



Characterization of the spontaneous hemolytic activity of *Rhamdia quelen* (Siluriformes: Heptapteridae) serum

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Abstract. Hemolytic assays are valuable screening tools to evaluate the functional activity of the innate immunity (complement-like) of fish and other animals. The aim of the present study was to evaluate and characterize the spontaneous hemolytic activity of silver catfish (*Rhamdia quelen*) serum. The hemolytic activity was inhibited by ethylenediamine tetraacetic acid (EDTA) and heat which are two classical inhibitors of the enzymatic complement cascade. In addition, the disruption of sheep erythrocytes by silver catfish serum was concentration, temperature, and time-dependent. Inhibition of sheep erythrocyte hemolysis by EDTA could be restored by the addition of excess Ca^{2+} , but not Mg^{2+} . Ethylene glycol-bis-tetraacetic acid (EGTA)-treated serum also showed significant reduced hemolysis against sheep erythrocytes. When red blood cells from different animal species were tested as targets, the highest hemolytic activity was obtained using rabbit erythrocytes, followed by sheep erythrocytes. Lastly, the *in vitro* effect of β -glucans, atrazine and citral on the spontaneous hemolytic activity of silver catfish serum was tested. All agents significantly reduced the hemolytic activity of fish serum. These results should be useful in establishing a simple and standardized assay to evaluate the silver catfish natural immunity.

Key words: fish, immune system, innate immunity, hemolytic assay, red blood cells

Resumo: Caracterização da atividade hemolítica espontânea de soro de *Rhamdia quelen* (Siluriformes: Heptapteridae). Ensaios hemolíticos são ferramentas valiosas de triagem para avaliar a atividade funcional da imunidade inata (sistema complemento) de peixes e outros animais. O objetivo do presente estudo foi avaliar e caracterizar a atividade hemolítica espontânea do soro de jundiás (*Rhamdia quelen*). A atividade hemolítica do soro de jundiás foi inibida na presença de EDTA e pelo tratamento do soro pelo calor, dois inibidores clássicos da cascata enzimática do complemento. Além disso, verificou-se que a capacidade do soro de jundiás em romper eritrócitos de ovinos foi dependente da concentração, temperatura e tempo de incubação. A inibição da hemólise dos eritrócitos de ovinos mediada pelo EDTA foi restaurada pela adição de Ca^{2+} em excesso, mas não de Mg^{2+} . O soro tratado com EGTA também apresentou atividade hemolítica reduzida contra as hemácias de ovinos. Quando diferentes hemácias foram testadas como alvo, a maior atividade hemolítica foi obtida com hemácias de coelho, seguido de hemácias de ovelha. Por fim, foi testado o efeito *in vitro* de vários agentes (β -glucanos, atrazina e citral) sobre a atividade hemolítica espontânea do soro de jundiás. Todos os agentes reduziram significativamente a atividade hemolítica do soro contra as hemácias de ovinos. Estes resultados são úteis no estabelecimento de um sistema de ensaio simples para mensuração da atividade do complemento a fim de avaliar os níveis de defesa natural da espécie.

Palavras-chave: peixe, ensaio hemolítico, hemácias, imunidade inata, sistema imune

Introduction

Innate and adaptive immunity rely on a network of cells and molecules to afford animals with protection to infecting organisms (Inglis *et al.* 2007). The complement system, composed by a large number of soluble molecules, is one of the main effectors of the innate response to pathogens. Complement proteins are found inactive in the blood and other body fluids; however, in response to the recognition of molecular components found on non-self antigens, complement proteins are activated in an enzyme cascade fashion in which one protein enzymatically cleaves and activates the next protein in the cascade (Janeway *et al.* 2001). The complement activation leads to the production of proinflammatory molecules, pathogen opsonization and destruction (Dunkelberger & Song 2010).

The complement system can be activated by three distinct routes designated classical, alternative and lectin pathways. These pathways depend on different molecules for their initiation, but they converge to generate the same set of effector molecules (Janeway *et al.* 2001). Classical serum complement activation requires the interaction of antibody with antigen. In this way, the classical pathway can be envisioned as a link between innate and adaptive immunity. The alternative and lectin complement pathways function independently, without the involvement of immunoglobulin molecules (Inglis *et al.* 2007).

