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Hematological and immune changes in *Piaractus mesopotamicus* in the sepsis induced by *Aeromonas hydrophila*

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ABSTRACT

The pathogenesis of sepsis involves complex systems and multiple interrelationships between the host and pathogen producing high mortality rates in various animal species. In this study, hematological disturbances, innate immunity and survival during the septic process in *Piaractus mesopotamicus* inoculated with *Aeromonas hydrophila* were studied. For this aim, fish blood samples were taken from control and infected groups 1, 3, 6, and 9 h post-inoculation (HPI). Leukogram showed reduction in the number of leukocytes and thrombocytes, followed by cessation of leukocyte chemotaxis 6 HPI and severe morphological changes in leukocytes and erythrocytes. At 3 HPI production of reactive oxygen species increased and at 6 HPI decreased. There was no change in serum lysozyme concentration and lytic activity of the complement system, despite the progressive increase in serum lytic activity and bacterial agglutination. Finally, the changes in clinical signs due to aeromonosis and increasing septicemia resulted in a reduction in survival to 57.14% after 36 HPI. It was possible concluded that these hematological and immune are crucial event in the worsening of sepsis in *P. mesopotamicus*, and these findings are utility for diagnosing and understanding the pathophysiology sepsis in pacu induced by *A. hydrophila*.

1. Introduction

Gram-negative sepsis is triggered by the activation of the immune and endothelial cells by infectious agents [1] mainly when endotoxins (LPS) are contacted to host monocytes and macrophages [2]. This response is complex, exacerbated and uncontrolled that leads to high rates of morbidity and mortality [3,4] altering inflammation response, hematological profile and morphology of leucocytes [5].

Unlike endothermic animals, ectothermic vertebrates are tolerant to endotoxic shock [6]. However, endotoxins are responsible for the pathogenicity of Gram-negative bacteria in several fish species [7,8]. *Aeromonas hydrophila* is an ubiquitous Gram-negative pathogen that causes extensive economic losses especially in intensive fish farming worldwide. This bacteria cause septic outbreaks with high mortality rates in several animal species and poses a serious threat to public health [9].

Piaractus mesopotamicus is a fish endemic to Parana-Paraguay Basin, and it is currently one of the most widely cultivated fish species in South America. It also has a great potential for aquaculture due to its characteristics [10,11]. In this context, it was studied the hematological disturbances, innate immunity and survival of fish during the evolution of the septic process in *Piaractus mesopotamicus* inoculated with *Aeromonas hydrophila* a potent immunogenic and has high lethality in this species of fish.

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2. Material and methods

2.1. Fish

A total of 144 pacus, *P. mesopotamicus*, from a commercial farm were used (180.71 ± 38.1 g/21.27 ± 6.7 cm), acclimatized during seven days in 18 fiber boxes (n = 8/250 L), filled with artesian well water (1 L/min flow) and constant aeration. Fish were fed with commercial diet (3% of biomass, 28% of crude protein and 4000 kcal of crude energy kg^{-1}) [11]. The study was carried out according to the Brazilian animal welfare standards and ISO - International Organization for Standardization (2006) and was approved by the Ethics Committee (protocol nº 008577/12). Water quality remained within the comfort range for fish throughout the experiment (DO = 5.2 mg/L; T = 28.87 °C; pH = 7.51 and electrical conductivity = 118.20 µS/cm) [12].

2.2. Bacteria

Aeromonas hydrophila (Accession number: CP007518-2) was isolated from naturally infected fish that presented clinical signs and lesions compatible with aeromonosis [13,14]. After euthanasia by deep anesthesia in 1: 10.000 aqueous solution of benzocaine. Fish were necropsied and tissues of brain, kidney and blood were aseptically sampled for bacteriological culture. The initial identification of colonies was performed according to Popoff [15] and Abeyta Junior et al. [16], supplemented by biochemical proofs made by means of the commercial kit Bactray (Bactray 3 - Laborclin®), according to the manufacturer recommendations.

