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RESEARCH ARTICLE

Acute Phase Response in Buffalo Calves Experimentally Infected with *Salmonella typhimurium*

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ABSTRACT

The aim of this study was to evaluate the changes that occur in the acute phase response (APR) of experimentally-induced salmonellosis in buffalo calves and to identify potential indicators of infection. Five buffalo calves received 10^9 colony-forming units of *Salmonella typhimurium*, orally. Another five animals were used as control group. Clinical examinations were performed and venous blood was sampled before and throughout 7 days after inoculation. Red blood cell count (RBC), packed cell volume (PCV), hemoglobin (Hb), plasma fibrinogen, serum haptoglobin, ceruloplasmin, transferrin and iron were measured. Severe diarrhea started 72-108 h after inoculation, in all animals. The infectious stimulus induced severe APR, characterized by pyrexia, lymphopenia ($4.99\pm1.73x10^9$ cells/L), increase in fibrinogen (24.7±4.91 µmol/L), haptoglobin (21.5±8.65 µmol/L) and ceruloplasmin (1370±374 mg/L), decreased transferrin concentration (3.90±0.69 g/L) and hypoferremia (15.2±11.2 µmol/L). These changes coincided with the onset of clinical signs. Alterations of lower intensity occurred in erythrogram. Based on the magnitude and duration of changes, it is suggested that the combined measurement of serum levels of iron and haptoglobin is a useful tool for identifying newborn buffaloes recently affected by bacterial disease and for monitoring the effectiveness of its treatment.

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INTRODUCTION

The highest mortality rates in buffalo herds are found until six months of age, most of these losses result from diarrhea (Fagiolo *et al.*, 2005; Zaman *et al.*, 2006), 25% of deaths are caused by *Salmonella* spp., where *S. typhimurium* is the most frequently isolated serovar (Borriello *et al.*, 2012; Kanwal *et al.*, 2015). In buffalo neonates, infection with *Salmonella* spp. in addition to acute diarrhea can cause, fever, anorexia, dehydration, sepsis and sudden death (Fagiolo *et al.*, 2005).

In diseases accompanied by inflammation, including infectious diseases, the host organism applies a defense mechanism, which starts a few hours after infection and is part of the innate immune response of the patient. This defense mechanism, called APR, is mediated by cytokines (IL1, IL6, TNFα), and results in a set of systemic changes, including alterations in leukocyte counts and in levels of acute phase proteins (APPs), which favor the restoration of homeostasis (Cray *et al.*, 2009). Also part of the APR is the activation of a mechanism for restricting the availability of iron, in which the APPs transferrin, lactoferrin, ferritin, hepcidin, haptoglobin and ceruloplasmin are involved (Schaible and Kaufmann, 2004). This series of changes that occur during inflammation also affects the erythrogram. The so-called anemia of inflammation (AI) can occur, among other situations, during bacterial, viral or fungal infections. This condition develops as a result of reduction in iron availability and inhibition of erythropoiesis, mediated by cytokines (Roy, 2010).

APPs are very useful in the diagnosis and prognosis of diseases and are being studied as potential biomarkers in various veterinary clinical situations (Eckersall and Bell, 2010). In cattle calves, salmonellosis causes an increase in serum levels of haptoglobin, ceruloplasmin, acid glycoprotein, and hypoferremia (Silva *et al.*, 2011). However, there are few studies on APPs in sick buffaloes (El-Deeb and Iacob, 2012; Tajik *et al.*, 2012; Kumar *et al.*,

2014), specially with simultaneous assessment of ferremic response (Horadagoda *et al.*, 2002).

The aim of this study was to evaluate the APR in buffalo calves affected by salmonellosis, verifying the usefulness of its components as indicators of the infection.

MATERIALS AND METHODS

Ethical standards: This research was approved by the Ethics Committee on Animal Use of Faculdade de Ciências Agrárias e Veterinárias, UNESP (protocol number: 004929/10).

Animals and experimental groups: 10 healthy Murrah buffalo calves (first month of life; average weight of 52 kg) were used in the experiment. Calves were obtained from commercial herds in the state of São Paulo (Brazil), after colostrum ingestion. The animals were housed in individual suspended shelters and fed with cow's pasteurized fresh milk, commercial feed, hay and water *ad libitum*.

