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Hemolytic activity of alternative complement pathway as an indicator of innate immunity in pacu (*Piaractus mesopotamicus*)

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ABSTRACT - The objective of this study was to evaluate the methodology to establish the hemolytic activity of alternative complement pathway as an indicator of the innate immunity in Brazilian fish pacu (*Piaractus mesopotamicus*), in addition to verifying the influence of β-glucan as an immunostimulant. Fish were fed with diets containing 0, 0.1 and 1% β-glucan, during seven days, and then inoculated with *Aeromonas hydrophila*. Seven days after the challenge, they were bled for serum extraction. The methodology consisted of a kinetic assay that allows calculating the required time for serum proteins of the complement to promote 50% lysis of a rabbit red blood cell suspension. The method developed in mammals was successfully applied for pacu and determined that the hemolytic activity of the proteins of the complement system (alternative pathway) increased after the pathogen challenge, but was not influenced by the β-glucan treatment.

Key Words: *A. hydrophila*, β-glucan, immune response, pacu

Introduction

The fish immune system is divided into innate or nonspecific defense, the first line of protection against pathogens, and the specific defense, characterized by the immune memory. Regarding the innate system, there are humoral components, such as the complement system, composed of about 35 proteins found in blood in inactive form. These proteins can be activated by three routes: classical pathway, activated by antigen-antibody complex; alternative pathway, activated by molecules of surface microorganisms and by antigen-antibody complex; and lectin pathway, activated by bacterial surface carbohydrate (Holland & Lambris, 2002). The complement system evaluation is widely used as an immune indicator due to its function in the organism defense, such as cellular activation, phagocytosis, chemotaxis, inflammatory reaction and lysis of foreign cells and pathogens (Robertsen, 1999; Bayne & Gerwick, 2001). The alternative pathway activity of complement system can be measured, among other techniques, by the determination of serum hemolytic activity, when this pathway is activated by foreign red blood cells (Yano, 1992). This analysis can be used to evaluate the effects of several factors on the lytic activity of complement system, such as infections, environmental impact and nutrition (Holland & Lambris, 2002).

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The standardization of techniques to measure the immune system indicators is a valuable tool for the assessment of fish organic protection, such as in the case of disease outbreaks caused by *Aeromonas hydrophila*, one of the most important opportunistic pathogens in fish described in Brazil (Godoy et al., 2008).

The expansion of fish farming has drawn the attention to the misuse of drugs as antibiotics (FAO, 2002). An alternative practice is the administration of immunostimulants; one example is β-glucan, which acts by increasing the fish resistance against disease outbreaks (Sakai, 1999; Selvaraj et al., 2005). Regarding pacu (*Piaractus mesopotamicus*), an important farmed Brazilian fish, there is a lack of knowledge of its immune system (Belo et al., 2005; Biller, 2008; Abreu et al., 2009). This study evaluated the methodology to measure the hemolytic activity of the complement system, alternative pathway, as an indicator of the innate immunity in pacu after being experimentally challenged with *A. hydrophila* and fed β-glucan.

Material and Methods

A total of 540 pacu (68.8±14 g and 14.9±0.8 cm) were distributed in 27,200-l tanks (20 fish per tank) with a continuous water and air flow system and constant temperature of 26.07±0.39 °C. Fish were fed with commercial

feed (28% crude protein and 3.600 kcal EB kg^{-1}) during 20 days for acclimatization to the laboratorial conditions; after they were fed with the experimental diets to apparent satiation in two daily meals.

The experimental diets were prepared from a commercial extruded feed that was triturated and into which 0.1% and 1% of β-glucan were added and then pelletized. The control feed was β-glucan-free. β-glucan (Macrogard®) is derived from *Saccaromyces cerevisiae* with 80% activity, according to the manufactory information (Biorigin, São Paulo, Brazil).

Fish received the experimental diets during seven days (three fish per tank, nine per treatment) and were anesthetized (benzocaine, 0.1 g L^{-1}) for blood collection through puncturing of the caudal vessels. After that, fish were weighed and measured. The remaining fish (seventeen fish per aquarium) were anesthetized and inoculated with *A. hydrophila* by intraperitoneal injection. Seven days after the inoculation, the surviving fish of all treatments were sampled as described above. Blood serum of pacu fed with the β-glucan-free diet was used for the standardization of the methodology to establish the hemolytic activity of the complement system, alternative pathway.

The water quality parameters were measured regularly to monitor the temperature, dissolved oxygen, pH (YSI 55 Oximeter) and total ammonia concentration by colorimetric method (Nessler reagent).

The *A. hydrophila* bacterium obtained according to Garcia et al. (2007) was provided by the Laboratory of Fish Pathology at Aquaculture Center of UNESP. Fish were challenged with 1×10^8 CFU mL^{-1}, which is the lethal dose (LD50) that causes 50% of fish death (Plumb & Bowser, 1983). After bacterium injection, fish remained in the aquaria for seven days for observation of clinical signs and mortality and then they were sampled again (three fish per tank, nine per treatment) as described before for determination of the hemolytic activity of alternative complement pathway.