For genetic characterization, the bacterial mass derived from pure colonies culture was submitted to a DNA extraction process, according to the manufacturer suggested methodology (Genomic DNA purification kit - Wizard®). DNA concentration was 1690.9 ng/µL and absorbance ratio 260/280 and 260/230, varying between 2.02 and 2.04. After obtaining the DNA, ribosomal gene 16 S RNA was amplified according to Sarkar et al. [17]. Sequences were analyzed by the BLAST algorithm (http://www.ncbi.nlm.nih.gov), which presented 99% similarity with *A. hydrophila*.

The pathogenicity of strain of *A. hydrophila* (Accession number: CP007518-2) was verified by three serial passages (n = 2/passage) and re-isolation in pacus, which were inoculated with 1.0 mL of bacterial suspension (2.4×10^8 CFU/mL) by coelom via. After the third passage, the bacteria re-isolated from fish tissue underwent the same tests of microbial and molecular confirmation described above.

2.3. Lethal dose (LD 50–96 h)

Forty-eight pacus (*P. mesopotamicus*) were stocked in six tanks (n = 8/250 L) in a completely randomized design. Forty of these fish were inoculated with 0.5 mL of increasing concentrations of *A. hydrophila*, corresponding to tubes 5 to 9 of the McFarland standards (T1 = 1.5×10^8; T2 = 1.8×10^8; T3 = 2.1×10^8; T4 = 2.4×10^8; T5 = 2.7×10^8 bacterial cells per mL) in the coelomic cavity. The control group animals received the same volume of sterile saline solution (0.65%).

During the exposure (96 h) the feed was suspended and the tanks were not siphoned. Fish that presented irreversible clinical signs were killed by deep anesthesia. The LD 50–96 h values were calculated by the "Trimmed-Sperman-Karber" method, and were 1.8×10^8, lower and upper limit at 1.64×10^9 and 2.3×10^9.

2.4. Induction of sepsis and collection of blood

Fish were anesthetized (benzocaine solution 1: 20,000, in alcohol 98°, 0.1 g/mL) [18] until the surgical plane [19]. The animals were divided into two groups (completely randomized design), one received 0.5 mL of sterile 0.65% saline (control group) and the other received 1.8×10^8 CFU/mL (corresponding to LD50%) dissolved in the same vehicle and volume per animal.

Samples were taken at 1, 3, 6, and 9 h post-infection (HPI) of both groups by puncture of the caudal vessel (3.0 mL blood/sample). An aliquot (2.0 mL) with heparin (10 U/mL) was used for hemogram, quantification of reactive oxygen species (EROS), immunofluorescence, and bacterial quantification. Another aliquot was centrifuged at 1200 g, for 5 min at 4 °C, to obtain the serum that was stored at - 80 °C.

2.5. Blood count, bacterial quantification and total serum proteins

Blood samples were stained with May-Grünwal-Giemsa-Wright method to carry out white blood cells and thrombocytes counts [20]. RBC and blood measurement indices were performed according to the direct methods of Tavares-Dias et al. [20] and Garcia et al. [13], respectively.

An aliquot of whole blood from each fish was used for quantification of *A. hydrophila* from blood culture at different times. Thus, 20 µL of whole blood were seeded in tryptic soybean agar (TSA), with ampicillin (10 mg/L) in duplicate and incubated for 24 h at 30 °C. Finally, the count was performed in bacterial colony counter.

Total protein concentrations (Biuret method) and albumin (green bromocresol method) were determined in the serum samples. Globulin concentrations were determined by subtracting the albumin concentration from the total proteins, and then the albumin/globulin ratio was calculated [21].

2.6. Analysis of innate immunity

2.6.1. Leukocyte respiratory burst

Using blood sampled from the caudal vein at the different evaluation times, the following were determined the respiratory burst, by means of turbidometric assay using nitroblue tetrazolium (NBT) to quantify the reactive oxygen species from blood leukocytes [22].

2.6.2. Anti-LAMP1 immunolabeling in peripheral blood cells by immunofluorescence: determination of leukocytes

Blood samples were collected with heparin (10 U/mL) and processed according to Brinkmann et al. [23] with modifications. Briefly, blood was centrifuged in Falcon tubes, containing similar quantity of 3 mL of Histopaque® −1119 and −1077 (Sigma Chemical Co., St. Louis, Mo.) sterile-filtered at 700G for 30 min at room temperature (25 °C). Then the layer of cells formed between the columns of Histopaque was collected and centrifuged again two times at 200 g for 10 min. Thus, a pellet was obtained (95% of the cells were leukocytes). In order to verify the vitality of these cells, the method of exclusion by staining with trypan blue solution followed by hemocytometer counting was used. Immediately, a pellet of 9×10^5 leukocytes was placed in glass slides [23]. Then, incubated with 2% Bovine serum albumin BSA (Gibco®) for 30 min at 37 °C with the primary antibody.

