-	-	-	-	-

*Note: (+) positive agglutination; (-) no agglutination.

Table 6. Mean dilution level of agglutination test of experimental treatments conducted on 3rd sampling.

Treatment	Dilution Level of Agglutination											
	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	1 2048	1 409
PMc	+	+	+	+	+	+	+	-	-	-	-	-
PMhs	+	+	+	+	+	+	-	-	-	-	-	-
PFc	+	+	+	+	-	-	-	-	-	-	-	-
PFhs	+	+	+	+	+	-	-	-	-	-	-	-
RMc	+	+	+	+	+	+	-	-	-	-	-	-
RMhs	+	+	+	+	-	-	-	-	-	-	-	-
RFc	+	+	+	+	-	-	-	-	-	-	-	-
RFhs	+	+	+	-	-	-	-	-	-	-	-	-

*Note: (+) positive agglutination; (-) no agglutination.

Table 7. Mean dilution level of agglutination test of experimental treatments conducted on final sampling.

Treatments	Dilution Level of Agglutination											
	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	1 2048	1 409
PMc	+	+	+	+	+	+	+	+	+	+	-	-
PMhs	+	+	+	+	+	+	+	-	-	-	-	-
PFc	+	+	+	+	+	+	+	-	-	-	-	-
PFhs	+	+	+	+	+	+	-	-	-	-	-	-
RMc	+	+	+	+	+	-	-	-	-	-	-	-
RMhs	+	+	+	-	-	-	-	-	-	-	-	-
RFc	+	+	+	+	-	-	-	-	-	-	-	-
RFhs	+	+	+	-	-	-	-	-	-	-	-	-

*Note: (+) positive agglutination; (-) no agglutination.

Discussion

On the study conducted by Wongsathein (2012), the results showed that there was a relationship between different stress-coping styles and immunity of Nile tilapia to *S. agalactiae* infection, with reactive or shy fish experiencing higher mortality rates than proactive or bold fish. This suggest that the bold fish was less susceptible to infection and had improved immunity on bacterial challenge than shy fish. The same result was obtained from the current study considering the two stress-coping style groups in which immune stimulation against *Aeromonas hydrophila* was higher in proactive individuals as they had better agglutination than reactive individuals. This was related to variation of individuals associated with coping-styles in terms of stress and immune responses leading to disease resistance or susceptibility and immunity stimulation or immunity depreciation (Koolhaas *et al.*, 1999).

Over the last decade, vaccination has become important for the prevention of infectious diseases in farmed fish (Gudding *et al.*, 1999). Vaccination with heat or formalin-inactivated bacterins provides some protection to a certain extent against *A. hydrophila* (Chandran *et al.*, 2002). Many studies reported the increase in agglutination after fishes are vaccinated. In a previous work, Nile tilapia presented higher agglutination after vaccination against *S. iniae* (Klesius *et al.*, 2000), and good immune stimulation after challenged with *S. iniae* on the tenth week post-infection (Shoemaker *et al.*, 2006). In a study by Selvaraj *et al.* (2004), carp (*Cyprinus carpio*) immunized with lipopolysaccharide against *A. hydrophila* also presented high agglutination titer, as well as sturgeons (*Acipenseridae*) intraperitoneally vaccinated against *A. hydrophila* that demonstrated good indexes until 29 days post-vaccination (Khoshbavar-Rostami *et al.*, 2007). The current study aimed to know the effect of handling stress upon utilization of formalin-killed *A. hydrophila* as a way of stimulating the immune system of experimental fish. Based on the result of the study, all treatments were observed to have positive agglutination in varying dilution levels of agglutination. Moreover, it was notable that as time progressed, the agglutination also increased which signified the effectiveness of vaccination in immune stimulation even handling stress was applied. It is demonstrated through the years that acute stress can alter the effect on immune function and disease resistance in fish as well as immunosuppressive effects (Maule *et al.*, 1987). Different strategies have been proposed as being potentially beneficial for reducing the immunological effects of stress in farmed fish, such as feeding elevated levels of vitamins (Jaffa, 1989; Hardie *et al.*, 1991). This addresses the application of formalin-killed *A. hydrophila* on the current study where it can be considered that upon vaccination, stress applied were not likely to influenced alteration in the immunity of fish. In the case of the current study, the FAC Selected Tilapia (FaST) strain was used as experimental fish. This strain possessed good aquacultural characteristics primary influenced by selective breeding. According to Huntingford and Adams (2005), there is further possibility that immunity can be manipulated or influenced through selective breeding or husbandry manipulations. That is why the selected tilapia strain used in the study possibly contributed on enhancing the immunity of experimental fish.

Conclusion

The study was conducted to determine the effect of handling stress application on the immune responses of Nile tilapia vaccinated with formalin-killed *Aeromonas hydrophila*. As time progressed the agglutination of anti-bodies increased which signified that immune stimulation had occurred even handling stress was present. Proactive individuals attained better immune stimulation against *A. hydrophila* than reactive individuals. Screening of Nile tilapia in terms of their stress-coping style obtaining the proactive individuals is a potential procedure to conduct in improving the immune stimulation of Nile tilapia against *A. hydrophilla* using formalin-killed vaccine even with the influence of handling stress.

Acknowledgement

The author would like to express his gratitude to the Department of Science and Technology – Accelerated Science and Technology, Human Resources and Development Program for the funding of this study.

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