The functional activity of complement can be assessed by hemolytic assays that are valuable screening tools for characterization and identification of deficiencies in the complement activity. The serum complement proteins do not discriminate between membranes of microbes and erythrocytes from other species; thus, hemolytic assays are used to detect and characterize the *in vitro* ability of the serum from one species to destroy red blood cells (RBC) from another species (Azzolini *et al.* 1997, Matheswaran *et al.* 2003, Zhang *et al.* 2003, Merchant *et al.* 2005, Ferronato *et al.* 2009, Merchant *et al.* 2010, Siroski *et al.* 2010, Major *et al.* 2011, Merchant *et al.* 2013), and this can be taken as a parameter to evaluate natural immunity. In the hemolytic assay, when blood serum of certain animal species is mixed with RBCs from other species (donor/target), the complement cascade is activated resulting in the assembly of the membrane attack complexes on RBC surface leading to hemolysis (Merchant *et al.* 2010). However, if the RBC were previously sensitized with specific antibodies, the classical complement pathway is credited with the hemolysis of the RBC, whereas the alternative pathway is generally believed to be involved in the spontaneous

hemolysis (SH) of untreated (unsensitized) RBCs (Lange & Magnadóttir 2003, Moreno-Indias *et al.* 2012).

Silver catfish (*Rhamdia quelen*, Quoy & Gaimard, 1824), is a siluriform species distributed from the South of Mexico to the center of Argentina. It is the most often raised native species in fish cultures in South Brazil, presenting good adaptation to different environments and artificial diets, resistance to handling, and good commercial acceptance (Baldisserotto 2009). In previous studies we demonstrated that several parameters of silver catfish innate immunity might be adversely affected by agrichemicals (Kreutz *et al.* 2011, Kreutz *et al.* 2012) or might be improved by immunomodulatory molecules (Domenico *et al.* 2017). However, several of those assays are laborious and as such prevent a routine analysis of fish immune status. Thus, an easy assay to evaluate silver catfish innate immunity that can be performed on a small blood sample is much needed. Thus, here we set up a series of experiments using the RBC hemolysis assay to characterize the natural hemolytic activity of silver catfish serum and to obtain relevant information to standardize a simple assay to evaluate the innate immunity of this species.

Material and Methods

Fish serum and red blood cells: Twenty silver catfish (number of voucher specimen: UFRGS 14114), approximately 150 g, were obtained and maintained at the Passo Fundo University Fish Farming Facility. They were acclimated for 7 days in continuously aerated tanks (1000 L), with controlled water parameters (water temperature at 22 °C, pH was 7.0-8.0, total ammonia-nitrogen was lower than 0.5 mg L⁻¹, and dissolved oxygen was not less than 6.0 mg L⁻¹). They were anesthetized (eugenol 50 mg L⁻¹) and blood from each fish was collected from the hemal arch in the caudal peduncle using 3-mL syringes (0.7 mm x 25 mm needles). The whole blood was allowed to clot at ambient temperature and then kept on ice up to serum separation by centrifugation (2500 x g for 10 min at 5 °C) and then stored at -80 °C until analysis. Equal volumes of the serum from all fish were pooled so that average values of the species could be obtained (Merchant *et al.* 2013, Merchant *et al.* 2014). Sheep (*Ovis aries*) red blood cells (SRBC), commonly used RBC for hemolytic assays (Costabile 2010, Moreno-Indias *et al.* 2012), were collected with Alsever's solution and kept refrigerated for at least 5 days prior to use.

Sheep red blood cells were washed with phosphate buffered saline (PBS, pH 7.4) several times until the supernatant was clear. Considering that the concentration of hemoglobin in RBC may be different among species or specimens, the washed RBC were prepared based on hemoglobin concentration instead of the percentage of RBC. Thus, for the standardization of the methodology, 100 μL of the SRBCs were lysed with 900 μL of deionized water (total volume 1000 μL) and the optical density (OD) determined at 540 nm. For all assays, the concentration of SRBCs was adjusted so that the final OD of the lysed SRBC (100 μL SRBC + 900 μL deionized water) was equal to 0.5. All hemolytic assays were performed based on studies on the characterization of the fish, reptile and amphibian complement system (Nakao & Yano 1988, Lange & Magnadóttir 2003, Ferronato *et al.* 2009, Siroski *et al.* 2010, Merchant *et al.* 2010, Major *et al.* 2011, Merchant *et al.* 2013, Merchant *et al.* 2014).

