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COMMUNICATION

Hematology and agglutination titer after polyvalent immunization and subsequent challenge with *Aeromonas hydrophila* in Nile tilapia *(Oreochromis niloticus)*

Hematología y título de aglutinación después de inmunización polivalente y desafío con *Aeromonas hydrophila* a tilapias del Nilo *(Oreochromis niloticus)*

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RESUMEN

Este trabajo evaluó el efecto de la vacuna polivalente sobre las respuestas hematológicas y inmunológicas de tilapias del Nilo desafiadas con *Aeromonas hydrophila*. Dos dosis, 1 x 10^4 y 1 x 10^8 Unidades Formadoras de Colonias (UFC)/mL, de vacuna conteniendo proporciones iguales de *Aeromonas hydrophila, Pseudomonas aeruginosa* y *Enterococcus durans* inactivadas con formalina, fueron testeadas por inyección intraperitoneal (i.p.). Los peces fueron desafiados 10 días después de la vacunación i.p. con la dosis ($DL_{50\text{-}96h}$) de 1 x 10^7 UFC de *A. hydrophila*/mL resuspendida en solución salina estéril. Por lo tanto, para analizar los parámetros hematológicos, la actividad antimicrobiana y aglutinante del suero de las muestras, fueron colectadas 48 h después del desafío. Antes de la infección experimental, el número de eritrocitos fue superior en los peces vacunados con 1 x 10^8 UFC/mL. Sin embargo, después del desafío, el número total de trombocitos fue mayor en los peces vacunados con la mayor dosis. Antes y después del desafío, el número total de leucocitos y el número de linfocitos presentaron mayores valores en los peces vacunados. El número de monocitos en los peces vacunados y en los inyectados con solución salina fue mayor antes del desafío. El mayor título de aglutinación frente *A. hydrophila*, *P. aeruginosa* y *E. durans* fue observado en los peces vacunados con 1 x 10^8 UFC/mL. Antes del desafío la actividad antimicrobiana del suero sanguíneo fue mayor en los peces no vacunados y en los vacunados con 1 x 10^8 UFC/mL, y mismo después del desafío los peces no vacunados y los inyectados con solución salina presentaron mayor actividad antimicrobiana. En este trabajo fue posible comprobar que después de 10 días la vacuna polivalente en la concentración de 1 x 10^8 UFC/mL estimuló la producción de eritrocitos, leucocitos, trombocitos y linfocitos circulantes reduciendo los niveles de glucosa.

Key words: Oreochromis niloticus, vaccination, *Aeromonas hydrophila*, challenge.
Palabras clave: Oreochromis niloticus, vacunación, *Aeromonas hydrophila,* desafío.

INTRODUCTION

The rapid development of aquaculture allied to intensive production systems favour stress conditions in captive fish, leading to diseases and economic losses (Schreck 1996). One of the most important challenges in aquaculture is the enhancement of performance and disease resistance in reared fish. In this sense bacterial diseases are one of the limitant factors (Abdel-Tawwab *et al* 2008, Martins *et al* 2009[a]). Bacteria are part of microrganisms present in the water of rivers and ponds, and its pathogenic potential may be altered under physical and chemical characteristics of the environment (Walters and Plumb 1980). Gram negative, worldwide and opportunist bacterium *Aeromonas hydrophila*, may cause disease in a several freshwater fish species for example in cyprinid as related by Rahman *et al* (1997).

Antibiotics are frequently utilized in aquaculture to increase larval resistance but this practice can also result in microbial resistance, residual accumulation in tissue (Vadstein 1997) and fish immunesuppression (Van Muiswinkel *et al* 1985). The immuneprophylaxis as the specific and non-specific immune system stimulation is the key for sustainable aquaculture development (Gudding *et al* 1999). Vaccination with inactivated antigens as well as the probiotic addition in the diet of cultured fish may contribute to reduction of chemicals and antibiotics in aquaculture (Gudmundsdóttir and Björnsdóttir 2007, Jatobá *et al* 2008).