Calves were randomly distributed into two experimental groups: control (n=5), that received 10 mL of Brain Heart Infusion (BHI) broth, and infected (n=5), that received 10^9 colony-forming units (CFU) of *Salmonella typhimurium* suspended in 10 mL of BHI broth.

***Salmonella typhimurium*'s inoculum preparation:** Inocula for induction of experimental infection were prepared from a *S. typhimurium* (IOC record: 6333/06) sample originally isolated from feces of infected cattle during an outbreak of salmonellosis and archived at Laboratory of Enterobacteria (FIOCRUZ, Rio de Janeiro, Brazil).

Inocula were prepared according to Fecteau *et al.* (2003). The concentration of colonies/mL was determined by Miles and Misra technique (1938). Each calf received orally approximately 10^9CFU, using a sterile syringe.

Feces collection and bacteriological isolation: Feces samples were collected with the aid of sterile swabs, in triplicate, directly from the rectum of all calves. The samples were collected immediately before inoculation (Day 0) and then daily, until the seventh day after inoculation (Days 1-7).

The isolation of *S. typhimurium* in feces samples was performed according to the recommendations of Santos *et al.* (2002) with some modifications. The swabs were incubated (24h/37°C) in tubes containing Rappaport-Vassiliadis broth (CM0866, Oxoid), selenite-cystine broth (CM0699, Oxoid), Muller-Kauffmann tetrathionate broth (CM0343, Oxoid). Subsequently, an aliquot of each selective enrichment broth was placed on agar XLT4 (223420/235310, BD Difco) containing nalidixic acid (50 µg/mL), and incubated at 37°C/48 to 96 hours. Colonies with the genus *Salmonella*'s characteristic morphology were subjected to biochemical tests (TSI, CM0277, and LIA, CM0381, Oxoid). After the biochemical proof, a slide agglutination test was performed (Probac Brazil).

Physical examination: Calves were subjected to clinical examination immediately before inoculation, and thereafter, twice daily over seven days after experimental infection or until the clinical regression of the disease (absence of fever and diarrhea).

Collection and analysis of blood samples: Blood samples were taken at Day 0 and Days 1-7 by puncture of the jugular vein using a vacuum collection system. Blood samples were collected into siliconized plastic tubes (containing EDTA) and tubes without anticoagulant. The PCV, RBC, Hb concentration and WBC were assessed using automatic device (pocH-100 iV Diff, Sysmex). Fibrinogen content was determined by the heat precipitation method (Millar *et al.*, 1971).

Concentration of serum total proteins (Biuret method) and iron (modified method of Goodwin) were evaluated using commercial kits (Labtest Diagnóstica) and semiautomatic spectrophotometer (Labquest, Labtest Diagnóstica). Serum concentrations of APPs were obtained by Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE), proposed by Weber and Osborn (1969). Protein fractions were determined by video densitometer (CS-9301PC, Shimadzu Corporation).

Statistical analysis: For data analysis Minitab 16 software was used and generalized linear model was applied with repeated measures, considering the group factor between subjects and time factor within subjects. When there was statistically significant effects ($P<0.05$), means were compared by the Tukey's test.

RESULTS

The control group did not shed *Salmonella* spp. in feces throughout the experimental period. In all calves that received the *S. Typhimurium* inoculum, the first bacterium isolation occurred between 24 and 72 hours after inoculation, and the fecal shedding of the agent was detectable for up to 16 days post-infection (PI). *S. typhimurium* samples isolated from rectal swabs showed identical antimicrobial susceptibility patterns to that of the sample used in inoculum preparation.

The signs of diarrhea began between 72 and 108 hours after infection in all inoculated animals. All infected calves had severe diarrhea with yellowish feces, mucus, blood and sometimes shreds of mucosa and fibrin. Clinical cure of the enteritis occurred spontaneously between 8 and 14 days PI in all animals. The median duration of diarrhea was 8 days. No animal was severely dehydrated. At 3 and 4 days PI, one of the infected animals showed reduced appetite. There were no apathy, respiratory signs or evidence of sepsis.

The infected group showed an increase in mean rectal temperature (Fig.1) in the morning and afternoon, reaching a maximum value in the afternoon of the day 3 PI ($P=0.0007$). In this group, the highest individual recorded temperature was 40°C in the morning of day 3 PI. In the control group, temperatures declined during the morning period ($P=0.0394$, day 4 PI). There were statistically significant differences between groups in the morning of day 1 PI ($P=0.0018$).