Samples were incubated overnight with the primary antibodies diluted in 1% BSA: rat polyclonal to LAMP1 antibody-Lysosome Marker (1: 200 anti-LAMP1, Abcam, ab24170). Then 5 washes of 5 min each were performed with PBS and slides incubated with the secondary antibodies for 1 h: donkey anti-rabbit Alexa 488 (1: 1000, Jackson Research Laboratories). For negative control, the primary antibody was omitted in the protocol. The coverslips were mounted on the slides with Fluoromount G medium (Electron Microscopy Sciences Cat. # 17984–25) and sealed with formalin-free enamel for observation under fluorescence microscopy (Leica TCS SP5- Leica Microsystems).

2.6.3. Serum lytic activity

First, it was performed a previous assay with the aim of standardize the growth of the bacterial strain and determine the colony count dilution factor between 30 and 300 CFU/plate. Six dilutions of *A.*

hydrophila were carried out in buffered peptone water, pH 7.2 (1.8×10^9 CFU, 1:10, 1: 100, 1: 1000, 1: 10000, 1: 100,000), seeded in TSA, added ampicillin (10 mg/L), and incubated for 24 h at 28 °C.

After the previous assay, it was established the dilution in 1: 100,000 in peptone water (pH 7.2) of the *A. hydrophila*. The bacteria (10^6 CFU) were washed and then, 50 μL of the *A. hydrophila* and 50 μL of serum were mixture and incubated for 1 h at 28 °C. Then, the suspension (100 μL) was seeded in TSA plates, added ampicillin (10 mg/L) and incubated for 24 h at 28 °C. For the reference group, 50 μL of sterile saline was mixture with 50 μL of the A. *hydrophila* suspension. Results were expressed in CFU were performed.

2.6.4. Alternative pathway activity of complement system: hemolytic activity

The standardization of the calibration curve of complement activity was done by means of 8 increasing dilutions of serum in 8 mM TEA-EDTA buffer and 2 mM Mg2+ with 0.1% gelatin (1: 2.3; 1: 3, 1: 4, 1: 5, 1: 9, 1: 12.3, 1:19). Then, added 40 μL of the red cell suspension, and reading at 700 nm, at 37 °C (SpectraMax M5 Microplate Reader®). As a negative control, a serum aliquot was heated at 56 °C for 30 min, followed by the same steps described above. The results were expressed using the variation of absorbance (ΔD initial – ΔD final) [24].

2.6.5. Determination of serum lysozyme concentration

Serum lysozyme concentration was determined by turbidimetric assay according to Marzocchi Machado et al. [25] and adapted by Castro et al. [22].

2.6.6. Direct agglutination

Using serum and plasma, the antibody concentration was determined by erythrocytes agglutination according to Biller-Takahashi et al. [26]. The results were expressed as $\log^2$ of the reciprocals of the serum titers.

2.7. Evaluation of clinical signs and survival analysis

Fifty-six pacus were used to evaluate clinical signs and survival. Fish were divided into 2 groups, one inoculated with 0.5 mL of *A. hydrophila* suspension (1.8×10^9 CFU), and the other one injected with the same volume of sterile saline (control). The evaluation was performed by direct observation for 5 days after the induction of sepsis.

2.8. Statistical analysis

It was performed analysis of variance in a completely randomized design (Kolmogorov-Smirnov, Anderson-Darling, Shapiro-Wilk and Watson), comparison means through Tukey test ($p < 0.05$) or Dunn's ($p < 0.05$) were compared. Survival followed the Kaplan-Meier method [27]. R software was used as statistical program for the calculations.

