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Proposed method for agglutinating antibody titer analysis and its use as indicator of acquired immunity in pacu, *Piaractus mesopotamicus*

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Abstract

Antibody can be assessed by agglutinating antibody titer which is a quantitative measure of circulating antibodies in serum from fish previously immunized. The antibody evaluation has been performed with different fish species, and is considered a reliable method that can be applied to confirm several hypothesis regarding acquired immunity, even in conjunction with precise methods to describe immune mechanisms. In order to provide appropriate analytical methods for future studies on the specific immune system of native fish, the present study standardized on assay to measure the serum agglutinating antibody titer produced after immunization with inactivated *A. hydrophila* and levamisole administration in pacu. It was possible to determine the agglutinating antibodies titer in a satisfactorily way in pacu immunized with inactive *A. hydrophila*, and the highest titers were observed on fish fed with levamisole.

Keywords: fish, methodology, immunology, acquire immunology, humoral immunity.

Aglutinante de anticorpos como indicador de imunidade adquirida de pacu *Piaractus mesopotamicus*

Resumo

Os anticorpos podem ser avaliados pelo título aglutinante de anticorpos, que é uma medida quantitativa de anticorpos no soro de peixe previamente imunizados. A determinação do título de anticorpos foi realizada com diversas espécies de peixes e é considerado um método confiável que pode ser aplicado para confirmar diversas hipóteses que envolvam o sistema adquirido de defesa, mesmo em conjunto com métodos precisos, para descrever mecanismos imunes. A fim de prover métodos analíticos adequados para futuros estudos sobre o sistema imune específico de peixes nativos, o presente estudo aperfeiçoou o ensaio para avaliar o título aglutinante de anticorpos em soro de pacu imunizados com *A. hydrophila* e alimentados com levamisol. Foi possível determinar o título aglutinante de anticorpos de forma satisfatória, em pacus imunizados com *A. hydrophila* inativa, e os maiores títulos foram observados em peixes alimentados com levamisol.

Palavras-chave: peixe, metodologia, imunologia, imunidade adquirida, imunidade humoral.

1. Introduction

Fish are the most primitive vertebrate group to present acquired immune system, and the ability to produce antibody after antigenic stimuli. Antibodies are glycoproteins classified as immunoglobulins (Ig) that can be expressed in the B lymphocyte membrane or be released by plasma cells (activated B lymphocytes post-connect with antigen) in several body fluids (Solem and Stenvik, 2006). Most of mammals have five classes of immunoglobulins: IgG, IgM, IgA, IgD and IgE, differing in structure and biological activities. However, in fish, the immunoglobulin IgM is the predominant immunoglobulin, and is secreted as a tetrameric form with eight sites for antigen binding. In addition to IgM, some low molecular weight Igs have been found in several fish species (Bogwald et al, 1991; Morrison and Nowak, 2002).

Aquaculture is an important economic activity for many countries, including Brazil. Thus, the development of effective measures to confer a better health status to fishes, such as the improvement of antibacterial or antiviral vaccines or by diet immune modulation can be useful for fish aquaculture (Muiswinkel, 2008). However, the development of these strategies depends on a better understanding of

fish acquired immune system, particularly the humoral immune responses (Solem and Stenvik, 2006; Vesely et al, 2006). In order to evaluate humoral immunity in fish, the concentrations of IgM in plasma have been usually determined, instead quantifying fish specific anti-antigen antibodies, due to lack of standardized serological techniques, or absence of immune-reagents, mainly for indigenous fish species, like those from our country (Misra et al., 2006). Moreover, it is important to quantify specific IgM antibodies, especially in studies addressing the immune modulation effects of diet components in fishes.

The assessment of agglutinating antibody titer is an easy approach to measure circulating antibodies in serum samples collected from fish previously immunized with particulate antigen preparations (Sugahara and Eguchi, 2012). The agglutination is a clumping reaction between specific antibodies and a particulate antigen, such as erythrocytes or bacterial cells suspension, and it is usually applied in the study of adaptive immune responses to evaluate the antibody production (Plumb and Areechon, 1990; Chen and Light, 1994; Yildirim et al., 2003). In spite of this, there are few studies on antibody immune responses of Brazilian fish species. Therefore, development and standardization of techniques to evaluate the humoral immune responses of native fish is of critical importance for the control of diseases in these species, particularly those used in aquaculture.

Regarding Brazilian native fish, the pacu *Piaractus mesopotamicus* (Holmberg, 1887) is one of the most significant fish in Brazilian aquaculture. Nevertheless, few studies exist on its immune responses (Abreu et al., 2009; Martins et al., 2009; Sado et al., 2010; Biller-takahashi et al., 2012, 2013). In order to provide appropriate analytical methods for future studies on the specific immune system of this native Brazilian fish, the present study aimed to standardize and applied the agglutination assay to measure the serum agglutinating antibody titer produced after immunization with inactive vaccine against *A. hydrophila* followed by the administration of the immune modulator levamisole.

