



Development of an indirect ELISA assay to evaluation of the adaptive immune response of pacu (*Piaractus mesopotamicus*)

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ABSTRACT

The pacu is one of the most important species for Brazilian fish farming and is considered emerging in the global aquaculture. Despite its importance, no effective tool for evaluation of the adaptive immune response of this species has been developed. Therefore, this study aimed the development and standardization of indirect ELISA for the measurement of pacu antigen-specific antibodies using polyclonal rabbit anti-pacu IgM used as detector antibody. For this purpose was isolated and purified of pacu IgM using mannose-binding protein affinity chromatography and produced specific polyclonal antibodies against heavy and light chains pacu IgM, that showed a molecular weight of 72 kDa and 26 kDa, respectively. Polyclonal antibodies obtained demonstrated specificity with heavy and light Ig chains of pacu serum in western blotting. These polyclonal antibodies allowed the development of an indirect ELISA assay of high sensitivity and specificity for the detection and quantification of pacu IgM antibodies immunized with bovine IgG. In conclusion, this approach has great potential to improve the monitoring of vaccine-induced immune responses and help develop immunodiagnostic and epidemiological studies of infectious diseases in pacu systems.

Key words: ELISA, Immunoglobulin, Pacu's immune system, Polyclonal antisera.

INTRODUCTION

Pacu (*Piaractus mesopotamicus*) is a native species of the Paraná-Paraguay River Basin in South America and one of the main fish species cultured in Brazil (Valladão et al. 2016). Pacu is also an

emerging species in global aquaculture because of its husbandry characteristics, ecological value, and marketability (MPA 2012) and it has also been produced in China (Lin et al. 2015) and the United States (Witmer and Fuller 2011). Moreover, pacu has been shown to be a good indicator of water quality and has been used as a model for ecotoxicity

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studies during the process of chemical registrations in Brazil (Castro et al. 2014).

The intensification of pacu culture has led to outbreaks of several diseases caused mainly by bacterial pathogens resulting in reduced yields and significant economic losses (Farias et al. 2016). In addition, the lack of information on the pacu's immune system is a limiting factor for the development of serological tests and vaccines for immunodiagnosis and monitoring of bacterial diseases treatment, which hinder the implementation of more effective biosecurity measures in intensive production systems.

Studies on the purification and characterization of teleost immunoglobulins have been described for several species (Lim et al. 2009) and, like mammals, these animals produce a dominant class of immunoglobulin (IgM) (Newman 1993). Different immunoglobulin isotypes have been detected in some fish species such as IgT (Hansen et al. 2005), IgZ (Danilova et al. 2005, Savan et al. 2005a) and IgH (Savan et al. 2005b). Nevertheless, to date no studies have been reported on the isolation of pacu immunoglobulins.

The purification and characterization of fish immunoglobulins can contribute to a better understanding of the immune system of different fish species. In addition, it enables the development of effective immunodiagnostic methods for the evaluation of vaccination and epidemiological studies of pathogenic microorganisms.

The enzyme-linked immunosorbent assay (ELISA) is an essential tool to monitor and measure antigen-specific antibody systemic and mucosal production in fishes (Grabowski et al. 2004). This immunoassay is simple, higher sensitivity, specificity and speed because it facilitates detection and quantitation of antibodies and the use of microplates allows for the testing of a large number of samples.

The ELISA assay requires the development of anti-specific species antibodies in a second

animal (Shelby et al. 2003). Although monoclonal antibodies (mAbs) are often used as secondary antibodies in such ELISAs, the production and use of specific polyclonal antibodies for the detection of fish immunoglobulins has been growing interest in because they are a viable, low-cost, and effective tool for determining the level of antibodies produced in response to antigens derived from pathogens or vaccines (Babu et al. 2008, Crosbie and Nowak 2002, Sudhagar et al. 2015). However, little has been investigated on the detection and measurement of pacu immunoglobulins, especially the antibodies against pathogenic microorganisms affecting this species.

This study aimed the development and standardization of indirect ELISA for the measurement of pacu antigen-specific antibodies using polyclonal rabbit anti-pacu IgM used as detector antibody.

MATERIALS AND METHODS

All experimental procedures involving animals were approved by the local animal welfare committee/CEUA of the School of Agricultural Sciences and Veterinary Medicine/FCAV at São Paulo State University/UNESP, Jaboticabal, São Paulo, Brazil under protocol number 027994/12 and performed in accordance with the guidelines of the Brazilian College of Animal Experimentation/COBEA.