3. Results

3.1. Blood count and total serum proteins

The evaluation of blood components of septic pacus was divided into erythrogram, leukogram and total plasma proteins. In the erythrogram was verified that at 9 HPI the mean globular volume increased ($p < 0.05$) in relation to control group. For others erythrocyte parameters analyzed, it was not observed differences ($p > 0.05$; Table 1).

Lymphocytes, monocytes, granulocytes (neutrophils and special granulocytic cells), thrombocytes, and eosinophils were observed on examination of the blood extensions. Eosinophils were not counted due to they were only present in the blood extensions of four fish.

In the leukogram, the inoculated animals showed a significant reduction in the number of leukocytes at all sampling times in relation to the control group ($p < 0.05$). Reduction in the number of lymphocytes and granulocytes 1 and 3 HPI ($p < 0.05$) and in the number of thrombocytes 3 HPI ($p < 0.05$) (Table 1).

In blood extensions, severe morphological changes were identified in erythrocytes, thrombocytes and leukocytes in septic pacus (Fig. 1), mainly anisocytosis, poikilocytosis, hypochromia, and binucleosis. It was also observed several immature cells with polychromasia and intense cytoplasmic basophilia. These changes were not present in the leukocytes of the control group.

3.2. Evaluation of innate immunity and bacterial quantification in blood

The evaluation of the innate immunity variables and the bacterial quantification in the blood during *A. hydrophila* induced sepsis are presented in Fig. 2. Anti-LAMP1 immunolabeling of peripheral blood leukocytes granules was positive for all groups challenged and control, and negative for negative control group. However, the concentration of reactive oxygen species (ROS) present in the blood leukocyte granules was higher ($p < 0.05$, Fig. 2A) 3 HPI comparing to 6 HPI and the control group. In this last time, it was observed the lower production ($p < 0.05$) in relation to the other groups. The lytic activity of the serum proteases was higher 6 and 9 HPI, differing statistically ($p < 0.05$) from the reference group and from 1 HPI group (Fig. 2B and C).

The activity of the membrane attack complex by activation of the alternative complement pathway and serum lysozyme concentration between the control group and the inoculated group at all times evaluated was similar ($p > 0.05$) (2D and 2E figures). The agglutination capacity of pacus antibodies against *A. hydrophila* increased ($p < 0.05$) at 3 HPI compare to control group (Fig. 2F).

The inoculation of the bacteria in the coelomic cavity allowed its dissemination in the bloodstream of the fish, as shown in Fig. 3G, confirming sepsis in the blood samples from the different groups, which was higher ($p < 0.05$) 6 and 9 HPI in relation the other sampling times and control group.

3.3. Evaluation of clinical signs and survival

After 9 HPI, infected fish presented petechiae and suffusions on the dorsal surface of the body near the inoculation site. After 24 HPI, it was observed systemic clinical signs of aeromonosis such as cutaneous bleeding, ulcerations, fins wounds, and lethargy. Others fish died without clinical signs.

Control group presented 100% of survival during the observation (5 days). The inoculated group presented survival of 85.71%, during the first 12 h, of 71.42% and 57.14% after 24 and 36 h, respectively (Fig. 3).

4. Discussion

Sepsis was induced by inoculation of *A. hydrophila* into pacus. This was evidenced by the hematological and immune changes, and the positive blood culture in blood. The cumulative effect of these changes led to clinical signs and decreased survival of fish. These data are similar to those found in different species of teleosts afflicted by Gram-positive and Gram-negative infections [8,28]. However, it was observed that at least 30% of the inoculated animals were resistant and/or tolerant to *A. hydrophila* infection.

This resistance/tolerance in Gram-negative infection is only observed in endotoxemia and not sepsis or septic shock. This hypothesis is based on the application of ultrapure LPS by the activation of toll-like receptor 4 (TLR4) inducing the innate immune response and releasing of proinflammatory mediators in teleosts [7,29]. LPS are potent inducers of cyclooxygenase-2 (COX-2) expression and proinflammatory cytokines in rainbow trout. The lipid portion of endotoxin that is

Table 1
Hematology and plasma proteins levels of *Piaractus mesopotamicus* after bacteria inoculation.

