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ORIGINAL ARTICLE

Erythrogram, leukogram, and acute phase protein reference intervals for healthy newborn Murrah buffalo calves (*Bubalus bubalis*) within the first month of life

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Abstract Establishing of reference intervals (RI) for hematologic variables and blood serum acute phase proteins (APP) of healthy newborn buffaloes is an important tool for monitoring alterations during infection and inflammation. Considering the scarcity of published data on newborns, the aim of the study was to establish RI for hematologic variables and APP from healthy newborn buffaloes. Blood samples from 28 healthy Murrah buffalo calves, 10–30 days old, were selected to determine RI. Fourteen hematologic and four blood APP variables were analyzed. Before collection of blood samples, calves were subjected to physical examination (rectal temperature, degree of dehydration, and fecal consistency) and only calves that were considered healthy were included in the study. The Anderson-Darling test was used to assess normal distribution of values. The Dixon test and Tukey test were used to identify outliers. RI and 90% CI were determined using standard/robust methods and Box-Cox transformation. RI for variables analyzed were the following: (1) hematologic variables: RBC 7.5–12.9 × 10^6/μL, HGB 10.6–19.0 g/dL, packed cell volume 33.1–54.8%, mean corpuscular volume 36.2–50.6 fL, mean corpuscular hemoglobin 12.1–17.3 pg, mean corpuscular hemoglobin concentration 28.1–42.9 g/dL, platelets 361–1081 × 10^3/μL, WBC 6.56–18.2 × 10^3/μL, lymphocytes 4.15–12.8 × 10^3/μL, segmented neutrophils 0.950–10.6 × 10^3/μL, band neutrophils 0–0.160 × 10^3/μL, monocytes 0–0.754 × 10^3/μL, eosinophils 0–0.326 × 10^3/μL, and basophils 0–0.149 × 10^3/μL and (2) APP variables: fibrinogen 2.49-9.50 g/L, haptoglobin 0.02-0.56 g/L, serum amyloid A (SAA) 3.70-97.51 μg/mL, and C-reactive protein (CRP) 0.02-2.78 μg/mL. In conclusion, hematologic and acute phase protein RI have been documented and can be used as a physiologic database to help the interpretation of laboratory results of newborn buffaloes during infection and inflammation conditions.

Introduction

The first month of life is a critical period for newborn buffaloes where two of the major causes of morbidity and mortality are diarrhea and pneumonia (Khan et al. 2009; Anwarullah et al. 2014; Naag et al. 2015). In particular, diarrhea, involving a complex etiology comprising infectious and parasitic pathogens (Anwarullah et al. 2014; Silva et al. 2015), can be responsible for until 23.7% of deaths that occur in neonatal (Sunil Chandra and Mahalingam 1994).

To address diseases in newborns, the clinical evaluation of neonates requires, besides the physical examination, the use of complementary laboratory tests such as measurement of blood serum components, which can enable the detection of anemia,

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inflammation, and infection conditions. For example, modifications of blood serum components in diarrheic newborn bovines infected with *Salmonella dublin* (Silva et al. 2010, 2011) and newborn buffaloes infected with *Salmonella thyphimurium* (Clemente et al. 2016) have been studied. Important alterations have been linked to inflammation and infection, such as increases in acute phase proteins (APP) fibrinogen, ceruloplasmin and haptoglobin, and leukocytosis. Studies with bovine calves during outbreak of respiratory disease caused by bovine respiratory syncytial virus have shown changes in haptoglobin, serum amyloid A (SAA), and LBP (Orro et al. 2011).

In buffaloes, studies with alterations in the hemogram and APP in newborn diseased animals are scarce (Clemente et al. 2016; Kabu and Sayin 2016). In adult buffaloes, studies have shown important alterations in blood serum components in animals with traumatic reticuloperitonitis and pericarditis (Saleh et al. 2008; El-Ashker et al. 2013; Neamat-Allah 2015), abomasal ulcer (Tajik et al. 2012), animals experimentally infected with *Pasteurella multocida* (Horadagoda et al. 2001, 2002), with uterine torsion (Ali et al. 2011), and in animals with fever, parasitic infestations, and respiratory disorders (Singh et al. 2013).

However, to use results of complementary laboratory tests to assess disease, the study of reference intervals (RI) for each of the tests used will help to establish a physiologic database and can help to minimize errors in clinical decision-making. The use of inappropriate RI can lead to both over- and underdiagnoses of disease, which can compromise the well-being of the animal and increase healthcare costs for the owner (Sample et al. 2015).