Animals in the infected group showed no significant difference in relation to their baseline values (day 0 PI) with respect to the PCV (Fig.2a), the RBC (Fig. 2b) and hemoglobin concentration (Fig. 2c).

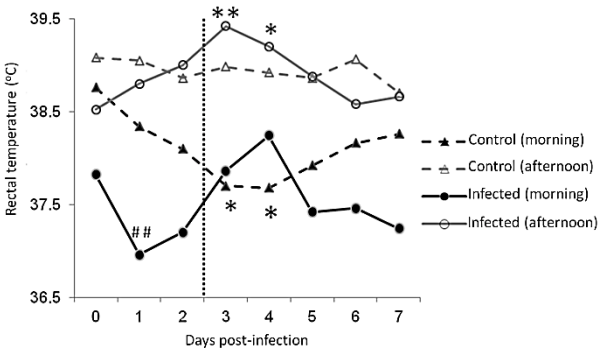

Fig. 1: Mean values of rectal temperature of buffaloes calves uninfected (control) and infected with 10^9CFU of *S. typhimurium* before inoculation (day 0) and over 7 days post-infection. Results of rectal temperature in the morning and afternoon were analyzed separately. The dotted vertical line indicates the onset of diarrhea episodes in animals of the infected group. Asterisks indicate statistically significant differences from the respective baseline value (Day 0) (*P<0.05; **P<0.01). Number signs indicate statistically significant differences from the value obtained in the control group at the same time (#P<0.05; ##P<0.01).

Leukocytes and segmented neutrophils counts were not statistically affected. However, it is possible to observe in Fig. 2d-e that, in the infected animals, these counts increased until days 2 and 3 PI, and then declined until the end of the trial. Following the mature neutrophils counts, the band neutrophils counts (Fig. 2e) in this group increased by 612% in day 3 PI. The number of lymphocytes (Fig. 2f) in the infected group decreased significantly in day 3 PI, with significant difference from the values obtained prior to inoculation (P=0.0277) and in the control group (P=0.0040). Then, the values increased, returning close to baseline.

APPs concentrations - fibrinogen, haptoglobin, ceruloplasmin and transferrin - had changes in diseased animals, as described below (Fig. 3a-e). Fibrinogen levels (Fig.3a) increased after inoculation with *S. typhimurium*, with a maximum value in day 5 PI, significantly higher than that obtained in the control group (P=0.0053). However, this increase was not evidenced by statistical analysis when the mean values were confronted with the one obtained prior to inoculation.

Regarding serum haptoglobin levels (Fig. 3b), there was an increase in the *Salmonella*-challenged animals from day 3 PI until the end of the trial. The highest average was recorded on day 6 PI and it was 476% higher

