HEMAGGLUTINATION ANTIBODY TITERS IN PACU, *Piaractus mesopotamicus,* AS AN INDICATOR OF ACQUIRED IMMUNITY

TÍTULO DE ANTICORPOS HEMAGLUTINANTES DE PACU, Piaractus mesopotamicus, COMO INDICADOR DE IMUNIDADE ADQUIRIDA

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SUMMARY

The evaluation of the hemagglutination titer is an option to evaluate the response of acquired immune system in order to assess the immunocompetence for antibodies production. The study was carried out to standardize the antibody titer against rabbit red blood cell in immunized fish and fed diets with levamisole (0; 250 and 500 mg.kg^{-1} levamisole). As a result, the cell cluster agglutination can be observed by the naked eye. Fish fed 250 mg.kg^{-1} of levamisole have shown the highest hemagglutination antibodies titer; however, fish fed 500 mg.kg^{-1} of levamisole have revealed titers equivalent to control group that was fed levamisole-free diet. This study has validated the methodology for determination of hemagglutination antibody titer of immunized fish and has found that antibody titers increased after feeding a diet containing 250 mg.kg^{-1} of levamisole during 10 days.

KEY-WORDS: Acquired immune system. Antibody. Immunostimulant. Methodology.

RESUMO

A determinação do título de hemaglutinação é uma alternativa para avaliar as respostas do sistema imune adquirido, ou seja, analisar a capacidade de produção de anticorpos circulantes do organismo. O estudo foi realizado a fim de padronizar a titulação de anticorpos produzidos contra hemáceas de coelho em peixes previamente imunizados e submetidos a dietas com diferentes concentrações de levamisol (0, 250 e 500 mg.kg^{-1} de levamisol). O resultado é um aglomerado celular que pode ser visualizado a olho nu. Peixes do presente estudo alimentados com 250 mg.kg^{-1} de levamisol apresentaram maiores títulos de anticorpos hemaglutinantes, entretanto os alimentados com 500 mg.kg^{-1} apresentaram títulos semelhantes ao grupo controle, alimentado com dieta sem levamisol. Este estudo validou a metodologia para determinação do título de hemaglutinação do soro de peixe nativo imunizados, após administração de levamisol e verificou um aumento da concentração de anticorpos hemaglutinantes após administração de 250 mg.kg^{-1} de levamisol por 10 dias.

PALAVRAS-CHAVE: Sistema imune adquirido. Anticorpo. Imunoestimulante. Metodologia.

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INTRODUCTION

The hemagglutination titer determination is an alternative to evaluate the responses of the acquired immune system, by analyzing the capacity of circulating antibodies in the body (KUMARI & SAHOO, 2005, 2006).

The immune system of fish is a defense mechanism divided into innate and acquired, both divided into cell and humoral mediated defense. The innate system is considered the first barrier against invading agents. The acquired system, on the other hand, needs the presence of the antigen to trigger reactions which culminate in specific increase of circulating antibodies, and result in immune memory (BAYNE & GERWICK, 2001; ELLIS, 2001).

The antibodies and lymphocytes comprise the acquired defense mechanisms, humoral and mediated by cells, respectively. The antibodies bind to microorganisms and can activate phagocytosis, promote the neutralization or opsonization of the pathogen, as well as complement activation and cell mediated cytotoxicity (ELLIS, 2001).

The immune responses of fish may be influenced by certain substances, such as levamisole. This compound is a synthetic anthelmintic drug commonly used in mammals, which has a powerful action on the specific innate immune system of fish (CHIRON, 2012). This immunostimulant promotes the increase of some parameters, such as the cytotoxic activity of leukocytes (CUESTA et al., 2002), number of phagocytes (MULERO et al., 1998; FINDLAY & MUNDAY, 2000), respiratory activity of macrophages (SIWICKI, 1989; MULERO et al., 1998) and enhances the specific immune responses (JENEY & ANDERSON, 1993; CUESTA et al. 2004).

Pacu, *Piaractus mesopotamicus,* belongs to the Characidae family and Myleinae subfamily, and has special features like ruggedness, easy feeding adaptation, rapid growth and ease artificial reproduction (OLIVEIRA et *al.,* 2004; QUEIROZ et al., 2005). However, there are few validated methods to assess the species immune responses, such as antibody production (BILLER et al., 2013) in addition to some studies on immune responses modulated by levamisole (SADO et al., 2010). This study aims to standardize a more simplified model based on using rabbit erythrocytes as antigen for induction and measurement of antibodies produced by pacu fed diets with different levamisole concentrations.