In healthy adult buffaloes, studies on the hemogram and APP have been performed in lactating and non-lactating buffaloes (Ciaramella et al. 2005; Gomes et al. 2010; França et al. 2011a; El-Ashker et al. 2013; Ellah et al. 2013b; Kumar et al. 2014), in pregnant and post-calving buffaloes (Fagliari et al. 1998a; Ellah et al. 2013a, c), and in buffalo heifers (Ellah et al. 2014).

In healthy newborns until 30 days of life, studies with erythrogram (Brun-Hansen et al. 2006; Benesi et al. 2012a), leukogram (Benesi et al. 2012b), and APP (Fagliari et al. 1998b, 2006; Rizzoli et al. 2006; Nikunen et al. 2007; Orro et al. 2008, 2011; Tóthová et al. 2015) have been widely assessed in bovines but very rarely in buffaloes (Fagliari et al. 1998b; Clemente et al. 2016), where studies with healthy calves have been performed in groups of animals ranging from 0 to 3 months and 0 to 6 months of life (Gomes et al. 2010; França et al. 2011a). Therefore, studies of the RI in newborn buffaloes with the age range between 0 and 30 days of life, a period in which the incidence of diseases is high, would be of great benefit in readily identifying health compromising conditions, especially as alterations in blood composition occur after the first month of life. Also, when comparing studies performed in calves and adult animals, it

is clear that the use of blood serum components of adult animals for analyzing calves is not accurate, since many alterations are known to occur with advancing age (Costa et al. 2000; Ciaramella et al. 2005; Gomes et al. 2010; França et al. 2011a).

Considering the lack of published works associated to newborn buffalo calves, the aim of the study was to establish RI for hematologic variables and APP from healthy newborn buffaloes in the first month of life, in order to help monitoring alterations during infection, inflammation, and trauma.

Materials and methods

Ethical standards

This research was approved by the Ethics Committee on Animal Use of Faculdade de Ciências Agrárias e Veterinárias, FCAV/UNESP (protocol number: 010885-08).

Animals and study area

Twenty-eight healthy Murrah buffalo calves, 10–30 days of life, from commercial herds localized in São Paulo state, Brazil, constituted the experimental group. Calves were kept together with the lactating buffaloes, which were kept in a semi-intensive system with diet based on roughage. Calves were fed with fresh buffalo milk and also had access to commercial feed, hay, and water ad libitum. All calves that participated in the experiment ingested colostrum.

Physical examination

Inclusion criteria Before collection of blood samples, all calves were subjected to physical examination. Feces were analyzed to check for signs of diarrhea, blood, and mucus. Rectal temperature was also measured to check for signs of hyperthermia. Degree of dehydration was also measured. A total of 28 newborn buffaloes that were normal following physical examination were included in the study. For these calves, rectal temperature ranged between 34.7 and 39.2 °C, indicating the absence of hyperthermia.

Blood sample collection and preparation

Blood sampling was performed by puncture of the jugular vein using a vacuum collection system (25 × 8 mm needles), after local antisepsis with iodized alcohol.

Blood samples were collected into siliconized plastic tubes containing EDTA (BD Vacutainer, 4.0 mL), to perform hemogram analyses and to analyze concentrations of the APP fibrinogen. To determine the fibrinogen concentration,

samples with EDTA were centrifuged at 1000×*g* for 5 min, to obtain the plasma to accomplish the analysis.

Blood samples were also collected into siliconized plastic tubes without anticoagulant (BD Vacutainer, 10 mL) prior to analysis of the APP using ABX Pentra 400 analyzer (haptoglobin) and ELISA methods (SAA, C-reactive protein (CRP)). These samples were centrifuged at 1000×*g* for 10 min after clot retraction, and 1.5-mL aliquots of serum were stored in Eppendorf tubes and frozen (−20 °C) until analysis was performed.

Laboratory analysis

Hemogram including RBC count, HGB concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), PLT and total WBC count was performed using automated hematology pocH-100iV Diff analyzer (Sysmex Corporation, Kobe, Japan). Differential WBC count was performed on blood smear stained with modified Rosenfeld dye by optical microscopy (Garcia-Navarro 1994). Plasma concentrations of fibrinogen were measured by heat precipitation method (Millar et al. 1971).

An ABX Pentra 400 (Horiba ABX SAS, Montpellier, France) was used to analyze serum concentrations of haptoglobin with the methods described in Eckersall et al. (1999) (Table 1). ELISA methods were used to analyze serum concentrations of SAA (Tridelta Development Limited, Maynooth, Co. Kildare, Ireland) and CRP (Life Diagnostics, Inc., West Chester, Pennsylvania, USA) (Table 1).