Serum hemolytic assay: The assay was performed to detect the total hemolytic activity of silver catfish serum and the effect of two classical inactivators of the serum complement: heat (56 °C for 30 min) and ethylenediamine tetraacetic acid (EDTA) at 5, 10, 20 or 40 mM. Equal volumes (100 μL) of serum and SRBCs were mixed and incubated for 30 min at room temperature (23 °C). Following incubation, the mixture was centrifuged at 2500 $\times g$ for 5 min. Then 100 μL of supernatant was transferred to a microtiter plate, and the OD was measured at 540 nm in a microplate reader (Synergy H1 Multi-Mode Reader). The percent of hemolysis was calculated by comparing between total hemolysis (100%: SRBCs + DI water) and no-hemolysis (0%: SRBCs + PBS) controls, as follows: % hemolysis = $[(A_{540 \text{ sample}} - A_{540 \text{ no-hemolysis}}) / (A_{540 \text{ total hemolysis}} - A_{540 \text{ no-hemolysis}})] \times 100$ (Suttili *et al.* 2016).

Concentration-dependent SRBC hemolysis: For the concentration-dependent hemolytic assay, silver catfish serum was diluted in PBS (10 –100% of serum). Then, 100 μL of each dilution was incubated with 100 μL of SRBCs. The hemolysis assay was performed as described above. A no-hemolysis control (%) was included for each serum dilution.

Time-dependent SRBC hemolysis: After the concentration-dependent assay, several aliquots of silver catfish serum at 10% (10 μL serum + 90 μL PBS) were mixed to 100 μL of SRBCs and incubated at 23 °C. At different time intervals (15 – 120 min) aliquots were removed and the supernatant

used to evaluate the percentile of spontaneous hemolysis as described above.

Temperature-dependent SRBC hemolysis: To determine the temperature-dependency of SRBC hemolysis by silver catfish complement, serum samples and SRBCs were incubated separately at different temperatures (5-45 °C, at 10 °C intervals) for 10 minutes to reach thermal equilibrium. SRBCs (100 μL) and 10% serum were mixed, and incubated at the respective temperatures for 30 minutes, followed by centrifugation. The percent of hemolysis was determined as described above.

Effect of Ca^{2+} , Mg^{2+} and EGTA: To determine the requirement for divalent metal ions (Ca^{2+} and Mg^{2+}), the serum was treated with 5.0 mM EDTA (final concentration, based on the first test). To reconstitute EDTA-depleted serum complement activity, a 10.0 mM CaCl_2 or MgCl_2 solution was added to the fish serum. The serum was also diluted in ethylene glycol-bis-tetraacetic acid (EGTA 10.0 mM), a chelating agent with lower affinity for Mg^{2+} . The serum at 10% concentration was then mixed to 100 μL of SRBCs. The assay was performed as described above (30 min of incubation at 25 °C).

Effect of different RBC donors: Erythrocytes from sheep, rabbit (*Oryctolagus cuniculus*), chicken (*Gallus gallus domesticus*), horse (*Equus caballus*), cattle (*Bos taurus*) and common carp (*Cyprinus carpio*) were evaluated as target cells in the hemolytic assay. The RBCs from each species were prepared as described for SRBCs (0.5 O.D. at 540 nm). Fish serum at 10% was mixed to 100 μL of RBCs (for each species) and then the assay was performed (30 min of incubation at 25 °C) and the percentile of hemolytic activity was determined as described above.

In vitro effect of different binding factors: Silver catfish serum samples were mixed (1:1) with each product solutions prepared with PBS. The products and final concentrations were: zymosan 10 $\mu\text{g mL}^{-1}$ (β -1.3-glucan known as activator of the alternative pathway); Macrogard® 10 $\mu\text{g mL}^{-1}$ (commercial β -1.3/1.6-glucan source used in aquaculture as health promoter); atrazine 1 $\mu\text{g mL}^{-1}$ (an herbicide used in crop production known to cause immunosuppression of fish) and citral 10 $\mu\text{g mL}^{-1}$ (monoterpenoid often present in several plant essential oils). The concentrations used were elected based on studies of Bridle *et al.* (2005), Kreutz *et al.* (2008) and Suttili *et al.* (2016). The mixtures were incubated for 30 min, at 25 °C. The control solution was only PBS. After the incubation period, the hemolytic activity of fish serum was determined using the supernatant of each

mixture separated by centrifugation (2500 ×g for 3 min). Considering the serum dilution when the fish blood was mixed to each product solution, the hemolytic assay was performed using 20 µL serum + 80 µL PBS. The hemolysis was measured and calculated as described previously.