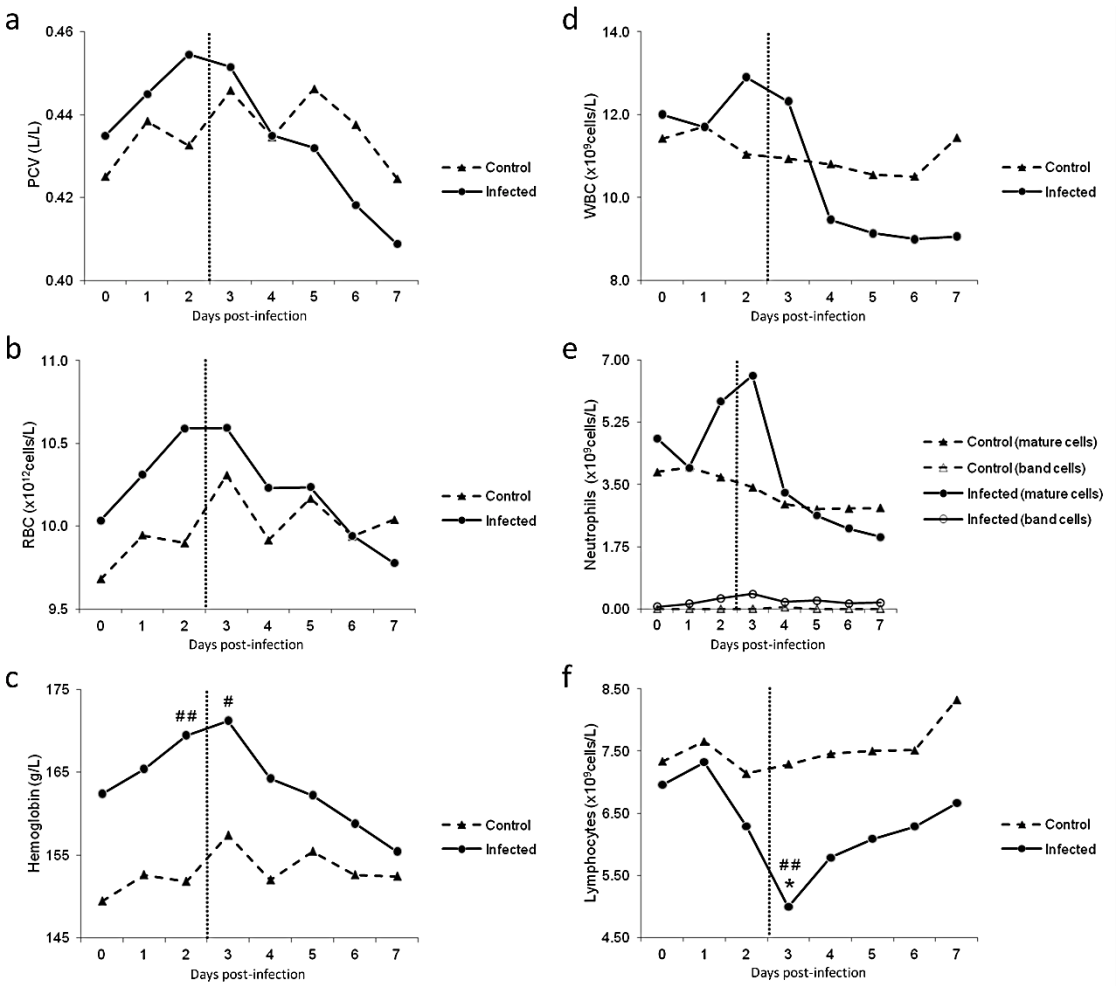

Fig. 2: Mean values of PCV (a), RBC (b), hemoglobin (c), WBC (d), neutrophils (e) and lymphocytes(f) in buffaloes calves uninfected (control) and infected with 10^9CFU of *S. typhimurium* before inoculation (day 0) and over 7 days post-infection. The dotted vertical line indicates the onset of diarrhea episodes in animals of the infected group. Asterisks indicate statistically significant differences from the respective baseline value (Day 0) (*P<0.05; **P<0.01). Number signs indicate statistically significant differences from the value obtained in the control group at the same time (#P<0.05; ##P<0.01). Band neutrophil counts were not statistically analyzed.