Variáveis	Groups				
	Control	1 HPI	3 HPI	6 HPI	9 HPI
TPP (g/dL)	3.8 ± 0.1A	3.3 ± 0.1A	3.4 ± 0.2A	3.5 ± 0.09A	3.7 ± 0.2A
Albumin (g/dL)	0.9 ± 0.03A	1.01 ± 0.03A	1.02 ± 0.03A	1.05 ± 0.02A	1.09 ± 0.03A
Globulins (g/dL)	2.8 ± 0.07A	2.4 ± 0.08A	2.6 ± 0.1A	2.5 ± 0.08A	2.6 ± 0.2A
A/G Ratio	0.35 ± 0.01A	0.41 ± 0.02A	0.4 ± 0.03A	0.41 ± 0.01A	0.41 ± 0.03A
Hct (%)	34.5 ± 3.6B	34.3 ± 1.7B	34.1 ± 2.7B	36.1 ± 1.5AB	38.6 ± 2.8A
Hb (g/dl)	10.5 ± 1.2A	10.6 ± 0.9A	10.2 ± 1.3A	10.2 ± 1.5A	11.4 ± 2.1A
RBC ($x10^6/mm^3$)	1.5 ± 0.1A	1.4 ± 0.2A	1.5 ± 0.2A	1.3 ± 0.3A	1.5 ± 0.2A
MCV (μm^3)	214.3 ± 28.8A	234.1 ± 43.3A	243.8 ± 31.3A	239.3 ± 39.1A	250.5 ± 28.2A
MCH (pg)	65.4 ± 9.3A	73.1 ± 16.1A	73.7 ± 13.3A	67.7 ± 14.5A	73.8 ± 13.7A
MCHC (%)	31.3 ± 5.9A	31.1 ± 2.9A	30.1 ± 2.4A	28.3 ± 4.1A	29.5 ± 4.7A
Total Leukocytes ($\times 10^3 \mu L^{-1}$)	7.8 ± 2.9A	4.8 ± 1.6B	3.2 ± 1.1B	4.5 ± 2.2B	4.5 ± 2.3B
Lymphocytes ($\times 10^3 \mu L^{-1}$)	1.7 ± 0.8A	0.6 ± 0.4B	0.8 ± 0.4B	1.4 ± 0.5A	1.1 ± 0.9A
Monocytes ($\times 10^3 \mu L^{-1}$)	1.3 ± 0.7A	0.4 ± 0.3B	0.6 ± 0.4A	0.7 ± 0.4A	0.8 ± 0.3A
Granulocytes ($\times 10^3 \mu L^{-1}$)	1.2 ± 0.7A	0.6 ± 0.4B	0.8 ± 0.6B	1.8 ± 1.5A	1.4 ± 1.2A
Thrombocytes ($\times 10^3 \mu L^{-1}$)	3.4 ± 2.1A	3.1 ± 1.4A	0.9 ± 0.7B	0.8 ± 0.7B	1.1 ± 0.7B

[1] Values (means ± SD) with different letters compare treatments in the column ($P < 0.05$). TPP: Total plasma protein; A/G: Albumin/globulin ratio; Hct: Hematocrit; Hb: Hemoglobin; RBC: Red blood cell count; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