Statistical analysis: Resulting data from all assays were expressed as the mean ± standard error of the mean (SEM) of six replicates. When necessary, the homogeneity of variances between groups was tested with the Levene's test. The one-way ANOVA test was used to determine significant differences among treatments, followed by Tukey's test. All the statistical analyses were performed using the Statistica® software 7.0 (StatSoft Inc., Tulsa, OK, USA). The Sigma Plot 10.0 (Systat Software, San Jose, CA) software was used for regression analysis. The minimum significance level was set at $p \leq 0.05$.

Results

The hemolytic activity was significantly reduced in heat-treated serum (56 °C/30 min) or by adding EDTA to the serum. The hemolytic activity was almost completely depleted with 40 mM EDTA (Fig. 1a). The incubation of SRBCs with fish serum diluted at 10% in PBS resulted in 40% (±6.2%) hemolysis. However, serum at concentrations ranging from 20 to 100% resulted in hemolysis above 90% (Fig. 1b). The kinetic character of SRBC hemolysis by silver catfish serum is displayed in Figure 1c. The hemolytic activity increased rapidly up to 60 minutes (80%). At 90 and 120 min the hemolysis of SRBC was higher than 90%. The temperature-dependence study indicated that the highest hemolytic activity was obtained at 25 °C (Fig. 1d).

The EDTA-inhibition of SRBC hemolysis could be restored, and significantly increased, by the addition of CaCl₂ (10 mM); however, in the presence of an excess of Mg²⁺ (MgCl₂, 10 mM) the SRBC hemolysis was significantly reduced. In addition, 10 mM EGTA reduced the average of spontaneous hemolytic activity of silver catfish serum by approximately 75% (Fig. 2a). Furthermore, the hemolytic activity of the serum varied depending on the target cells used in the assay. The mean hemolytic activity of silver catfish serum was higher when rabbit and sheep RBC were used in the assay and much lower hemolysis was observed when cattle, horse, chicken or carp RBCs were used, either after at 30 or 60 min incubation period (Fig. 2b). Lastly, the effect of various agents such as β-glucans, atrazine and citral on the spontaneous

hemolytic activity of silver catfish serum was tested. All agents significantly reduced the hemolytic activity of silver catfish serum against SRBCs by approximately 40 to 60% when compared to control group (Fig. 2c).

Discussion

The complement system is a major component of vertebrate immune system. In addition to microbial lysis, complement activation generates several inflammatory mediators that attract phagocytic cells to the site of infection (Janeway *et al.* 2001; Holland & Lambris 2002). For aquaculture species, the lysing activity of complement, measured on RBC of another animal species, can be used as an indicator of the ability of the fish to cope with invading pathogen. The ideal condition for *in vitro* assessment of complement activation, however, should be determined for each species. Here, we used different assays with unsensitized SRBCs to characterize the hemolytic activity of silver catfish serum. The hemolytic activity of the serum observed in our experiments was dependent on the complement system as demonstrated by heating the serum or by addition of EDTA, which are known complement inhibitors (Zhang *et al.* 2003; Siroski *et al.* 2010). In addition, activation of the classical complement pathway requires the presence of both Ca²⁺ and Mg²⁺ ions, whereas the alternative system only needs Ca²⁺ ions. Ethylenediamine tetraacetic acid is a potent chelator of divalent metal ions such as Ca²⁺ and Mg²⁺ and can be used to inhibit both pathways (Holland & Lambris 2002).