Due to the unavailability of polyclonal anti-pacu IgM, a first part of this study reports the isolation of pacu IgM and the subsequent production of polyclonal anti-pacu IgM. These polyclonal antibodies were used for the standardization of the indirect ELISA for the detection and quantification of pacu IgM antibodies.

FISH AND OBTENTION OF PACU IgM

To obtain polyclonal sera, the antigen (pacu's IgM) must first be isolated from serum samples with

high antibody levels, purified and then used for hyperimmunizations in laboratory animals.

Seventy pacu juveniles (100.0 ± 30.0 g) obtained from a commercial fish farm (Itajobi, São Paulo, Brazil) were maintained in 500 L plastic tanks with continuous water flow (~ 16 L min⁻¹) at a stocking rate of 10 fish per tank and allowed to acclimate to experimental conditions for 15 days. No differences were observed in water quality parameters during the study period (temperature: 26.0 ± 2.0 °C, pH: 7.9 ± 0.1 , dissolved oxygen: 6.7 ± 0.69 mgL⁻¹ and ammonia: 0.1 ± 0.1 mgL⁻¹). The fish were fed a commercial feed (32% crude protein- Trouw Nutrition, The Netherlands) twice daily to apparent satiety.

To obtain hyperimmune serum, 10 fish were anesthetized with clove oil (100µL⁻¹) and immunized intraperitoneally with bovine IgG purified with diethylaminoethyl (DEAE) cellulose (250 µg fish⁻¹) and emulsified 1:1 (v/v) in Freund's complete adjuvant (FCA) (Intervet, Brazil). Booster doses were administered 14 and 28 days after the first immunization using the same route and dose of bovine IgG emulsified in Freund's incomplete adjuvant (FIA) (Intervet). (Howard and Kaser 2013). Two weeks after the second booster, blood was collected by caudal vessel puncture, maintained at 4 °C for 1 h, and centrifuged at 1000 x g for 15 min for serum separation, which was stored at -20 °C.

PURIFICATION OF PACU IMMUNOGLOBULINS

Serum immunoglobulin from fish immunized with bovine IgG were purified according to a protocol adapted from Shin et al. (2007) using the IgM purification kit (Pierce, USA) with a mannose-binding protein (MBP) affinity column. Briefly, the 5 mL pre-packed MBP affinity column was pre-washed with 5 mL of preparation buffer and equilibrated with 20 mL of binding buffer. Pacu serum samples were mixed with binding buffer

(1:1), added to the MBP-Sepharose column, and allowed to stand for 1 h at 4 °C. After unbound molecules were removed by passing 42 mL of binding buffer, the column was loaded with 3 mL of elution buffer and incubated overnight at room temperature, and the elutes were collected in 1 mL fractions in sterile microtubes. The concentration of purified protein fractions eluted from the MPB-Sepharose column was determined by the Bradford method (Bradford 1976).

CHARACTERIZATION OF PURIFIED PACU IMMUNOGLOBULINS

The purified pacu immunoglobulins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Column elute samples were diluted in sample buffer containing 62.5 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerol, 0.001% bromophenol blue, and 1% 2-mercaptoethanol. After boiling, the samples were loaded in 12% polyacrylamide gel. After electrophoretic separation, the polypeptide bands were detected by Coomassie Brilliant Blue R-250 staining. The molecular weight of pacu IgM was estimated by comparison with standard molecular weight proteins (PageRuler Unstained Protein Ladder, Thermo Scientific, USA).

PRODUCTION OF RABBIT ANTI-PACU IgM ANTIBODY

Three adult rabbits, New Zealand White inbred, were immunized intramuscularly with 1 mL of a solution containing 500 µg of purified pacu IgM emulsified in FCA (1:1 v/v). Four booster doses of pacu IgM emulsified in FIA at the same concentration were administered at 15-day intervals (Howard and Kaser 2013). The animals were bled from the lateral ear artery every 15 days. The collected blood was allowed to clot at room temperature and then allowed to stand overnight at 4 °C. The serum samples were separated after centrifugation and stored at -20 °C.