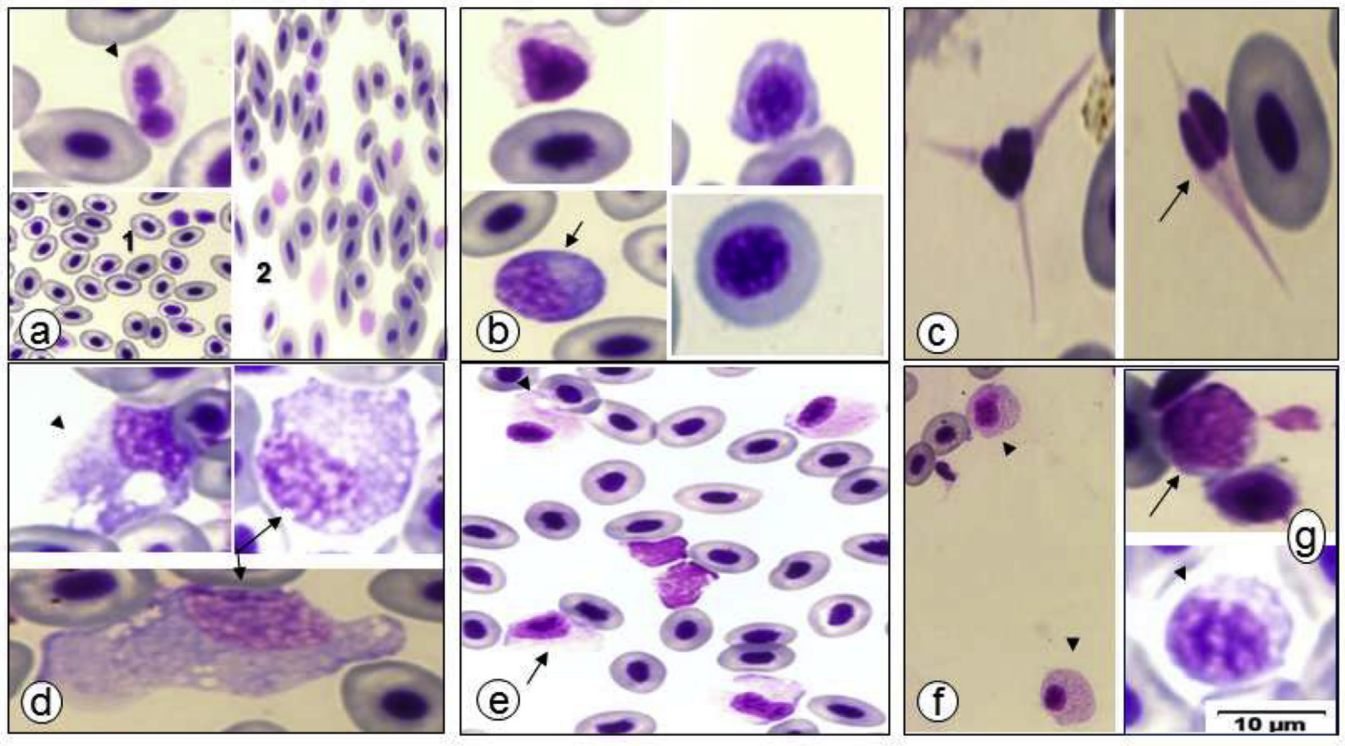

Fig. 1. Morphological abnormalities of pacus blood cells inoculated with *Aeromonas hydrophila*. (a) Binucleate erythrocyte (arrowhead), hypochromia (1) and anisocytosis (2); (b) immature cells with poikilocytosis and basophilia (arrow); (c) Thrombocytes with binucleation (arrow); (d) monocyte with different degrees of vacuolization (arrow) and toxic changes (arrowhead); (e) granulocytes with different forms, light vacuolization (arrowhead) and toxic changes (arrow); (f) phagocytic activity in granulocytes (arrowhead); (g) basophilic (arrow) and vacuolized (arrowhead) lymphocytes. May Grünwald-Giemsa-Wright stained. Bar = 10 µm.

responsible for the activity in mammals, did not induce expression of IL-1 or IL-6 in the same study [6].

In contrast, the results of this study demonstrate that *A. hydrophila* is a potent immunogenic and has high lethality, developing host responses immediately after inoculation, with reduction in the number of leukocytes in blood from 1 HPI. This effect is likely due to the massive release of proinflammatory mediators by leukocytes and endothelial cells that occur within the initial 30 min of infection inducing the expression of adhesion molecules [30].

In teleosts, such as pacu, proinflammatory mediators induce the accumulation of cells in the inflammatory focus [31]. Nevertheless, it was observed in the analysis of the kinetics of blood leucocytes of septic pacus, that first, fish presented blood leucopenia and finally, return to basal levels of monocytes, lymphocytes and granulocytes. This suggests a greater activity of replacement of these cells from reserve blood depots [32].