Statistical analysis

RI were determined according to the guidelines of the American Society for Veterinary Clinical Pathology (ASVCP) (sample size: $20 \leq \times < 40$) (Friedrichs et al. 2012). The Anderson-Darling test was used to assess normal distribution of values with a $p < 0.05$ (Reference Value Advisor) (Geffré et al. 2011). The Dixon test and Tukey test (3xIQR, 1.5xIQR) were used to identify outliers and suspect outliers

(Reference Value Advisor) (Geffré et al. 2011). RI and 90% CI for lower and upper limits were determined using untransformed data, when data distribution was Gaussian (standard) or symmetric but not Gaussian (robust). When data was not symmetric by using untransformed standard and robust methods, the Box-Cox transformation was performed so to normalize data (Reference Value Advisor).

Results and discussion

All variables were analyzed using 28 samples except haptoglobin, SAA, and CRP. For haptoglobin, SAA, and CRP, 20 samples were used. However, considering that the study included 20 or more samples for each variable, RI were able to be calculated using the Reference Value Advisor (Geffré et al. 2011) according to the guidelines of the ASVCP for sample size between 20 and 40 (Friedrichs et al. 2012). Therefore, RI for 14 hematologic variables and 4 blood serum APP variables were calculated and are arranged in Tables 2 and 3, respectively.

The hemogram is a simple and cheap method to assess blood alterations associated to pathological conditions, such as anemia, dehydration, inflammation, and infection, and can be a valuable complementary data for diagnosis, prognosis, and evaluation of infectious diseases. In buffaloes, studies with the hemogram have been focused on adult animals, greater than 1 year old and under different conditions, such us lactating buffaloes (Ellah et al. 2013b), non-lactating buffaloes (Ciaramella et al. 2005), pregnant and post-calving buffaloes (Fagliari et al. 1998a; Ellah et al. 2013a, c), and buffalo heifers (Ellah et al. 2014). However, it is clear that the use of blood serum constituents of adult animals for analyzing calves is not accurate, since many alterations occur with advancing age (Costa et al. 2000; Gomes et al. 2010; França et al. 2011a).

Gomes et al. (2010), comparing the erythrogram between 0–3-month-old and 1–5-year-old buffaloes and França et al. (2011a), comparing the erythrogram between 0–6 month-old and 1–2-year-old buffaloes, obtained statistically different results among groups, observing a decrease in PCV, RBC, and HGB with advancing age. This

Table 1 Analytic methods for APP variables measured in newborn buffalo calves (*Bubalus bubalis*)

Variables	Method	Absorbance required (nm)
Fibrinogen	Heat precipitation and refractometer reading[a]	–
Haptoglobin	Hemoglobin binding[b]	600
SAA	ELISA[c]	450
CRP	ELISA[d]	450

[a] ATAGO

[b] Based on the method of Eckersall et al. (1999) on an ABX Pentra 400, Horiba ABX SAS, Montpellier, France

[c] Catalog number: TP 802, Tridelta Development Limited, Maynooth, Co. Kildare, Ireland

[d] Catalog number: 2210-8, Life Diagnostics, Inc., West Chester, Pennsylvania, USA

Table 2 Hematologic reference intervals (RI) for buffalo calves from 10 to 30 days of life, analyzed using automated hematology analyzer (pocH-100iV Diff, Sysmex Corporation, Kobe, Japan)