In Brazil, studies on fish immunology are scarce. The administration of vaccines against bacterial and viral diseases has demonstrated good results in relation to scientific and economic approaches by reducing the chemical use (Costa 2004). Several factors can interfere on fish immune response such as developmental stage, size, nutritional status, water

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quality and vitamin supplementation (Tatner and Manning, 1983, Bly *et al* 1997, Nakanishi and Ototake 1997, Moraes and Martins 2004, Martins *et al* 2008[a]). On the other hand, the efficacy of the vaccine is strongly influenced by the type of administration, dose and nature of antigens, adjuvant addition, environment and water temperature (Tatner 1987, Lillehaug *et al* 1993, Bly *et al* 1997). Thus, information on the infectious agent and the host response must be considered for the development of an efficient vaccine (Gudmundsdóttir and Björnsdóttir 2007).

The resistance of fish can be evaluated by analyzing the survival rate after experimental infection (Wasson and Kelly 1990) as well as hematological and immunological parameters (Martins *et al* 2009[a]). A combined vaccine against *Streptococcus iniae* administrated intraperitoneally in tilapia has demonstrated more protection compared to a single isolate (Klesius *et al* 2000). The authors argued that antigen heterogenicity in *Streptococcus* exists and a combined vaccine may enhance fish response.

Park and Jeong (1996) reported improved resistance in tilapia injected with protein-bound polysaccharide (PS-K) against *Edwardsiella tarda*. Sarder *et al* (2001), found that lysozyme and phagocytic activities in tilapia were higher after challenge with *A. hydrophila,* but no difference was noted in the differential counting of leukocytes. On the other hand, Castro *et al* (2008) observed good results in turbot *Scophthalmus maximus* vaccinated against *E. tarda* characterized by the highest survival rate and antibody levels for at least six months after immunization.

According to Balfry *et al* (1997) lymphocyte number decreased after challenge with *Vibrio parahaemolyticus* but no difference in thrombocyte, neutrophill and monocyte was found. The study of Harikrishnan *et al* (2003) in carp infected by *A. hydrophila* showed an increase in the leukocyte number. But the opposite was foud in pacu *Piaractus mesopotamicus* experimentally infected with *A. hydrophila* (Garcia *et al* 2007). Martins *et al* (2008[b]) have reported that *Enterococcus*-injected tilapia showed not only a high number of thrombocytes, but also an increase in white blood cell and lymphocyte when compared to non-injected. Phagocytyc activity and circulating lymphocyte number were also enhanced after *Enterococcus* injection (Martins *et al* 2009[a]).

This study evaluated the effects of polyvalent vaccination containing *A. hydrophila*, *Pseudomonas aeruginosa* and *E. durans*, on the hematological and serum agglutination responses in Nile tilapia challenged with *A. hydrophila*.

MATERIAL AND METHODS

The experiment was carried out at the Aquaculture Department, Laboratory AQUOS-Aquatic Organisms Health, CCA, UFSC, Florianopolis, Santa Catarina State, with GIFT tilapia from "Fundação 25 de Julho", Joinville, SC, Southern Brazil.

The strains of *A. hydrophila* ATCC 7966, *Enterococcus durans* ATCC 19492, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 used in this assay were donated by Institute André Tossello (Campinas, São Paulo State), and cultured at the Microbiology Laboratory of Marine Shrimp Laboratory, UFSC, Florianópolis, SC.

LETHAL DOSE ($LD_{50\text{-}96H}$) TO *AEROMONAS HYDROPHILA*

Fish with 196.97 ± 25.00 g weight and 18.53 ± 1.20 cm length were distributed in 150 L tanks with constant aeration and water renewal 30% a day, and fed *ad libitum* with a commercial diet 28% crude protein twice a day. After acclimation for 5 days, fish were intraperitoneally (i.p.) injected with 0; 1 x 10^5; 1 x 10^6; 1 x 10^7 and 1 x 10^8 Forming Colony Units (FCU)/mL per fish diluted in 1 mL esterile saline solution, in triplicates with 5 fish in each repetition. The water quality was daily monitored as follows: dissolved oxygen 5.54 ± 0.44 mgL; temperature 24.93 ± 2.49 ºC; pH 7.29 ± 0.30; total ammonia 0.45 ± 0.48 mg/L.

After 96 h of mortality observation, $DL_{50\text{-}96h}$ was estimated for *A. hydrophila* by the method Trimmed Spearman-Karber (Hamilton *et al* 1977). Bacterial concentration that provoked 50% mortality in 96 h was utilized to challenge the fish. Furthermore, as an experimental vaccination, a previously reported lower dose (Martins *et al* 2008[b]) and a time collection that caused no severe mortality were used.