Decrease in neutrophil migration may be due to TNF-alpha and IL-8 mediation as observed in laboratory rodents [33] and/or due to the reduction of leukocyte-endothelial adhesion caused by nitric oxide [34]. It is not possible to say which mechanisms modulate leukocyte

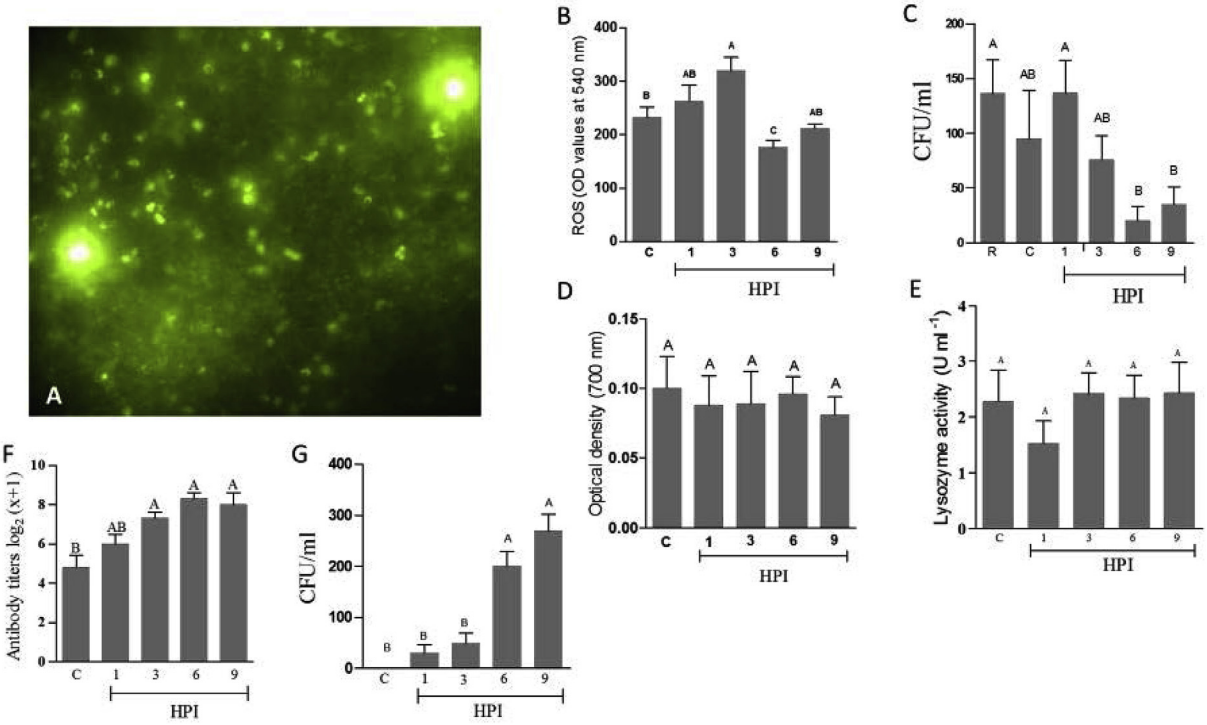

Fig. 2. Evaluation of innate immunity and bacterial quantification of peripheral blood. (A) Anti-LAMP1-immunolabeling in leukocytes by immunofluorescence. (B) Concentration of reactive oxygen species (ROS) in blood leukocytes. (C) Lithic activity of serum. (R) Reference group - saline solution (0.65%) + *A. hydrophila*; Control - serum of control animals + *A. hydrophila*. (D) Alternative pathway activity of complement system: hemolytic activity of pacus serum ΔDO. (E) Quantification of serum lysozyme concentration. (F) Values of natural antibody titers. (G) Bacterial quantification in peripheral blood. * Vertical columns represent means of each group in the sampling times. Vertical bars represent the standard error of the mean. Columns with letters in common do not differ by the level of 5% by Tukey test (B, D, C and F) and Dunn (C and G). HPI - hours post-inoculation; C - control group; CFU - colony forming unit.

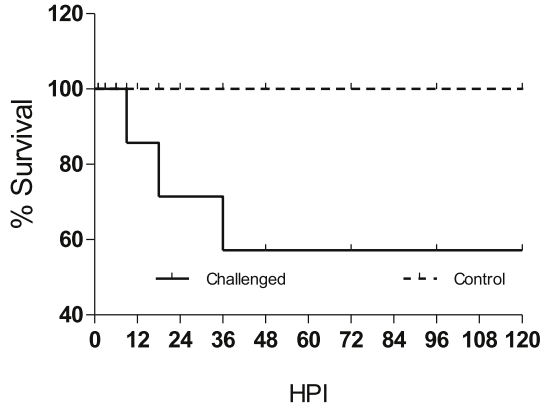

Fig. 3. Survival of *Piaractus mesopotamicus* after induction of sepsis by *Aeromonas hydrophila* and control group.

migration due to the complexity of the sepsis process [30,33]. However, these events were crucial for clinical signs and pacu survival similar to the observed in laboratory rodents [34].