MATERIAL AND METHODS

Animals, experimental design and sampling

We used 180 pacu, *Piaractus mesopotamicus,* averaging 218.92 ± 47.74 g weight and 21.36 ± 2.15 cm total length. The fish were divided into 18 polyethylene tanks with 100 liters capacity, placed in open circulation system, equipped with continuous water from an artesian well at a constant temperature (approximately 29°C). The fish were fed commercial levamisole-free diet (28% crude protein, 3% lipid, 1% fiber) for 20 days for adaptation to laboratory conditions. Subsequently, they were fed the experimental diets to apparent satiation, two times daily. The experimental diets consisted of the commercial diet added 0, 250 and 500 $mg.kg^{-1}$ levamisole. The physico-chemical parameters of the water remained within the values recommended for the species (URBINATI & GONÇALVES, 2005): temperature, 28.82 ± 0.67°C; dissolved oxygen, 5.96 ± 0.89 $mg.L^{-1}$; NH_4, 0.41 ± 0.22 $mg.L^{-1}$; and, pH, 7.09 ± 0.11.

Fish from all treatments were fed experimental diets for ten days, and after this period, fish from each aquarium were inoculated with a 10% rabbit erythrocyte suspension and during this period, the fish were fed a commercial diet. After 15 days, two fish from each treatment aquarium (12 fish per treatment) were anaesthetized with benzocaine (0.1 $g.L^{-1}$) to undergo blood sampling by caudal vein puncture. The serum after clotting was subjected to hemagglutination antibodies titration.

Hemagglutination Antibody Titers

In order to titrate the antibodies against rabbit erythrocytes, a serum agglutination reaction was carried out. These are cellular flocculation reactions, in which the antigen is comprised of stable cells. The result is a cell cluster that can be viewed with the naked eye.

Rabbit erythrocyte suspension and inoculation

An aliquot of rabbit whole blood was mixed with the same volume of Alséver solution (pH 6.1 anticoagulant) and the resulting solution was filtered through sterile gauze. Upon use, the red cells were re-suspended, washed and centrifuged (refrigerated centrifuge at 3000g for 3 min) 3 times with sterile phosphate saline buffer (PBS, consisting of 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 Milli-Q distilled water excipient qsp 1 liter), pH 7.4. The suspension was diluted to 1% (optical density between 0.8 and 0.9 at 700 nm) for microplate testing and 10% for fish inoculation.

Serum-agglutination reaction

Initially, the fish hyperimmune serum, obtained 15 days after erythrocyte inoculation at 10%, was inactivated at 56°C for 20 minutes to denature complement proteins, which are heat sensitive and have great ability to lyse erythrocytes. In sterile acrylic microplates with 96 wells, 50 µL of PBS were distributed in the wells using a multichannel pipette. Subsequently, 50 µL of inactivated serum was placed in the first column, and from this solution the serum was two-fold serial diluted in PBS buffer of the following well until the penultimate well since the last one was the negative control, containing only 50 µL PBS buffer to maintain 50 µL final volume per well and the following serum dilutions: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, 1/2048. Lastly,

50 μL of 1% rabbit erythrocyte suspension was added to all wells using multichannel pipette, the microplates were covered with plastic film and incubated for 16-18 hours at room temperature. The hemagglutination antibody titer was defined as the last serum dilution showing visible agglutination, and values are expressed as the log10 of the dilution reciprocal.

Design and statistical analysis

The results were submitted to analysis of variance (ANOVA) and means were compared by Tukey test (5%). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Fish fed 250 $mg.kg^{-1}$ levamisole had higher hemagglutination titers while those fed 500 $mg.kg^{-1}$ had the same titers as the control group fed diet without levamisole (Figure 1). Levamisole action has been evaluated in other species and Cuesta et al. (2004) reported high IgM concentrations in *Sparus aurata* after administering levamisole for two weeks while the effect persisted for more than six weeks. Hung et al. (1997) also reported that in Japanese eel, the immunoglobulin concentrations displayed peaks three to four weeks after immunization.

The improvement of the resistance against various etiological agents after administration of levamisole was observed in fish challenged with *Vibrio anguillarum* (KAJITA et al. 1990), *A. hydrophila* (BABA et al., 1993), *Paramoeba sp.* (FINDLAY et al. 2000; MUNDAY & ZILBERG, 2003), *Edwardsiella tarda* (SAHOO & MUKHERJEE, 2002), *Photobacterium damselae* (LEANO et al., 2003), as well as the nematodes *Anguillicola crassus* (GEETS et al., 1992). However, levamisole immunomodulatory responses on antibody production are dependent on the dose and time of administration; it is, therefore, necessary to investigate these two parameters in future studies to evaluate in more depth the action of this compound on the responses of the immune system (KIRON, 2012).