The hemolytic activity of silver catfish serum was concentration- and time-dependent; for both, the regression analysis showed a line with good fit for a first-order equation. Similar curves were observed for SRBC hemolysis by the serum of the Komodo dragon (*Varanus komodoensis*) (Merchant *et al.* 2012) and crocodilians from Mexico (*Crocodylus acutus*, *Crocodylus moreletii* and *Caiman crocodilus*) (Merchant *et al.* 2014) and Central Africa (*Osteolaemus tetraspis* and *Mecistops cataphractus*) (Merchant *et al.* 2013). At lower concentrations, the complement components cooperativity might not be observed due to reduced contact between the enzymes and, as a consequence, the onset of activity initiates much later (Merchant *et al.* 2012). However, silver catfish serum showed substantial hemolytic activity at the lowest serum concentration tested. Sheep erythrocytes incubated with silver catfish serum diluted to 10% and incubated for 30 min had a hemolysis rate of

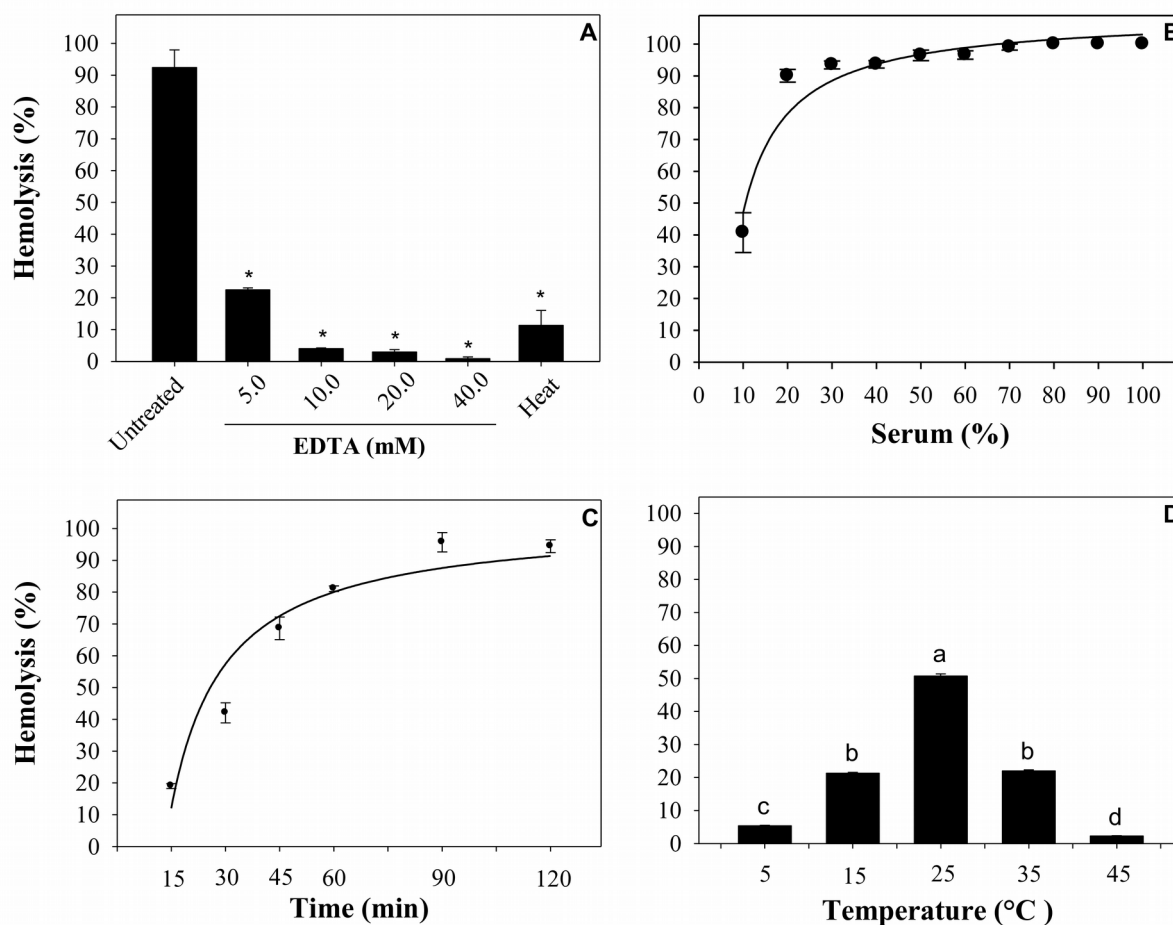


Figure 1. (A) Effects of EDTA and heat (56°C for 30 min) treatment on the hemolysis of sheep red blood cells (SRBC). (B) Concentration-dependent hemolysis, (C) Time-dependent hemolysis and (D) Temperature-dependent hemolysis of SRBCs by silver catfish serum. The results represent the means $\pm$ standard error of mean. Concentration- and time-dependent analysis fit to a first order equation: B – $y=109.2+(-622.4/x)$; C – $y=102.6+(-1359.2/x)$. * indicate significant difference related to untreated group and different letters indicate a significant difference between treatments (ANOVA and Tukey's test, $p \leq 0.05$).

approximately 40%; this serum dilution was then elected for the following tests.