Serum complement hemolytic activity, alternative pathway, was established in accordance with the methodology described by Ferriani et al. (1990) and adapted to pacu serum in this study. Initially, a sample of total rabbit blood was mixed with equal volume of Alsevers solution (anticoagulant pH 6.1), filtered in sterile gauze for withdrawal of suspended material and then stored at 4-8 °C in order to avoid the natural lysis of RRBC. Following, the filtered solution was added to an equal volume of TEA-EDTA chelating buffer (triethanolamine-ethylenediaminetetraacetic acid) 10 nM, pH 7.4 and 0.1% gelatin, incubated for 15 min at 37 °C, and then centrifuged at 2500 rpm for 10 min at 4 °C, to separate the red cells. RRBC were then mixed and washed for three times in TEA-Mg buffer 2 mM, pH 7.4 after successive centrifugations (2500 rpm for 10 min at 4 °C). After this procedure, the RRBC were stored in Alsevers solution at 10 °C up to 15 days. At the moment of RRBC utilization, a portion of the red cells suspension was washed twice in TEA-EGTA buffer (triethanolamine-ethyleneglycol tetraacetic acid) 8 mM with Mg 2 mM and 0.1% gelatin (2500 rpm for 10 min at 4 °C) and then diluted in 1% TEA-EGTA buffer in a cell concentration between 0.8 and 0.9 of optical density, at 700 nm.

The standardization of the methodology was held with pacu serum diluted (1:10; 1:15 and 1:20 of the final volume of 600 µL) in TEA-EGTA 8mM e Mg^{2+} 2 mM with 0.1% gelatin, which consisted of the standard curve. In order to determine the best dilution, the hemolytic activity of alternative complement assay was carried out by the mixture of 200 µL of each diluted serum with 400 µL of the RRBC suspension, prepared previously, and the absorbance was analyzed in spectrophotometer (Beckman DU-70S) at 700 nm for 10 min, at 27 °C. After standard curve establishment, 60 µL of fish serum, diluted at 1:10, were mixed with 140 µL TEA-EGTA buffer and then mixed with 400 µL of the RRBC suspension. This final solution was read in spectrophotometer, as previously described. Serum aliquots were heated for 30 min at 56 °C to provide a negative control of the alternative complement system activity.

The experiment was carried out with six treatments: three β-glucan levels (0, 0.1 and 1%) and two samplings, before and after *A. hydrophila* challenge, with three replications per treatment. Data were submitted to one-way ANOVA. If results were significant, a Duncan Test was applied for means comparisons by SAS statistical program (9.2). Differences between treated and control groups were considered statistically significant at $P<0.05$.

Results and Discussion

The physical and chemical water parameters remained within the values described as appropriate for pacu (Urbinati et al., 2010) with temperature at 26.07±0.39 °C; dissolved oxygen at 5.6±0.5 mg L^{-1}, pH at 7.6±0.09 and total ammonia at 0.23±0.65 mg L^{-1}.

The hemolytic activity of alternative complement pathway of pacu was assessed by a kinetic test, which allows the calculation of time in minutes required for serum proteins promote 50% of lysis (ACP 50 T1/2) of a rabbit RRBC suspension (Polhill et al., 1978; Ferriani et al., 1990). In order to standardize the methodology for fish, several serum dilutions were performed (Figure 1).

After the comparison of the curves obtained with the different serum dilutions, the dilution of 1:10 was chosen due to its effectiveness for determining the time of 50% hemolysis. The highest amount of blood serum promoted the lysis of RRBCs, by reducing the optical density, was faster than the other dilutions. The study confirmed the viability of the method, regularly applied to mammals (Ferriani et al., 1990), for pacu serum and suggest that it can be employed in fish immunological studies.

The static method is widely applied in fish for determination of the hemolytic activity of ACP and it determines the amount of serum tho lyse 50% of RRBC suspension in a known concentration (Yano, 1992). The standardized methodology in this study, however, consists of a kinetic test and differs from the method proposed by Yano (1992), because it determines the time required for serum, in a fixed amount, to lyse 50% of a known concentration of a RRBC suspension.

Comparatively, the hemolytic activity of complement in fish is much higher than in mammals (Yano, 1996), which could indicate either a higher importance of the alternative pathway in this group of animal or a species specific difference in the sensibility of the target molecules (Holland & Lambris, 2002). Another difference between fish and mammals is the temperature required for the complement activation and inactivation (Sakai, 1981). Fish complement has its highest activity at 15-26 °C and is still active at 0-4 °C, while the optimum temperature for mammals complement is 37 °C. In coldwater fish, the complement can be inactivated at 40-45 °C for 20 min (Ingram, 1987), while in warmwater fish, the inactivation of complement occurs between 45 and 54 °C (Sakai, 1981). These temperatures seem to reflect the temperature of fish environment and indicate that the complement system is still functional even in lower temperatures when the acquired immunity is reduced, emphasizing the importance of the innate immune system in fish (Le Morvan et al., 1998).