WESTERN BLOT ANALYSIS

The specificity and immunochemical analysis of polyclonal rabbit anti-pacu IgM was assessed by western blot (Towbin et al. 1979). Briefly, after SDS-PAGE, the separated protein fractions of whole pacu serum were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, USA) at 350 mA for 1 h. The membrane was blocked with 1.5% ovalbumin in carbonate-bicarbonate buffer for 1 h, incubated with rabbit anti-pacu IgM polyclonal antibodies diluted 1:2000 in PBS-T with 1.5% ovalbumin for 1 h, and washed three times with PBS-T. Antibody binding was detected by adding goat anti-rabbit IgG peroxidase conjugated antibody diluted 1:1000 in PBS-T + 1.5% ovalbumin to the pre-washed membranes. The antigen-protein bands were detected by adding 3,3'-diaminobenzidine-tetrahydrochloride-dihydrate (DAB) peroxidase substrate (Sigma-Aldrich, USA). All steps were performed at room temperature and pre-immunized rabbit and chicken sera were used as controls.

MEASUREMENT OF ANTI-PACU IgM ANTIBODIES BY CHECKERBOARD TITRATION

Detection of rabbit anti-pacu IgM antibodies was performed by indirect ELISA (checkerboard titration) (Crowther 2000). Microtiter plates (96 wells, Corning Costar, USA) were coated with different concentrations of purified pacu IgM (1.0, 0.5, 0.25, 0.125, 0.0625, 0.03 and 0.015 $\mu\text{g mL}^{-1}$) diluted in carbonate-bicarbonate buffer and incubated overnight at 4 °C. The wells were then washed and blocked with 1.5% ovalbumin in carbonate-bicarbonate buffer for 45 min at 37 °C. After blocking, the wells were incubated with a solution of rabbit serum serially diluted (1:500 to 1:16000) in PBS-T + 1.5% ovalbumin for 60 min at 37 °C, washed, and incubated with goat anti-rabbit IgG-peroxidase conjugate (Sigma-Aldrich, USA) diluted (1:1000) in PBS-T with 1.5% ovalbumin

for 2 h at 37 °C. After another washing cycle, the wells were incubated with a solution of substrate/chromogen (hydrogen peroxide - H_2O_2 / ortho-phenylenediamine OPD - Dako, USA) for 15 min and the colorimetric reaction was stopped by adding 2N HCl. All reagents were added at 50 μL /well and the wells were washed four times between each step with PBS + 0.05% Tween-20 (PBS-T). The optical density was read at 490 nm on a Model 550 microplate reader (Bio-Rad, USA).

SAMPLES OF PACU SERUM IMMUNIZED WITH BOVINE IgG

To evaluate the reactivity and the possibility of use the polyclonal rabbit anti-pacu IgM by the indirect ELISA method, pacu serum samples immunized with bovine IgG were collected. Fifty pacu individuals were injected intraperitoneally with bovine IgG (400 μg of bovine IgG) emulsified in FCA and boosted after 15 days with the same dose of antigen emulsified in FIA. Ten control fish received FCA and FIA emulsions prepared with PBS only intraperitoneally. Blood samples were collected by caudal vessel puncture prior to and at 7, 15, 21, 30, and 60 days post-inoculation (dpi), and serum was separated from the blood by centrifugation and stored at -20 °C.

INDIRECT ELISA

Determination of antigen concentration and control sera

A new checkerboard titration was performed by indirect ELISA method to identify the dilution of use of control sera (positive and negative) and antigen concentration (bovine IgG). The anti-bovine IgG antibodies in pacu serum were determined by following the general recommendations of Crowther (2000) and using three different antigen (bovine IgG) concentrations (0.5, 0.25 and 0.125 $\mu\text{g/mL}$) to react with six two-fold serial dilutions of serum from pacu immunized with bovine IgG (1:200 to

1:6400). First, the microplate wells were coated with different dilutions of antigen solution (bovine IgG) and reactions were incubated overnight at 4 °C. The wells were washed as described above and blocked with 1.5% ovalbumin + 10% rabbit serum diluted in carbonate-bicarbonate buffer for 45 min at 37 °C, washed, and incubated with different dilutions of serum samples from immune and non-immune pacu in PBS-T + 1.5% ovalbumin + 10% normal rabbit serum for 1 h at 37 °C. After another washing cycle, the wells were incubated with rabbit anti – pacu IgM (detector antibody) diluted 1:2000 in PBS-T + 1.5% ovalbumin + 10% normal rabbit serum, for 1 h at 37 °C followed by incubation with goat anti-rabbit IgG-peroxidase conjugate (Sigma-Aldrich) at a dilution of 1:1000 in PBS-T+ 1.5% ovalbumin + 10% normal rabbit serum for 2 h at 37 °C. The color development was stopped and the reaction was read as described above.