2. Material and Methods

A total of 120 pacu (*P. mesopotamicus*) (218.92 ± 47.0 g; 21.36 ± 1.44 cm) was distributed in twelve100 l tanks (10 fish per tank) with a continuous water flow system and aerated with compressed air diffused through air stones. The water quality parameters were monitored daily and were within the values described for the species (Urbinati et al. 2010): temperature 27.02 ± 0.89 °C; dissolved oxygen 5.08 ± 0.49 mg l^{-1} and pH 7.76 ± 0.07. Fish remained in these conditions during 20 days for acclimatization, being fed to apparent satiation twice a day with commercial diet (28% protein, 3% fat, 1% fiber, levamisole free). The experimental diets were prepared with commercial feed to which were added 0 and 250 mg.Kg^{-1} of levamisole.

Fish were randomly distributed into tanks and after acclimatization period fish from all treatments have received their experimental diets during seven days. After this period fish from control and levamisole fed groups were immunized with inactive *A. hydrophila* and remained in their aquarium up to 15 days. Subsequent to this period 12 fish per treatment were anesthetized in benzocaine (0.1 g L^{-1}) and blood was drawn for serum extraction. Serum was stored at –70° C until use to preserve thermo labile proteins. A sample of whole blood was obtained for haematological determinations.

In order to titer the serum agglutinating antibodies against *A. hydrophila*, a serum agglutination reaction was attempted. The assay was a cell flocculation reaction, in which the antigen consists of stable cells, in this experiment *A. hydrophila* colony forming units (CFUs) were used, and the result was a visible clumping reaction between particulate and specific antibodies.

Serum of pacu was used to standardize the methodology of agglutinating antibody titer, adapted according to Plumb and Areechon (1990), Chen and Light (1994) and Yildirim et al. (2003). Initially, an *Aeromonas hydrophila* strain from Laboratório de Patologia de Organismos Aquáticos (*LAPOA-CAUNESP-Jaboticabal*) (Genbank indetification as ATCC 7966-http://www.ncbi.nlm.nih.gov/genbank/) was cultured in tryptone soya broth (TSB), at 25° C, for 24 h incubation. After this period, the bacterial suspension was washed and centrifuged at 3000 g (3 min) in sterile phosphate buffer solution (PBS) (NaCl, 0.137 M; KCl, 2.7 mM; KH_2PO_4, 1.5 mM; Na_2HPO_4, 8.1 mM; $CaCl_2$, 0.9 mM; $MgCl_2$, 0.49 mM in 1 l Milli-Q distilled water), with pH 7.4, for three times until complete TSB removal. The bacterial suspension was inactivated with 3% formalin treatment, washed three times and diluted in sterile PBS. The efficacy of inactivation procedure was performed by culturing the suspension of vaccine. The dilutions were adjusted by turbidity score at 1×10^9 CFU following Mc Farland scale to adequate the concentration (Vandepitte et al., 1993).

The bacterial suspension was inoculated into the abdominal cavity by intraperitoneal injection. After this procedure, fish were fed commercial diet during 15 days (28% PB, and 3,000 kcal EB. kg^{-1}, levamisole free), and then sampled and blood was drawn for serum extraction in order to titer the agglutinating antibodies.

The serum agglutinating titer was determined in a 96-well microtiter plate with round bottom wells. The assay was initiated with a dilution of 1:1 (50 mL of phosphate buffer: 50 mL of serum) and consequently a two-fold serial serum dilutions were made by adding 50 mL of diluted serum into the remaining wells with 50 mL of PBS. 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 and 1/2048. Thereafter, 50 mL of inactive *A. hydrophila* (1×10^9 CFUs) suspension was added to each well and then micro plates were covered with plastic film and incubated at room temperature for 16-18 h. The agglutination end point was established as the last serum dilution where agglutination was visible. Agglutination antibodies titers were expressed as log2 (x+1) of the reciprocal of the highest serum dilution showing

visible agglutination as compared to the positive control. The last well was used as a negative control, where there were only 50 mL PBS buffer.

The haematological determinations included the red blood cell, total and differential leukocytes counts were determined in blood smears stained by May–Grünwald–Giemsa (Rosenfeld, 1947). Total leukocytes number was calculated using the formula: leukocytes/ml = (leukocyte number in the smear x erythrocyte number/ml)/2,000 erythrocytes counted in the smear, as described by Ishikawa et al. (2008).

Data were submitted to one-way ANOVA. If results were significant, Tukey test was applied for means comparison. Differences were considered significant at $p < 0.05$ throughout SAS (9.2) statistic program.

3. Results

In the present study, the immunization with *A. hydrophila* and administration of 250 mg kg^{-1} of levamisole for seven days have enhanced the agglutination antibody titer against *A. hydrophila* (Figure 1). In the Figure 1 the negative control (serum free) has displayed the zero titer once there was no serum, and no agglutination reaction has been observed.

The haematological determinations of immunized pacu have shown an increase in the number of lymphocytes, the responsible cell for antibody production (Table 1). In the Table 1 the augment of lymphocytes is followed by an enhancement of red blood cell (RBC) and leukocytes.