VACCINATION AND CHALLENGE

Bacterial strains of *A. hydrophila*, *P. aeruginosa* and *E. durans* were reproduced in separate tubes of 10 mL containing BHI broth (Brain and Heart Infusion, Difco), incubated at 30 ºC for 24 h under continuous agitation. After verifying the bacterial population, equal portions of bacteria were added to formalin 0.5%, being incubated again under agitation at 30 ºC for 24 h. Bacterial cultures were centrifuged at 4,000 *g* for 30 min, the supernatant with formalin was discarded and the pellet re-suspended in sterile saline solution. The vaccine was determined to be sterile by lack of growth in TSA (Tryptic Soy Agar, Difco) medium culture at 30 ºC for 24 h.

Fish with 255.52 ± 21.16 g weight and 21.28 ± 1.93 cm length were distributed in 150 L tanks with constant aeration and water renewal 30% a day. Fish were acclimated for 10 days and fed *ad libitum* with a commercial diet 28% crude protein twice a day. Water quality was daily monitored as follows: dissolved oxygen 5.43 ± 0.48 mg/L; temperature 26.09 ± 3.13 ºC; pH 7.02 ± 0.32 and total ammonia 0.90 ± 0.67 mg/L.

The experiment was entirely randomized with i.p. vaccinated fish with 1 x 10^4 and 1 x 10^8 FCU/mL polyvalent vaccine formalin-inactivated; control saline-injected fish and non-injected ones, 10 fish per treatment, in triplicates.

Before inoculation and for blood collection fish were anethestized with a benzocaine solution (50 mg/L) (Ethic Committee n° 23080.024659/2007-99 CEUA/ UFSC). Fish were maintained in starvation for 24 h before challenge by i.p. 1 mL of $DL_{50\text{-}96h}$ *A. hydrophila* (1 x 10^7 UFC/mL). This trial was carried out 10 days after immunization.

HEMATOLOGY AND GLUCOSE

Blood samples were collected before and 48 h after challenge with *A. hydrophila*. After fish anesthetizes, the blood was withdrawn from the caudal vein using a 3 mL (21G) syringe with 10% EDTA and a syringe without anticoagulant. Blood collected without anticoagulant was left to clot for 2 h at 25 ºC, and then centrifuged at 1,400 *g* for 10 minutes. Serum aliquot was taken with assist of a micropipette and stored at -20 ºC until analysis. Serum of 3 fish from the same experimental aquarium was pooled for immunological analyses. The blood collected with anticoagulant was used to produce duplicates of blood ex tensions stained with Giemsa/MayGrunwald (Rosenfeld 1947), for differential counting of leukocytes and total counting of thrombocytes and leukocytes. One aliquot was used to determine hematocrit (Goldenfarb *et al* 1971) and the rest was stored in glass flasks on ice to quantify the total number of erythrocytes in a hemocytometer. Total number of thrombocytes and leukocytes were counted in blood extension by the indirect method described by Ishikawa *et al* (2008). One aliquot of serum was used to determine glycemic index in spectrophotometer (Biotécnica® kit) at 505 nm.

In the first collection (ten days after immunization), three fish per each treatment were used for blood collection and euthanized. For the second collection (48 h after challenge) the other seven fish were utilized finally used.

AGGLUTINATION TITER

The test for agglutination titer was realized individually for each bacterium strain (*A.hydrophila*, *E. durans* and *P. aeruoginosa*) according to Yildirim *et al* (2003). The concentration of inactivated cells used in the test was of 0.8 in 550 nm wave length (DO_{550nm}). Briefly, each well of a 96-round well microplate was plated with 50 µL of phosphate-buffered saline (PBS 0.2M monobasic phosphate, 0.2M dibasic phosphate, 0.11M sodium chloride, pH 7.4) and then 50 µL of serum was added to the first well, and diluted serum was serially diluted into the remaining wells. After that, 50 µL of each bacterium strain was added to each well and incubated in humidified air at 25 ºC for 18 h. The agglutination was measured as the amount of a required substance to react with a given amount of other substance, that was considered as reciprocal of the last dilution that presented agglutination.