Because fish are ectothermic vertebrates, their biochemical and physiological processes are largely affected by external temperatures. One important physiologic activity influenced by environmental temperatures is the serum complement system (Siroski *et al.* 2010). The mean water temperature in South Brazil, where silver catfish is mostly cultured, ranges within 14-28 °C (Garcia *et al.* 2008) and the upper lethal temperature for this species is 34.5 °C (Chippari-Gomes *et al.* 1999). The hemolytic activity of silver catfish serum was significantly reduced at 35 °C and almost inactivated at 45 °C. The thermal analysis showed that the SRBC hemolysis was maximal at 25 °C and silver catfish best zootechnical performance occurs at temperatures around 24 °C (Piedras *et al.* 2004).

Mammalian complement function activation is known to require both Mg^{2+} and Ca^{2+} , while the complement activation in more ancient vertebrates appear to require either Ca^{2+} or Mg^{2+} (Major *et al.* 2011; Merchant *et al.* 2012). Ethylenediamine tetraacetic acid, a known Ca^{2+} and Mg^{2+} chelator, reduced the hemolytic activity of silver catfish serum. In addition, because the classical pathway requires Ca^{2+} , only the alternative pathway should be active using EGTA, a Ca^{2+} chelator with lower affinity for Mg^{2+} (Moreno-Indias *et al.* 2012). In our study, however, a similar reduction in hemolysis was observed in serum treated with either EDTA or EGTA, and the EDTA-inhibition of SRBC hemolysis was restored by the addition of Ca^{2+} , but not Mg^{2+} , indicating specificity for Ca^{2+} ions.

Similar effect of EGTA on fish serum, as observed in our study, was also verified in other fish