The HA is a reaction between particulate antigens, a homogenous cell suspension of either bacteria or rabbit erythrocytes and serum from previously immunized fish (TIZARD, 2002; KUMARI & SAHOO, 2005, 2006). The production of antibodies by the immunized fish arises from the recognition of the particulate antigen, initially by the innate immune system, by the antigen-presenting cells (macrophages or dendritic cells) which process the antigen into particles and, at first, will trigger immune and proliferation responses; and subsequently, together with the specific defense compounds, trigger memory response (ABBAS & LICHMAN, 2004). Cellular and humoral factors from the innate and acquired systems work together to induce an increase of circulating antibodies (IWANA & NAKANISHI, 1996; MAGNADOTTIR et al., 2011).

Evaluation of antibody production is very important because the humoral factor of the acquired system is essential for pathogen recognition and triggers responses that culminate in the destruction of the agent, as well as the immune memory. The onset of diseases, despite body defenses, can occur when there is excess organic matter in the water, stress or presence of parasites (POST, 1987). Fish farms in Brazil report great economic losses due to outbreaks caused by microorganisms, including bacteria, fungi and parasites while even the indiscriminate use of antibiotics in subtherapeutic doses added to fish diet result in increased resistance of bacteria to antibiotics worldwide (SUHET et al., 2011; BELEM-COSTA & CYRINO, 2006; VIVEKANANDHAN et al., 2002).

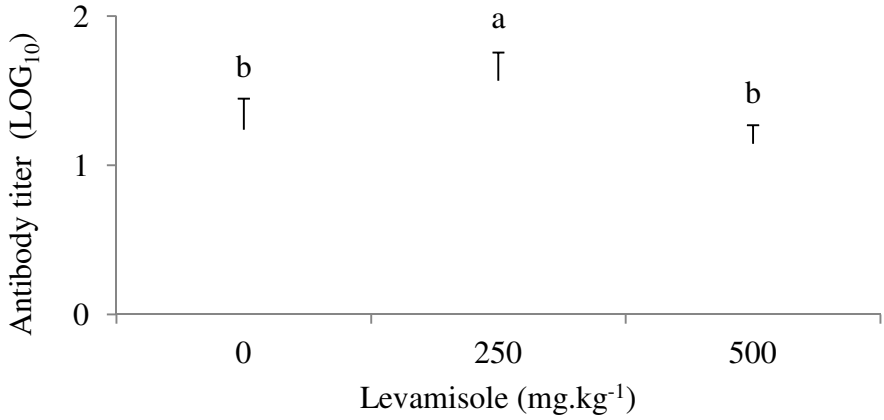

Figure 1 - Hemagglutination antibody titers against rabbit erythrocytes in pacu *Piaracus mesopotamicus* previously immunized (mean ± standard deviation). Significant differences are indicated by different letters ($P < 0.05$).

Currently, defense against microorganisms can be stimulated through immunization and the use of immunostimulants, with consequent increase of circulating antibodies concentration, survival and production performance (POUEY et al., 2011; SUHET et al., 2011; TIZARD, 2002). In aquaculture, immunization can be an alternative to antibiotics, since disease prevention is critical to the development of the activity (ROMANO & MEJÍA, 2003; PLANT & LAPATRA, 2011).

In this respect, methods to evaluate immunocompetence are useful in comparative studies, particularly those which use tiny samples. Additionally, the method for determining the hemagglutination antibody titer of pacu described in this work is a simple method that analyzes the capacity of antibodies production of immunized fish. However, in order to avoid losses in the hemagglutination titer determination is important to denature proteins of the complement system, since these proteins have a natural affinity for red blood cells and consequently promote cell lysis (BILLER et al., 2012).

The hemagglutination is a reaction with particulate antigens and such reaction occurs only with antigens located on the cell surface and often the identification of these antigens can occur naturally without the need for prior immunization, as verified by Kumari & Sahoo (2005), Sahoo et al. (2005) and Dash et al. (1993). Antibodies that perform the hemagglutination are usually multivalent whilst the isotypic class - IgM has higher binding activity. As fish have higher IgM concentrations, the hemagglutination reaction can be observed very effectively (BILLER et al., 2014; DASH et al., 1993).

CONCLUSION

The standardization of techniques to assess indicators of the immune system of native fish is of great importance to the national research scenario. This study described the methodology for determining the HA titers of sera from immunized native fish as an indicator of acquired immunity after administration of levamisole and found an increase of hemagglutination antibody concentration after administering 250 $mg.kg^{-1}$ levamisole for 10 days.

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