STATISTICAL ANALYSIS

Data were analized using the Bartlett test and hematological parameters with no homogenicity in variance were transformed in log (x + 1) prior to analysis of variance with parcels subdivided in time ($\alpha < 0.05$). Differences in means were detected by the Student Newman Keuls test (SNK), and agglutination titer data was $\log_2$ (x + 1) transformed prior to analysis.

RESULTS AND DISCUSSION

LETHAL DOSE

The first mortalities occurred 11 h after challenge in all experimental units of the dose 1 x 10^8 UFC/mL (table 1). Except for saline-injected fish, all dead animals showed typical symptoms such as reddish on ventral body surface, scaleness, fin and integument hemorrhages with exposition of muscular tissue. After 23 h the first mortality was detected in fish that received the dose 1 x 10^7 UFC/mL. Park and Jeong (1996) found mortality in tilapia 48 h after infection with *E. tarda*. Our results were similar to the findings of Sarder *et al* (2001) who observed tilapia mortality 12 h after infection with *A. hydrophila*. Balfry *et al* (1997) reported 28.9% mortality in black strain tilapia 24 h after challenge with *V. parahaemolyticus*. The mortality in saline-injected fish (6.66%) has probably been caused by injection handling stress corroborating the findings of Sarder *et al* (2001). The stress due to saline injection was related in the studies of Martins *et al* (2006) in pacu (*Piaractus mesopotamicus*).

Except for the increased mortality rate in fish that received 1 x 10^5 CFU/mL in relation to those that received 1 x 10^6 CFU/mL, the mortality was proportional to the concentration of pathogen inoculums. Fish inoculated with 1 x 10^7 CFU/mL showed 50% mortality ($DL_{50\text{-}96h}$ *A. hydrophila*). Contrarily to here observed, Wang and Wang

Table 1. Mortality rate in Nile tilapia 96 h after intraperitoneal injection of saline solution and crescent dosis of *Aeromonas hydrophila*/mL, to determine the $DL_{50\text{-}96h}$.

Tasas de mortalidad en tilapia del Nilo después de 96 h de la inyección intraperitoneal inyección de solución salina y la creciente dosis de *Aeromonas hydrophila*/mL, para la determinación de la $DL_{50\text{-}96h}$.

Colony forming units of *Aeromonas hydrophila*/mL	Mortality (%)
Saline solution	6.66
1 x 10^5	39.96
1 x 10^6	26.64
1 x 10^7	53.28
1 x 10^8	79.92

(1997) found that injection with 1 x 10^7 CFU *A. hydrophila*/mL in tilapia and carp caused 100% mortality (DL_{100}). It depends on several factors as environmental conditions and bacterial strain.

HEMATOLOGY AND GLUCOSE

As a result of stress, normal hematological parameters could be changed in infected fish (Martins *et al* 2008[b]). In this study, after challenge with *A. hydrophila*, the number of thrombocytes in the circulating blood of 1 x 10^8 vaccinated tilapia was higher than the number observed in non-vaccinated ones (table 2).

The results showed an increase in the number of total leukocyte and lymphocyte in 1 x 10^8 vaccinated fish in relation to other treatments either after immunization or challenge (table 3). In the studies of Harikrishnan *et al* (2003) in carp (*Cyprinus carpio*) infected i.p. with *A. hydrophila*, the number of leukocyte increased until 30 days after infection. In contrast to that observed by Harikrishnan *et al* (2003), in this work erythrocyte number and hematocrit did not alter after challenge. On

Table 2. Blood characteristics in the blood of Nile tilapia, before (6 days after immunization) and 48 h after challenge with *Aeromonas hydrophila*. Non-vaccinated fish, fish injected with 1 mL saline solution, fish vaccinated with 1 x 10^4 and 1 x 10^8 CFU of polyvalent vaccine/mL. Different letters indicate significant difference among the treatments in each time of collection by SNK test of mean comparison ($P < 0.05$).

Características de la sangre de tilapia del Nilo, antes (6 días después de la inmunización) y 48 h después de infectarlas con *Aeromonas hydrophila*. Peces no infectados, e inyectados con 1 mL solución salina y peces vacunados con 1 x 10^4 y 1 x 10^8 CFU con la vacuna polivalente/mL. Las letras distintas indican diferencia significativa entre los tratamientos en los distintos tiempos de colecta por el test de comparación de medias SNK ($P < 0.05$).