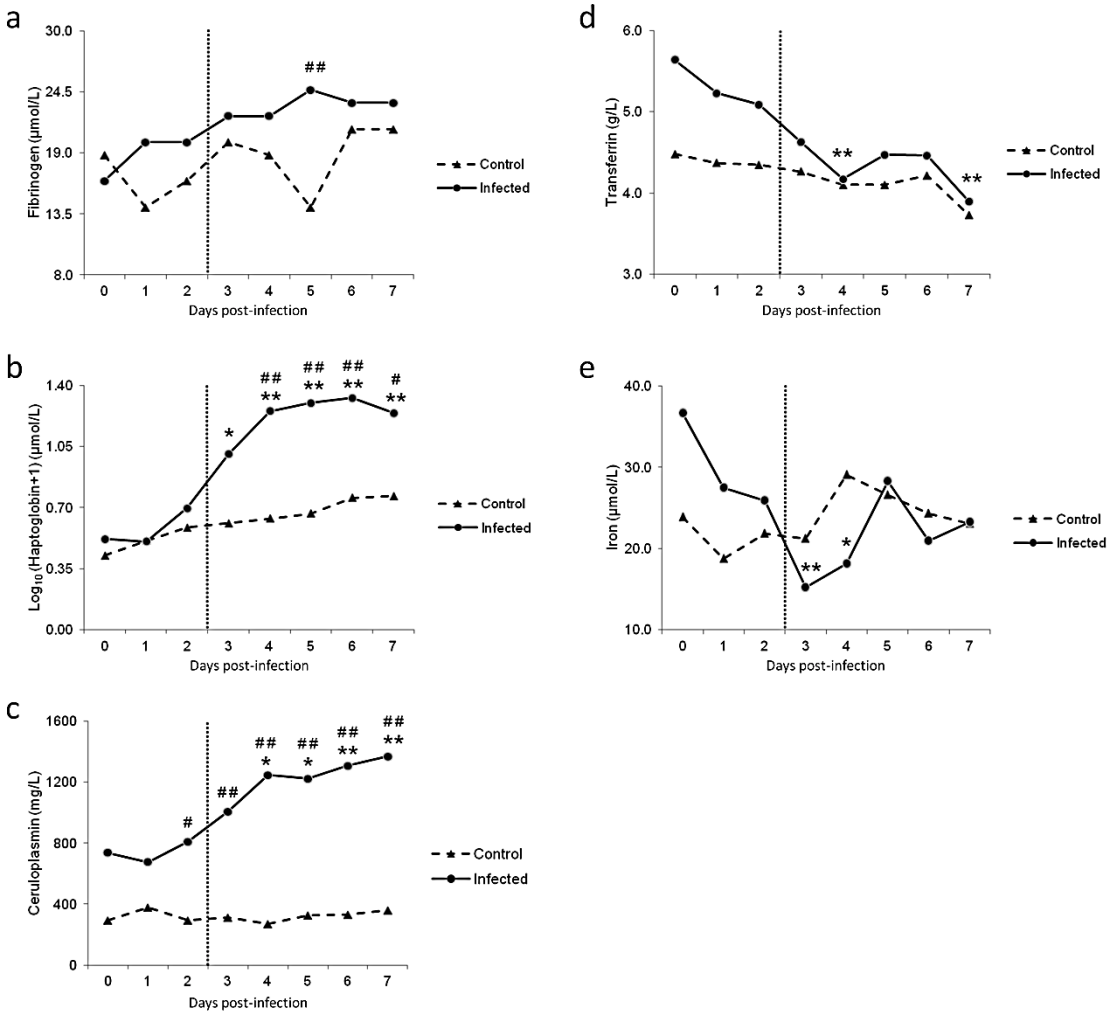

Fig. 3: Mean plasma fibrinogen concentration (a), and mean serum concentrations of haptoglobin (b), ceruloplasmin (c), transferrin (d) and iron (e) in buffalo calves uninfected (control) and infected with 10^9CFU of *S. typhimurium* before inoculation (day 0) and over 7 days post-infection. The dotted vertical line indicates the onset of diarrhea episodes in animals of the infected group. Asterisks indicate statistically significant differences from the respective baseline value (Day 0) (*P<0.05; **P<0.01). Number signs indicate statistically significant differences from the value obtained in the control group at the same time (#P<0.05; ##P<0.01).

than that obtained before inoculation. On that day, it was found a statistically significant difference when compared to day 0 PI (P=0.0000). Likewise, in sick animals there was an increase in ceruloplasmin level (Fig.3c), starting on day 2 PI, when there was a significant difference between groups (P=0.0106). This condition persisted until the end of the study. On the last day, there was an average ceruloplasmin content 85% higher than baseline (P=0.0006).

Transferrin concentration (Fig.3d) was reduced in infected animals throughout the trial. This reduction was statistically significant on days 4 (P=0.0088) and 7 PI (P=0.0008).

Concomitantly with increases in haptoglobin and ceruloplasmin levels and decrease in transferrin level, serum iron concentration (Fig.3e) decreased dramatically after *S. typhimurium* inoculation. Serum iron concentration was reduced in 58% in day 3 PI(P=0.0081).

DISCUSSION

The experimental model used, with inoculation of 10^9CFU of *S. typhimurium*, was effective in inducing acute enteritis. The infectious stimulus caused an APR, whose components were detected as soon as the diarrhea started.

Clinical signs were similar to those described in experimental models with cattle using *Salmonella typhimurium* serovar (Fecteau *et al.*, 2003; Ávila *et al.*, 2011; Srinivasan *et al*., 2015). As well as in buffalo calves, these authors also reported predominantly enteric signs, although these signs took place earlier in cattle than in buffalo calves. Moreover, Fecteau *et al.* (2003) also reported deaths in cattle due to the infection, which did not happened with the buffalo calves. It must be considered that factors other than animal species and *Salmonella* spp. serovar influence the severity of the infection, as the virulence of the strain, infectious dose, geographical region, age and immune status of the host. The animals were not severely dehydrated thanks to adequate fluid replacement, as they ingested large amounts of water voluntarily.