| Analyte | Units | Descriptive statistics | | | | RI within 90% CI | | Reference Value Advisor | |
		Mean	SD	Median	Min-Max	Lower limit (90% CI)	Upper limit (90% CI)	n	Method
RBC	$\times10^6/\mu L$	10.3	1.29	10.2	7.69–12.4	7.50 (6.88–8.27)	12.9 (12.3–13.5)	28	BCTRD
HGB	g/dL	15.1	2.00	15.3	11.3–18.9	10.6 (9.10–12.2)	19.0 (17.9–19.9)	28	BCTRD
PCV	%	43.5	5.10	44.6	31.7–50.8	33.1 (30.0–37.1)	54.8 (52.2–57.8)	28	RUD
MCV	fL	42.4	3.40	41.6	36.8–49.0	36.2 (35.1–37.3)	50.6 (47.5–53.2)	28	BCTRD
MCH	pg	14.7	1.20	14.8	11.8–17.5	12.1 (11.4–12.9)	17.3 (16.6–18.0)	28	BCTRD
MCHC	g/dL	34.9	3.30	36.1	29.2–38.5	28.1 (26.0–31.0)	42.9 (41.3–45.0)	28	URD
Platelets	$\times10^3/\mu L$	675	173	665	372–1047	361 (300–433)	1081 (946–1213)	28	BCTRD
WBC	$\times10^3/\mu L$	12.1	2.78	12.2	6.30–19.1	6.56 (5.24–8.13)	18.2 (16.5–19.9)	28	BCTRD
Lymphocytes	$\times10^3/\mu L$	7.46	2.10	7.12	4.62–12.4	4.15 (3.80–4.73)	12.8 (10.9–15.0)	28	BCTRD
SN	$\times10^3/\mu L$	4.19	1.95	3.98	0.61–7.64	0.950 (0.640–1.69)	10.6 (8.36–13.2)	28	BCTRD
BN	$\times10^3/\mu L$	0.034	0.059	0	0–0.160	0 (0–0)	0.160 (0.110–0.190)	28	USD
Monocytes	$\times10^3/\mu L$	0.259	0.180	0.281	0–1.10	0 (0–0)	0.754 (0.513–0.999)	28	URD
Eosinophils	$\times10^3/\mu L$	0.064	0.125	0	0–0.504	0 (0–0)	0.326 (0.168–0.463)	28	USD
Basophils	$\times10^3/\mu L$	0.030	0.057	0	0–0.174	0 (0–0)	0.149 (0.096–0.194)	28	USD

n number of animals, *Min* minimum, *Max* maximum, *CI* confidence interval, *SN* segmented neutrophils, *BN* band neutrophils, *URD* untransformed robust data, *BCTRD* Box-Cox transformed robust data, *USD* untransformed standard data

occurs probably due to a decrease in bone marrow hematopoietic activity and production of thyroid hormones with advancing age (Ciaramella et al. 2005). In the same way, when comparing results for PCV, RBC and HGB of adult animals (Fagliari et al. 1998a; Ciaramella et al. 2005; Gomes et al. 2010; França et al. 2011a; Ellah et al. 2013a, b, c; Ellah et al. 2014) with the results obtained in this work (Table 2), it was observed that concentrations are higher in our study. Also, it was observed that PCV, RBC, and HGB concentrations are higher in our study (0–30 days old) when comparing with results of 0–3-month-old buffaloes (Gomes et al. 2010) and 0–6-month-old buffaloes (França et al. 2011a). This is likely to be because the age range used in these studies is older and wider than in the present study and therefore modifications in erythrogram can occur. This reinforces the importance of analyzing reference intervals specifically

using calves with age ranging from 0 to 30 days of life for comparisons leading to health assessment in such young buffaloes.

WBC of buffalo calves is higher in 0–6-month-old animals when comparing with 12-month-old animals. However, after the first year, WBC increase again and stabilize in value similar to the values detected in 0–6-month-old animals (França et al. 2011a). When comparing results for WBC of adult buffaloes (Fagliari et al. 1998a; Ellah et al. 2013a, b) with the results obtained in this work (Table 2), it was observed that concentrations are higher in buffalo calves. However, when compared with studies in adult buffaloes performed by Ellah et al. (2014) and França et al. (2011a), it was observed that concentrations were similar between buffalo calves and adult buffaloes.

França et al. (2011a) compared the leukogram values between 6- and 12-month-old buffaloes and observed a decrease

Table 3 Blood serum acute phase protein (APP) reference intervals (RI) for buffalo calves from 10 to 30 days of life

| Analyte | Units | Descriptive statistics | | | | RI within 90% CI | | Reference Value Advisor | |
		Mean	SD	Median	Min-Max	Lower limit (90% CI)	Upper limit (90% CI)	n	Method
Fibrinogen	g/L	6.07	1.68	6.00	2.00–10.0	2.49 (1.59–3.58)	9.50 (8.55–10.5)	28	BCTSD
Haptoglobin	g/L	0.17	0.11	0.20	0.05–0.41	0.02 (0.02–0.04)	0.56 (0.42–0.67)	20	BCTSD
SAA	µg/mL	49.9	21.9	45.9	23.3–85.8	3.70 (0.0–18.3)	97.51 (82.3–113)	20	BCTSD
CRP	µg/mL	0.84	0.62	0.82	0–2.59	0.02 (0–0.10)	2.78 (1.93–3.67)	20	BCTSD

n number of animals, *SAA* serum amyloid A, *CRP* C-reactive protein, *BCSTD* Box-Cox standard transformed data, *BCTSD* Box-Cox transformed standard data;

in neutrophil count and increase in lymphocyte count with advancing age. However, after the first year, lymphocyte count decreased and neutrophil count increased and stabilized in values similar to or below to the values detected in 6-month-old buffaloes. In this sense, when comparing results for neutrophil and lymphocyte count of adult buffaloes with the results obtained in this work (Table 2), it is observed that concentrations in or study are slightly higher or similar to other studies (Fagliari et al. 1998a; França et al. 2011a; Ellah et al. 2013a, b; Ellah et al. 2014).