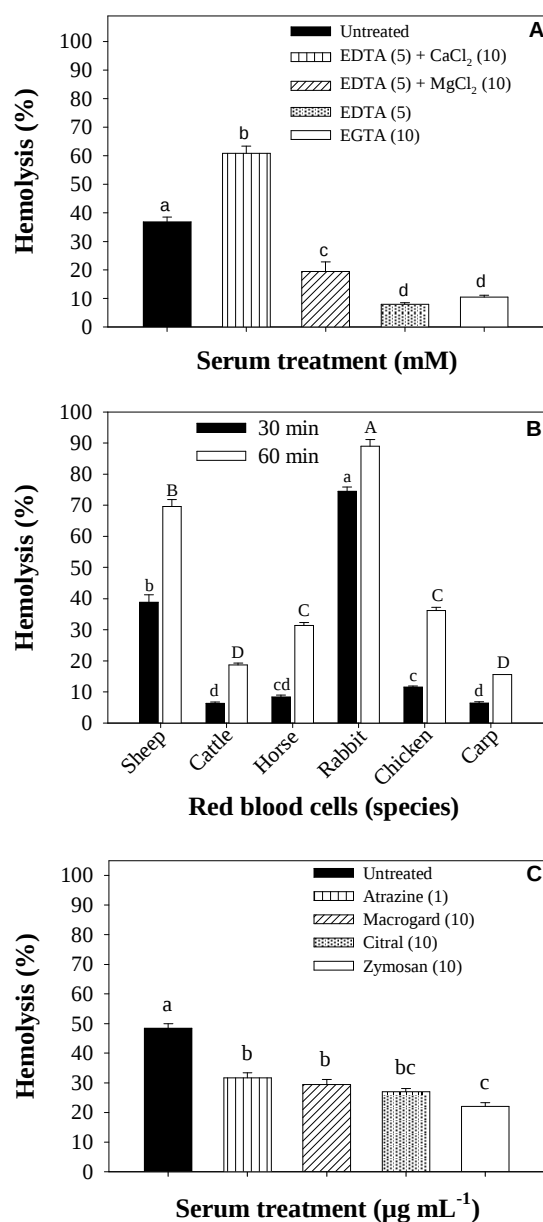


Figure 2. (A) Effect of the ions Ca²⁺ and Mg²⁺ and EGTA on the hemolysis of sheep red blood cells (SRBCs) by silver catfish serum. (B) The ability of silver catfish serum to lyse erythrocytes from different animal species. (C) *In vitro* effect of different agents on the hemolysis of SRBCs by silver catfish serum. The numbers within brackets indicate the concentration of the molecules. The results represent the means $\pm$ SEM. Different letters indicate a significant difference between treatments (ANOVA and Tukey's test, $p \leq 0.05$).

species. Ethylene glycol-bis-tetraacetic acid at 10 mM reduced the mean spontaneous hemolytic activity of *Hippoglossus hippoglossus* serum by approximately 70% and 1 mM EGTA reduced the mean hemolytic activity of *Dicentrarchus labrax*

serum by approximately 75% (Lange & Magnadóttir 2003). Ingram (1987) also reported reduced hemolytic activity of *Salmo trutta* serum against unsensitized sheep RBC after EGTA treatment. However, Magnadóttir (2000) reported no effect of EGTA (up to 10 mM) on the spontaneous hemolytic activity of *Gadus morhua* serum against SRBCs. In addition, in the same study, EDTA, which binds both Ca²⁺ and Mg²⁺ and should inhibit both the classical and alternative pathways, enhanced the hemolytic activity.

The hemolytic activity of the serum varied depending on the target cells used. All RBCs tested activated the hemolytic activity of silver catfish serum. A higher mean hemolytic activity was observed when rabbit and sheep RBCs were used in the assay, indicating that these cells are more sensitive to the natural hemolytic activity of silver catfish serum than the RBCs of the other species used in our study. Higher hemolytic activity against sheep and rabbit RBCs was also found with *Hippoglossus hippoglossus* serum, however, *Dicentrarchus labrax* serum had higher mean hemolytic activity with rabbit RBC and relatively lower activity with sheep and chicken RBCs (Lange & Magnadóttir 2003). Sheep, rabbit and chicken RBCs are good target cells for different fish species (*Ictalurus punctatus*, *Ictiobus cyprinellus*, *Carassius auratus*, *Lepisosteus osseus* and *Amia calva*) (Legler et al. 1967). The poor sensitivity of carp RBC to silver catfish serum could be explained by the closer evolutionary relationship between the two fish species. However, once similar low activity was found for mammals (cattle and horse) and chicken, there is no clear relationship between the phylogeny of the target cells and degree of hemolysis.

The effect of various agents on the spontaneous hemolytic activity of silver catfish serum was tested. All tested agents significantly reduced the hemolytic activity of silver catfish serum, although not completely. Zymosan and MacroGard are β -glucans primarily known as complement activators via the alternative pathway. Once activated, complement components are consumed, showing the reduced hemolysis found in the *in vitro* test. *In vivo*, these agents are known as fish immune enhancers (Vetvicka et al. 2013) even for silver catfish (Pavan et al. 2016; Domenico et al. 2017). Some molecules are not known as specific complement inhibitors but may inappropriately activate and consume the complement system. Atrazine is an herbicide used in crop production. This herbicide, when added to the fish water, had no effect on the natural complement

hemolytic activity in silver catfish after 24 h or 10 days of exposure (Kreutz *et al.* 2012). However, in our *in vitro* study, atrazine reduced the hemolytic activity of silver catfish serum against SRBCs. Similar inhibition was found for citral, a monoterpene often present in several plant essential oils. Also, *Cymbopogon flexuosus* essential oil (citral-rich) at 16 µg mL⁻¹ mixed to whole blood of *Sciaenops ocellatus* significantly inhibited the complement activity after 1 h of exposure (Suttili *et al.* 2016).

Taken together, the results presented in this study show that silver catfish serum exhibits potent and rapid hemolytic activity. The hemolytic activity reported here has features common to the complement system of other fish and reptiles, occurring in a volume-, time- and temperature-dependent manner. Sheep and rabbit RBCs appear as the best options for hemolytic assays. Our results provide meaningful information for the standardization of hemolytic tests for the species and are important to better understand the immune system of silver catfish and other native fish species from Brazil.

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