As in this study, Garcia and Moraes [14] observed similar clinical signs, reduction in the number of total leukocytes, lymphocytes, and eosinophils, and increased numbers of neutrophils and monocytes in pacus. In both studies, this leukocytes reduction favored bacterial spread as verified by the increase of the bacterial spread in blood. The reduction in the number of blood leukocytes showed an unfavorable prognosis for pacus related to reduction of survival, as occurs in human patients [35].

In the current study, the morphological alterations identified in erythrocytes, leukocytes and thrombocytes in blood extensions were associated with the acute inflammatory response [36], positive blood culture [37] and increased neutrophil activity [38]. It is probable that bacterial products and chemical mediators present in blood activated leukocytes, producing more ROS with vacuolization and toxic lesions facilitating bacterial spread.

Labeo rohita after stimulation with different levels of *Escherichia coli* LPS, increased ROS, myeloperoxidase and lysozyme concentrations at the lowest concentrations of LPS and decreased in the higher concentrations of LPS [7]. The kinetics of ROS production in *Dicentrarchus labrax* macrophages stimulated with LPS and activated by phorbol-12-myristate-13-acetate (PMA) demonstrated that the maximum release occurred up to 3 HPI, followed by a decrease [39]. The results of this assay corroborated both observations regarding the peak time of leukocyte ROS release and increased bacterial spread, followed by a decrease in the number of leukocytes in blood and ROS production.

Serum bacterial lysis is explained to the bactericidal action of substances present in serum released mainly by leukocytes [40,41]. The maximum values of serum lytic activity were observed at 6 and 9 HPI, following by a decrease of leukocyte chemotaxis. However, there was no difference in the serum lysozyme concentration, pathway alternative and classical (there was no lytic activity of pacus serum) complement pathway. Thus, the rise of serum bacterial activity was likely due to other bactericidal enzymes present in serum, leukocyte granules [41], and/or due to activation of the complement system by lectin pathway [42].

The bacterial agglutination activity in this study can be attributed the production and release of IgM [43], which even in normal conditions occurs rapidly [44] and presents immediate action on bacteria and virus [45]. The increase in serum bacterial agglutination activity observed presented similar results with those found in hybrid surubim (*Pseudoplatystoma corruscans x P. fasciatum*) after experimental infection by *A. hydrophila* [46].

Serum protein remained unchanged during the time evaluated

showing that the analysis of total proteins has little influence on *A. hydrophila* sepsis in pacus. However, it disagrees with some results where proinflammatory cytokines stimulate the release of total plasma proteins and increase the complement and coagulation system activity [1,14]. The unchanged serum proteins and complement system can be explained due to both leukocyte dysfunction and hepatic injury (data not shown).

The decrease of thrombocytes 3 and 9 HPI can be explained in part by the consumption of these cells by the disseminated intravascular coagulation that accompanies sepsis [47]. Thrombocytopenia, increased hematocrit and petechiae on the body surface suggest a coagulation disorder [4,10]. It was not possible to determine prothrombin, activated partial thromboplastin and serum fibrinogen due to the formation of fibrin in citrated plasma, consequently the clotting did not form in the blood coagulometer during these analyzes.

The results of this work suggest that the inoculation of *A. hydrophila* in the coelomic cavity of *P. mesopotamicus* caused leucopenia, thrombocytopenia, reducing chemotaxis and phagocytosis that facilitated bacterial dissemination. These alterations added to the morphological changes identified in blood cells with unchanged complement system and serum lysozyme concentration led to reduction of survival to 57.14%, despite the increase in lithic activity and bacterial agglutination in pacus serum. It was possible concluded that pacu is susceptible to sepsis and septic shock and that these hematological and immune are utility for diagnosing and understanding the pathophysiology sepsis in pacu induced by *A. hydrophila*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.044.

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