The optimal concentration for the antigen was determined by the lowest concentration which can demonstrate the positivity of the reaction at any dilution of the positive serum. The optimal dilution of the serum was presented by the greatest difference in reading between the positive and negative serum in the optimal antigen concentration.

Response kinetics of anti-bovine IgG antibodies in immunized pacu

To determine the production kinetics of anti-bovine IgG antibody in immunized pacu, indirect ELISA was performed as described in the previous item using serum collected at 0, 7, 15, 21, 30 and 60 dpi. The dilution of serum use was determined from the results obtained in the previous assay.

RESULTS

CHARACTERIZATION OF PACU IgM

The affinity-purified pacu immunoglobulin revealed on SDS-PAGE had two bands (Fig. 1)

with molecular weights of approximately 72 kDa and 26 kDa, possibly corresponding to the heavy and light chains of pacu IgM, respectively. The protein concentration of pacu serum was 25 mg/mL and that of the purified pacu Ig fraction was 0.204 mg/mL.

WESTERN BLOT ANALYSIS

Western blot analysis showed a strong reaction of rabbit anti-pacu IgM antiserum with two fractions of pacu total serum. One fraction corresponds to a band of approximately 72 kDa and the other to a band of 26 kDa (Fig. 2). The rabbit anti-pacu IgM antibodies did not react with the heterologous serum proteins from chicken and rabbit sera (data not shown).

INDIRECT ELISA

The titration of rabbit anti-pacu IgM antibodies determined by indirect ELISA showed high level of specific antibodies against the purified pacu IgM and a positive reaction was detected up to the highest serum dilution tested (1:16000). In view of this result, the ideal condition of the polyclonal antibody produced in rabbit to be applied in the indirect ELISA was defined dilution of 1: 2000 (Fig. 3).

In addition, the titration of anti-bovine IgG antibodies in immunized pacu showed a dose-response relationship between the antibody concentrations and the optical density (OD) values, e.g., the highest OD values were obtained for the highest concentration of the antigen tested (bovine IgG) and for the lower serum dilutions of immunized pacu (Fig. 5). This enzyme immunoassay has effectively distinguished the positive (bovine IgG-immunized) and the negative (non-immunized) pacu sera up to the dilution of 1:400 and at an antigen concentration of 0.25 µg/mL of bovine IgG (Fig. 4).

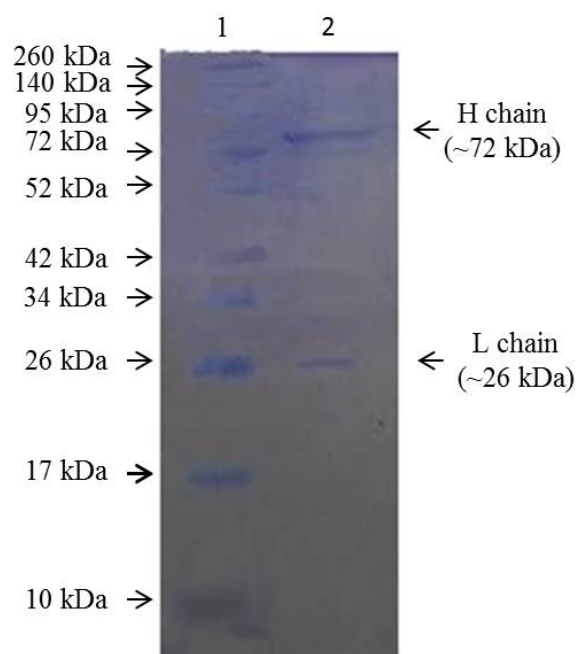


Figure 1 - SDS-PAGE of purified pacu (*Piaractus mesopotamicus*) IgM. Molecular weight marker (lane 1) and purified pacu IgM (lane 2).

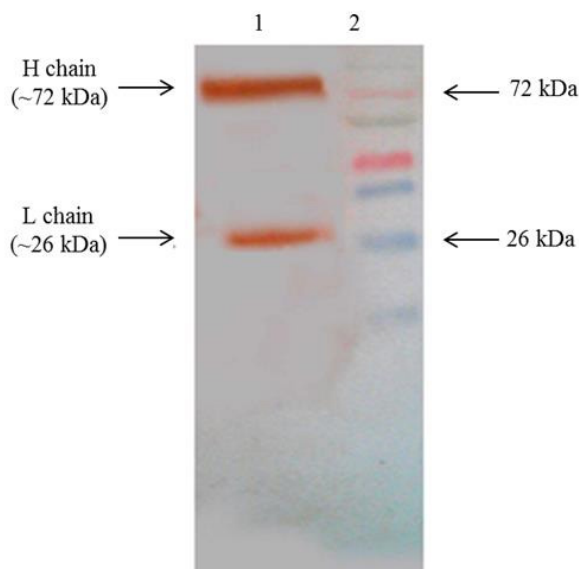


Figure 2 - Western blot analysis of rabbit anti-pacu IgM hyperimmune serum. Pacu total serum (lane 1) and molecular weight marker (lane 2).