Collection	Treatment	Glucose (mg/dL)	Hematocrit (%)	Erythrocyte (10^6/µL)	Thrombocyte (10^3/µL)	Leukocyte (10^3/µL)
Before challenge	Non-vaccinated	85.62 ± 45.88[a]	18.83 ± 4.19[a]	1.02 ± 0.30[b]	35.20 ± 5.41[a]	18.19 ± 1.50[b]
	Saline	75.56 ± 55.00[a]	24.78 ± 2.80[a]	1.25 ± 0.14[ab]	46.66 ± 12.68[a]	21.80 ± 5.65[b]
	1 x 10^4	21.91 ± 21.77[b]	26.83 ± 2.80[a]	1.34 ± 0.28[ab]	46.66 ± 6.49[a]	26.35 ± 5.20[b]
	1 x 10^8	42.81 ± 18.69[b]	23.33 ± 4.93[a]	1.70 ± 0.22[a]	49.41 ± 10.41[a]	34.72 ± 10.09[a]
After challenge	Non-vaccinated	90.79 ± 38.75[a]	22.19 ± 2.82[a]	1.16 ± 0.07[a]	18.13 ± 10.77[b]	17.46 ± 9.58[b]
	Saline	58.48 ± 44.25[a]	22.25 ± 3.26[a]	1.12 ± 0.08[a]	27.39 ± 5.21[ab]	19.12 ± 1.12[b]
	1 x 10^4	34.17 ± 25.79[b]	22.33 ± 2.04[a]	1.17 ± 0.28[a]	30.44 ± 8.84[ab]	26.42 ± 3.28[b]
	1 x 10^8	21.05 ± 17.24[b]	21.86 ± 1.91[a]	1.19 ± 0.12[a]	42.46 ± 4.21[a]	37.66 ± 4.21[a]

Table 3. Differential counting of leukocytes in the blood of Nile tilapia, before challenge (6 days after immunization) and 48 h after challenge with *Aeromonas hydrophila*. Non-vaccinated fish, fish injected with 1 mL saline solution, fish vaccinated with 1 x 10^4 and 1 x 10^8 CFU of polyvalent vaccine/mL. Different letters indicate significant difference among the treatments in each time of collection by SNK test of mean comparison ($P < 0.05$).

El conteo diferencial de leucocitos en la sangre de tilapia del Nilo, antes del desafío (6 días después de la inmunización) y 48 h después del desafío con *Aeromonas hydrophila*. Peces no vacunados, peces inyectados con 1 mL de solución salina y peces vacunados con 1 x 10^4 y 1 x 10^8 UFC de vacuna polivalente/mL. Las letras distintas indican diferencia significativa entre los tratamientos en los distintos tiempos de colecta por el test de comparación de medias SNK ($P < 0.05$).

Collection	Treatment	Basophill (10^3/µL)	Neutrophill (10^3/µL)	Lymphocyte (10^3/µL)	Monocyte (10^3/µL)
Before challenge	Non-vaccinated	0.58 ± 0.55[a]	7.83 ± 3.72[a]	9.51 ± 4.46[b]	0.27 ± 0.23[b]
	Saline	0.26 ± 0.13[a]	11.08 ± 0.98[a]	9.55 ± 5.76[b]	0.91 ± 0.06[a]
	1 x 10^4	0.33 ± 0.13[a]	9.50 ± 1.40[a]	14.21 ± 3.17[b]	1.66 ± 0.88[a]
	1 x 10^8	0.47 ± 0.20[a]	8.51 ± 6.02[a]	22.63 ± 1.02[a]	0.98 ± 0.35[a]
After challenge	Non-vaccinated	0.27 ± 0.39[a]	6.92 ± 4.38[a]	10.11 ± 5.16[b]	0.08 ± 0.07[a]
	Saline	0.05 ± 0.08[a]	10.62 ± 1.88[a]	8.44 ± 2.93[b]	0.00 ± 0.00[a]
	1 x 10^4	0.00 ± 0.00[a]	9.36 ± 6.39[a]	10.89 ± 4.39[b]	0.19 ± 0.25[a]
	1 x 10^8	0.17 ± 0.16[a]	9.99 ± 8.78[a]	20.28 ± 2.70[a]	1.08 ± 1.07[a]

the other hand, reduced glucose concentration either before or after challenge corroborated the findings of Harikrishnan *et al* (2003).