The ACP 50 hemolytic activity of fish inoculated with *A. hydrophyla* was higher than that of the not challenged fish, indicated by the shortest time for serum proteins to promote ACP 50 lysis through the hemolysis of RRBC, suggesting that this indicator of innate immunity was activated by the bacterium, although the immunostimulant did not influence the parameter measured (Figure 2).

Innate immunity is the first line of defense and includes several humoral components such as lysozyme, complement system, antibodies and other lytic proteins that prevent bacterial adhesion and colonization and consequently, disease outbreaks (Robertsen, 1999). The results obtained with pacu serum indicate that the bacterial challenge promoted the activation of innate immunity parameters, reflected by the highest hemolytic activity of the inoculated fish. The activation of the alternative pathway is triggered by the association of the complement system proteins with polysaccharides of bacteria, fungi and viruses walls (Ellis, 2001). As a consequence, the *A. hydrophila* injection in fish may have led to complement activation due to its bacterium polysaccharides that have facilitated their opsonization to phagocyte and/or lyse the bacterium. In addition, an inflammatory reaction and a consequent increase of the acute phase proteins of inflammation may have occurred, including complement

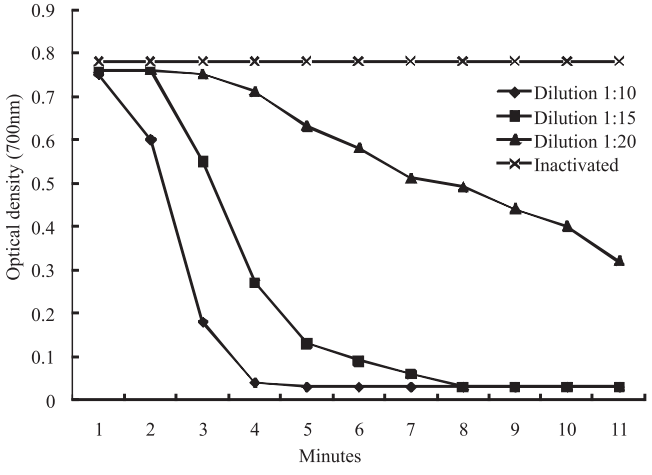

Figure 1 - Standard curve of serum dilution for hemolytic activity of ACP 50 of pacu (*P. mesopotamicus*). The values of T1/2 for each serum dilution are specified.

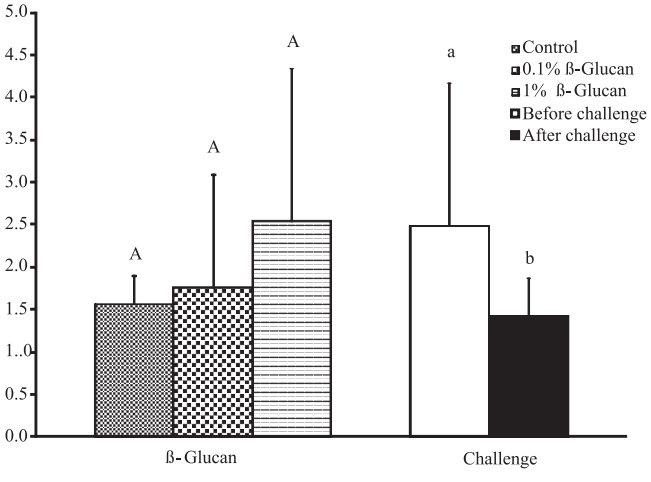

Figure 2 - Hemolitic activity of ACP 50 of pacu (*P. mesopotamicus*) fed β-glucan and challenged with *A. hydrophyla*. Significant differences are indicated by different letters (mean±standard deviation; P≤0.5).

system components, such as C2, B factor, C3, C4, C5 and C9 (Robertsen, 1999).

In this study, the administration of β-glucan did not promote changes in the hemolytic activity of complement system. Different results have been described on the β-glucan influence on immunity of fish. While many authors have observed that β-glucan increases the activity of lysozyme (Paulsen et al., 2001; Sahoo & Mukherjee, 2001; Bagni et al., 2005), alternative complement system (Misra et al., 2006; Siwicki et al., 2009) and respiratory activity of macrophages (Verlhac et al., 1996; Sakai, 1999; Sahoo & Mukherjee, 2001; Cook et al., 2003; Bagni et al., 2005; Selvaraj et al., 2005), others have observed that the immunostimulant does not promote increase in innate immunity, including the complement system (Verlhac et al., 1998; Cook et al., 2003; Selvaraj et al., 2005; Kumari & Sahoo, 2006), as in the present study. The differences found in each study may be explained, in part, by different fish species and protocols utilized (concentrations and administration periods and methods).

Conclusions

The results of this research showed an increase of ACH 50 after *A. hydrophyla* challenge, indicating that the method can be applied as a tool for the assessment of the innate immune response in this species.

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