Salmonella has LPS, which trigger an immunological cascade that leads to fever. In infected animals, there was an increase in rectal temperature, just on days 3 and 4 PI. Buffaloes have their body temperature strongly influenced by environmental conditions, since the low sweat gland density hampers thermoregulation. This explains the

higher mean values in the afternoon when compared to the morning.

The initial increase in segmented neutrophil counts occurred as an inflammatory response to intestinal infection. Consumption of these cells caused a regenerative bone marrow response, which generated significant increase in the number of circulating band neutrophils. Santos *et al.* (2002) describe a similar condition in bovine calves. Although neutrophilia with a left shift in the acute phase of bacterial infections occurs (Morris, 2009), many calves with salmonellosis have leukopenia with neutropenia when severely affected. This happens because there is an intense migration of circulating neutrophils to the intestinal mucosa and the marrow granulocyte reserve is small (Santos *et al.*, 2002). That is what happened in this study, beginning from day 4 PI. Another finding was the marked decrease in lymphocyte count observed on day 3 PI, corroborating Morris's statement (2009) that severe bacterial infections may be the cause of lymphopenia.

Fibrinogen and haptoglobin, which are the main APP in ruminants, are good indicators of inflammation in buffaloes (Khan *et al.*, 1997). In this study, the increase in fibrinogen concentration was progressive, but slower than that of other APPs, reflecting its delay of a few days as acute phase reactant (Gruys *et al.*, 2005). On the other hand, haptoglobin was the APP that showed most consistent changes. As described by Deignan *et al.* (2000), the significant increase in haptoglobin levels occurred within 3 days of infection, when clinical signs started, and got worse as all animals presented diarrhea (day5 PI). This APP binds to free hemoglobin in the blood, preventing the loss of iron and making it unavailable to bacteria (Cray *et al.*, 2009). In cattle, serum haptoglobin increase was positively correlated with the severity of clinical signs of salmonellosis (Deignan *et al.*, 2000; Silva *et al.*, 2011). These findings indicate that haptoglobin is a useful non-specific marker of *Salmonella* spp. infection in ruminants.

There was also an increase in ceruloplasmin concentration from day 2 PI. This protein oxidizes the iron that was mobilized out of the cell and facilitates its incorporation into transferrin (Schaible and Kaufmann, 2004). Therefore, its concentration in the blood is inversely related to the iron levels. This hypothesis is supported by the fact that, in this study, the largest increases in ceruloplasmin levels occurred after the marked fall in ferremia, in an attempt to reestablish it.

Transferrin, the iron transporting protein involved in its intestinal absorption and cell internalization (Schaible and Kaufmann, 2004), is a negative APP in most animal species (Murata *et al.*, 2004). Thus, as observed in this study, its levels fall progressively during salmonellosis. This reduction is still unclear. It has been suggested that hepatic synthesis of transferrin is reduced to minimize intestinal iron absorption, or its degradation by the reticuloendothelial system is increased to capture the iron bound to this protein (Jurado, 1997).

Modification of hepatic synthesis of APPs is directly linked with the drastic decrease in ferremia observed on day 3 PI. The downward trend in iron concentration could already be observed before the onset of diarrhea. The complete reestablishment to baseline levels was not observed, since the infection remained (as shown by bacteriological analysis). However, after 48 hours of low levels, they began to be restored, confirming that hypoferremia is limited to the acute phase of infection.

In the erythrogram, it was noted that PCV, RBC and Hb tended to increase in the first days of infection. This indicates the hemoconcentration caused by the mobilization of fluids into the intestinal lumen. The decline that followed in those three parameters reflects the fecal blood loss and the impaired erythropoiesis resulting from hypoferremia, insufficient erythropoietin production, decreased responsiveness of the erythroid precursors to erythropoietin and reduction in RBC survival (Jurado, 1997). The hemoconcentration may have masked the presence of anemia. This phenomenon is particularly important in neonates.

Conclusions: Clinical and laboratory results showed a strong and durable APR to *S. Typhimurium* infection and results suggest that the combined measurement of haptoglobin and iron constitutes a useful tool for identifying newborn buffaloes recently affected by bacterial disease and for monitoring the effectiveness of its treatment.

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Author's contribution: VC, AMS, DGS and JJF conceived and designed the study. VC, AMS, DGS, CRAS and LJLP executed the experiment and analyzed the blood and serum samples. VC and ZC analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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