Kinetic analysis of anti-bovine IgG antibody production in immunized pacu revealed a sharp increase in antibody response against bovine IgG and the absence of humoral immune response in non-immunized fish in all post-immunization periods analyzed (Fig. 5). There was a gradual increase in the production of anti-bovine IgG antibodies from 7 dpi up to a peak at 30 dpi, followed by a gradual decrease two months after immunization, when the anti-bovine IgG antibody titers returned to the levels detected at 7 dpi.

DISCUSSION

The preparation of a rabbit anti-pacu IgM antibody from the purified pacu IgM fraction is essential to perform immunoassays for detecting and measuring pathogen- and vaccine-induced humoral immune response in this fish species.

Pacu IgM was effectively purified by affinity chromatography with the lectin known as mannose binding protein (MBP). Furthermore, the serum yield of purified pacu IgM using the MBP affinity chromatography column was 0.2 mg/mL, which is within the range for immunoglobulins purified by affinity chromatography (0.02–0.9 mg/mL) in other fish species such as barramundi (*Lates calcarifer*) (Crosbie and Nowak 2002), tuna (*Thunnus maccoyii*) (Watts et al. 2001a), olive flounder (*Paralichthys olivaceus*) (Shin et al. 2007), Nile tilapia (*Oreochromis niloticus*) (Al-Harbi et al. 2000), black rockfish (*Sebastes schlegeli*) (Shin et al. 2006) and guppy (*Poecilia reticulata*) (Lim et al. 2009).

The yield of purified pacu IgM using lectin affinity chromatography is affected by glycosylation levels and the amount of mannose in the Fc regions of these Igs, which differ across fish species (Crosbie and Nowak 2002, Shin et al. 2007). Variations in the mannose content of fish immunoglobulins can also be related to differences in the age/size of animals, environmental conditions, and other factors that affect Ig levels in serum samples (Pucci et al. 2003).

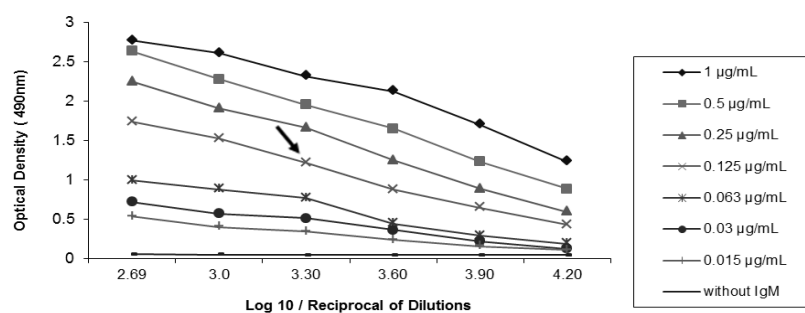


Figure 3 - Titration curves in indirect ELISA of rabbit anti-pacu IgM antiserum that was serially diluted (1:500 to 1:16000) and tested against different coating concentrations (1.0 to 0.015 µg/mL) of purified pacu IgM. The arrow indicates dilution of use for polyclonal antibody (detector 1:2000).

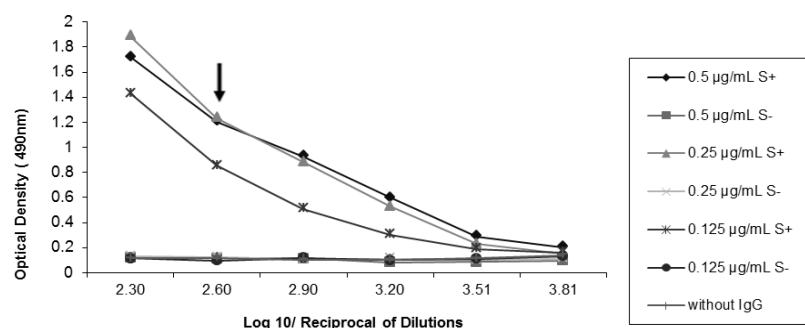


Figure 4 - Titration curves in an indirect ELISA assay sera from bovine IgG-immunized and non-immunized pacus. The pacu sera were serially diluted (1:200 to 1:6400) against bovine IgG antigen tested at different coating concentrations (0.5 to 0.125 µg/mL) and using an anti-pacu IgM polyclonal antibody (1:2000) as detector antibody. S+: Positive serum; S- : Negative serum. The arrow indicate the concentration of use for bovine IgG (antigen) and the dilution of use for pacu serum (1:400).