Reduced number of erythrocytes and hemoglobin concentration was reported in cichlid (*Etroplus suratensis*) infected by ulcerative epizootic syndrome (Pathiratne and Rajapakshe 1998). Although no difference was observed in erythrocyte number of *A. hydrophila* infected fish, our results showed an increase after immunization when compared to unvaccinated fish.

According to Haney *et al* (1992), a decrease in the erythrocyte number and hemoglobin concentration may be caused by bacterial agent. A decrease in the number of erythrocyte and hematocrit percentage suggests that erythrocyte has been destructed by leukocytic activity. On the other hand, increased hematocrit is a result of oxygen depletion (Kirk 1974). In the present study, the infection and/or vaccine did not cause alteration in hematocrit percentage.

Similarly to the present results, an increase in the number of thrombocyte, lymphocyte and hematocrit percentage was also found in tilapia after challenge with *Enterococcus* sp. (Martins *et al* 2008[b]). In contrast to the observations in this assay, Martins *et al* (2008[b]) did not relate influence of infection on glucose levels and erythrocyte number. They argued that this event occurred due to either insufficient inoculums to provoke these blood alterations or hematopoiesis. Contrarily to our results, Lamas *et al* (1994) and Balfry *et al* (1997) found a reduced number of lymphocytes in the blood of rainbow trout and tilapia, infected with *V. anguillarum* and *V. parahaemolyticus*, respectively. Our results with 1 x 10^8 bacteria-injected fish can be explained by the fact that lymphocytes have migrated to injured site, as found by Martins *et al* (2009[b]).

After immunization the number of monocytes was significantly lower ($P < 0.05$) in non-vaccinated fish (table 3). Monocytes have phagocytic activity and differentiate in macrophages in order to migrate to an inflammatory site like a defense response (Griffin 1984, Martins *et al* 2009[a]). This is especially true by the fact that vaccinated and saline-injected fish showed an increase in these cells confirming their action on the fish defense system.

Glucose concentration can increase in short periods in order to supply the energetic demand in stress situations as commented by Barton (2000). In this assay glucose levels did not alter before and after challenge in non-vaccinated and saline-injected fish. In 1 x 10^4 and 1 x 10^8 vaccinated fish glucose decreased after challenge (table 2). On the other hand, in infected carp treated with herbal extract an increase in the glucose concentration was observed 30 days after infection (Harikrishnan *et al* 2003). Our results confirmed the comments of Barton (2000). In fact, after both immunization and challenge, the fish was trying to supply the energetic demand in this situation of bacterial stress.

AGGLUTINATION TITER

After immunization and challenge fish vaccinated with 1 x 10^8 CFU inactivated bacteria/mL stimulated the agglutination titer against *A. hydrophila*, *P. aeruginosa* and *E. durans* (table 4). An increase in the agglutination titer in fish after immunization was reported by other authors. In the Studies on *Seriola quinqueradiata* vaccination

Table 4. Agglutination titer ($\log_2$ (x +1)) in the serum of Nile tilapia, before challenge (6 days after immunization) and 48 h after challenge with *Aeromonas hydrophila*. Non-vaccinated fish, fish injected with 1 mL saline solution, fish vaccinated with 1 x 10^4 and 1 x 10^8 CFU of polyvalent vaccine/mL. Different letters indicate significant difference among the treatments in each time of collection by SNK test of mean comparison ($P < 0.05$).

Título de aglutinación ($\log_2$ (x +1)) en el suero de tilapia del Nilo, antes del desafío (6 días después de la inmunización) y 48 h después del desafío con *Aeromonas hydrophila*. Peces no vacunados, peces inyectados con 1 mL de solución salina y peces vacunados con 1 x 10^4 y 1 x 10^8 UFC de vacuna polivalente/mL. Las letras distintas indican diferencia significativa entre los tratamientos en los distintos tiempos de colecta por el test de comparación de medias SNK ($P < 0.05$).