4. Discussion

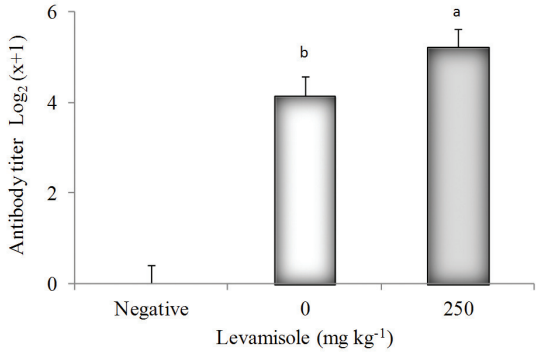

Figure 1. Serum agglutinating antibodies titer against *Aeromonas hydrophila* of immunized *Piaractus mesopotamicus* (mean ± sd). Significant differences are indicated by different letters ($p<0.5$).

The immunization with *A. hydrophila,* in this study, has been effective in induce a benefic response, such as the increase in antibody titer, since this may protect fish against bacterial disease outbreak. The immunogenic compound used in the vaccine is not pathogenic, but may come from pathogenic microorganisms. In aquaculture, immunization can be an alternative to antibiotics administration, once vaccinated fish became resistant against the inoculated agent (Parslow and Bainton, 2004; Thorarinsson and Powell, 2006).

The increase of antibody concentration found in immunized pacu may protect against disease prompted by *A. hydrophila*, the etiologic agent of an important infectious disease that causes severe outbreaks in Brazilian aquaculture. It is considered a serious economic problem, in addition to the fact that led to the indiscriminate use of antibiotics, which has increased resistance of bacteria to antibiotics worldwide (Vivekanandhan et al., 2002; Vieira, 2003).

The antibody evaluation has been performed with different fish species, and an increase in agglutination titer, as well in this study, has been reported in serum of *Oreochromis niloticus* challenged with *A. hydrophila* or immunized with a polyvalent vaccine against *A. hydrophila, P. aeruginosa* and *E. durans* (Bailone et al., 2010). The agglutination titer has supported Sugahara and Eguchi (2012) hypothesis in order to confirm that warm water treatment has influenced on immunization of *Plecoglossus altivelis* besides the effectiveness of different vaccines. This shows that the agglutination titer is a reliable method and can be applied to confirm several hypothesis regarding acquire immunity, even in conjunction with accurate methods as RT-PCR to characterize immune mechanisms (Takano et al., 2010).

Conversely, fish immune responses can also be influenced by some diet components such as levamisole. The compound included on pacu diet is a synthetic anthelmintic extensively applied in veterinary and human treatments, and has shown a potent immune modulatory action on innate and acquired immune systems of several species, such as *Sparus aurata*, *Salmo salar, Oncorhynchus mykiss* and *Cyprinus carpio* (Siwicki, 1989; Jeney and Anderson, 1993; Mulero et al., 1998; Findlay and Munday, 2000; Cuesta et al., 2002, 2004). Therefore, levamisole has prompt resistance against various etiological agents, such as *Vibrio anguillarum, A. hydrophila, Paramoeba sp*., *Edwardsiella tarda, Photobacterium damselae* and nematodes as *Anguillicola crassus* (Kajita et al., 1990; Geets et al., 1992; Baba et al., 1993; Findlay et al., 2000;

Table 1. Haematological parameters of immunized *Piaractus mesopotamicus* (mean ± sd)*.

	Levamisole (mg kg^{-1})	
	0	**250**
RBC (10^6/mm^3)	2.92±0.34 a	3.15±0.28 a
Leukocytes (10^3/mm^3)	31.42±15.39 b	61.28±20.89 a
Lymphocytes (%)	79.82±4.99 b	90.04±4.11 a

*Significant differences are indicated by different letters ($p<0.5$).

Sahoo and Mukherjee, 2002; Leano et al., 2003; Munday and Zilberg, 2003).

As a consequence, several studies have been done to demonstrate the efficacy of immunization and levamisole administration. Jeney and Anderson (1993) have observed in rainbow trout, increase in acquired and innate parameters besides greater protection against *A. salmonicida*. Additionally, Cuesta et al. (2004) have found in vaccinated *Sparus aurata* elevation in IgM concentration after two weeks of levamisole administration and the effect has persisted for more than six weeks.

The antibody concentration and persistence in serum can differ according to species, age, sexual maturity and physiological events. However the antigen stimulation by artificial immunization or due to chronic infection clearly increases the concentration of circulating antibodies (Hordvik, 2002; Zhao et al., 2008; Jerônimo et al., 2011).

The identification of antibodies produced after immunization by quantitative or qualitative techniques have already been described, including bacterial agglutination, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot, Western blotting, viral neutralization and plaque-forming assays. Among the serological tests, the agglutination assay applied is considered quick, simple and inexpensive, in addition to be less prone to generate false reactivity, due to IgM low specificity, multivalence or cross-reactivity (Morrison and Nowak, 2002).

5. Conclusion

In conclusion, this study standardized the methodology of agglutinating antibody titer from pacu serum samples, an important Brazilian farmed fish, after *A. hydrophila* immunization added of levamisole as immune modulator, and could contribute with an appropriate analytical method for future studies on the specific immune system of native Brazilian fish.

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