Moreover, the production of pacu anti-bovine IgG hyperimmune serum, which was added to the MBP column, contributed to the yield of purified pacu IgM. This is supported by our observations that bovine IgG have high immunogenicity for pacu and was able to induce a high production of IgM antibodies in the immunized fish, and similar observations from others who studied purification of immunoglobulins from other fish species (Palenzuela et al. 1996, Shin et al. 2007, Watts et al. 2001a, b).

The analysis of purified pacu IgM by SDS-PAGE revealed the presence of two protein bands of approximately 72 kDa and 26 kDa. These

proteins are within the molecular weight range of heavy and light Ig chains from different teleost fish species, which range from 60–77 kDa and 23–26 kDa, respectively (Bag et al. 2009). The purity of the pacu IgM fraction separated in the MBP column was also confirmed in this study, because SDS-PAGE revealed no protein bands other than purified pacu IgM and no reactivity of rabbit antiserum with other pacu serum proteins was observed by western blotting.

The rabbit anti-pacu IgM antiserum presented high antibody titer against this immunoglobulin in indirect ELISA. In addition, a high specificity for

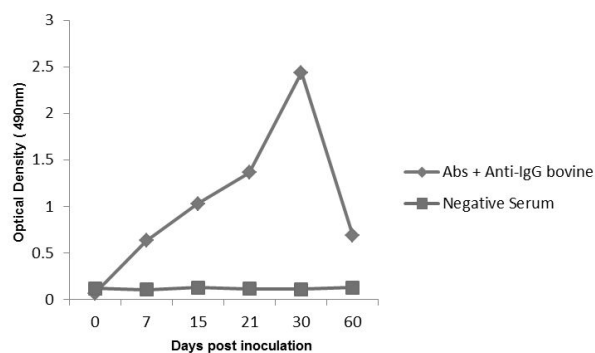


Figure 5 - Response kinetics of anti-bovine IgG antibodies in immunized pacu and non-immunized pacu (negative serum). Dilution of pacu serum (1:400). Data are presented as mean optical density $\pm$ SD (n= 10 per group). dpi: days post-inoculation.

this rabbit antiserum was also observed by western blotting, and reactivity was only detected with two protein fractions, which corresponded to the heavy and light Ig chains of pacu serum.

By using rabbit anti-pacu IgM antiserum as detection antibody, indirect ELISA was optimized and proved to be an effective method for measuring pacu antibodies against bovine IgG, detecting these antibodies at high serum dilution (1:3200) and low antigen (bovine IgG) concentration (0.125 μ g/mL). A similar study reported high titers of guppy IgM antibodies (up to 2560) in guppy fish immunized with *Pseudomonas fluorescens* (Lim et al. 2009). However, Rathore et al. (2008) detected significantly higher antibody titers in carps immunized against *Edwardsiella tarda* antigens (51200) in a study with monoclonal antibodies specific for carp immunoglobulin that used a different ELISA method to monitor humoral immune response. The higher antibody titer in that study may have been due to the higher reactivity of anti-carp Ig monoclonal antibodies, the higher humoral immune response induced by these bacteria, or the higher concentration of bacterial antigens used in the ELISA method.

The kinetics of the humoral immune response in pacu immunized with bovine IgG was similar to that observed in Japanese eels immunized with

goat IgG (Hung et al. 1997) and to the kinetics of antibody production in striped bass immunized with bovine serum albumin (Shelby et al. 2003).

In conclusion, this study provides the first description of pacu IgM purification in conjunction with the production of specific polyclonal antibodies against this purified immunoglobulin, which were successfully used for the standardization and development of an indirect ELISA to measure pacu antibodies. This approach has great potential for the evaluation of the effectiveness of vaccination programs and the development of novel approaches for immunodiagnosis and epidemiological studies of infectious diseases causing relevant mortality and economic losses in pacu systems.

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