Collection	Treatment	Agglutination titer		
		Aeromonas hydrophila	*Pseudomonas aeruginosa*	*Enterococcus durans*
Before challenge	Non-vaccinated	2.64 ± 0.92[b]	0.00 ± 0.0[b]	3.51 ± 1.39[b]
	Salina	2.89 ± 0.49[b]	0.00 ± 0.0[b]	3.57 ± 1.79[b]
	1 x 10^4	3.17 ± 0.55[b]	0.00 ± 0.0[b]	5.05 ± 0.97[b]
	1 x 10^8	8.34 ± 0.58[a]	5.38 ± 1.12[a]	7.01 ± 0.99[a]
After challenge	Non-vaccinated	2.08 ± 0.43[c]	0.53 ± 0.92[b]	4.73 ± 0.55[b]
	Salina	3.23 ± 1.57[bc]	0.77 ± 1.34[b]	4.09 ± 0.00[b]
	1 x 10^4	4.73 ± 0.55[b]	1.83 ± 0.43[b]	4.41 ± 0.55[b]
	1 x 10^8	11.33 ± 0.58[a]	6.04 ± 0.57[a]	6.68 ± 0.57[a]

against *Photobacterium damsela piscicida*, Gravningen *et al* (2008) detected increased antibody levels three and four weeks after immunization.

Similar to our results, higher antibody levels for two weeks were reported in tilapia vaccinated intraperitoneally with *A. hydrophila* (Ruangpan *et al* 1986). On the other hand, Swain *et al* (2007) have related the efficacy of poli or monovalent vaccine in cyprinid fish (*Labeo rohita*) with the maximum values of agglutination titer after 4 weeks. In this assay, the highest agglutination titer was found after injection with 1 x 10^8 CFU bacteria/mL.

In conclusion, polyvalent vaccine at a concentration 1 x 10^8 CFU/mL stimulated the erythrocyte production, total leukocyte counting, monocyte and lymphocyte number, important cells involved on the specific and nonspecific immunological process.

Fish vaccinated with the highest dose also stimulated the thrombocyte and leukocyte production after challenge. An increase in the agglutination titer against *A. hydrophila, P. aeruginosa* and *E. durans* was found either after immunization or challenge with *A. hydrophila.* However, further studies must be developed to solve the defense mechanisms in the Brazilian cultured fish to enhance the vaccine efficacy. Moreover, studies should be carried out to evaluate the cumulative mortality in fish treated with different vaccine doses regarding to field application and the duration of vaccine efficacy.

SUMMARY

This study evaluated the effects of polyvalent vaccination on the hematological and serum agglutination responses in Nile tilapia challenged with *Aeromonas hydrophila*. Two dosis, 1 x 10^4 and 1 x 10^8) Colony Forming Units (CFU)/mL, of vaccine containing the same amount of *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Enterococcus durans* formalin-inactivated were tested by intraperitoneal (i.p) injection. Fish were challenged ten days after vaccination i.p. with a $DL_{50\text{-}96h}$ of 1 x 10^7 CFU *A. hydrophila*/mL. Samples were collected 48 h after challenging fish to check the hematological parameters, antimicrobial activity and agglutination titer of serum, samples were collected 48 h after challenge. Before challenge, the number of erythrocytes was higher in fish vaccinated with 1 x 10^8 CFU/mL. After challenge, total number of thrombocytes was higher in fish that received the greatest dose of vaccine. Before and after challenge, total number of leukocytes and the number of lymphocytes showed the highest values in vaccinated fish. Before challenge, increased number of monocytes in vaccinated and saline-injected fish was observed. The highest agglutination titer against *A. hydrophila*, *P. aeruginosa* and *E. durans* was related in 1 x 10^8 CFU/mL vaccinated fish. Before challenge, high values of antimicrobial activity in non-vaccinated fish and 1 x 10^8 CFU/mL vaccinated ones was also related. Therefore, after challenge, non-vaccinated fish and saline-injected ones showed the highest antimicrobial activity. This study showed that 10 days after immunization with a polyvalent vaccine at a concentration 1 x 10^8 CFU/mL, there was an increase on erythrocytes, leukocytes, thrombocytes and circulating lymphocytes production, while the glucose levels were reduced.

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