Costa et al. (2000) and França et al. (2011a) in studies assessing the effect of age in the hemogram of bovine and buffalo calves, respectively, observed an increase in eosinophil count with advancing age. They concluded that this increase, especially in buffalo calves, can be due to immune response to parasites (Jain 1993), common in this age (Naag et al. 2015; Silva et al. 2015). When comparing results of eosinophil count of adult buffaloes (Fagliari et al. 1998a; França et al. 2011a; Ellah et al. 2013a, b; Ellah et al. 2014) with the results obtained in this work (Table 2), it was observed that concentrations are much lower in the present study, to about 6 to 10 times fold, which reinforces the conclusion that age causes important effects in this cell type.

Costa et al. (2000) and França et al. (2011a) observed a decrease in monocyte count with advancing age, while Jain (1993) observed no significant change with advancing age. Although some studies show a decrease in monocyte count within advancing age (França et al. 2011a), results for adult buffaloes (Fagliari et al. 1998a; Ellah et al. 2013a, b; Ellah et al. 2014) were similar or slightly higher than the concentrations analyzed in our study (Table 2).

APP are blood proteins primarily synthesized by hepatocytes as part of the acute phase response (APR). The APR is part of the innate immune system, which is triggered by different stimuli including trauma, infection, stress, neoplasia, and inflammation (Cray et al. 2009). Investigations over the last few years have shown that the quantification of APP in serum can provide valuable diagnostic information in the detection, prognosis, and monitoring of disease (Eckersall 2000). In ruminants, haptoglobin and SAA are considered major APP (10–100-fold increase in response to stimuli), while CRP and fibrinogen are moderate APP (2–10-fold increase in response to stimuli) (Murata et al. 2004; Eckersall and Bell 2010) and albumin a negative APP (Ceciliani et al. 2012).

Haptoglobin is a major APP in ruminants. In healthy adult cattle, the serum haptoglobin concentrations are lower than the detection limit or between 0.02 and 0.35 g/L, depending on the study (Salonen et al. 1996; Horadagoda et al. 1999; Eckersall et al. 2001; Gronlund et al. 2003; Huzzey et al. 2009; Eckersall and Bell 2010), but can increase to more than 2.0 g/L within 2 days of infection (Eckersall and Bell 2010). Haptoglobin has already been shown to be effective in the diagnosis and prognosis of mastitis, enteritis, peritonitis, pneumonia, endocarditis,

and endometritis (Murata et al. 2004; Petersen et al. 2004). In this study, the RI for haptoglobin in serum of healthy buffalo calves ranged between 0.02 and 0.56 g/L, with average of 0.17 g/L (Table 3). These results are higher than measured in 3–11-month-old healthy buffaloes (average of 0.008 g/L) (Kabu and Sayin 2016) and adult buffaloes (average of 0.11 g/L) (El-Ashker et al. 2013) but lower than the observed in newborn bovine calves, with average of 0.23 g/L (Nikunen et al. 2007) and 0.27 g/L (Tóthová et al. 2015). Also, studies in healthy adult cattle showed that haptoglobin blood serum concentrationcan range between 0.02 and 0.10 g/L (Eckersall et al. 2001) and that concentrations below 0.35 g/L (Horadagoda et al. 1999) indicates that animals are healthy, different from the buffalo calves from this study (Table 3), where upper limit for haptoglobin were higher (0.56 g/L).

SAA is also a major APP in ruminants. In healthy adult cattle (Horadagoda et al. 1999; Eckersall et al. 2001; Gronlund et al. 2003) and adult buffaloes (El-Ashker et al. 2013; Kumar et al. 2014), SAA concentrations have similar ranges. However, when comparing SAA concentrations of adult cattle and adult buffaloes with newborn bovines (Tóthová et al. 2015) and newborn buffaloes from this study (Table 3), SAA concentrations of newborn animals are at least fourfold higher than the adult animals. Additionally, SAA upper limits are similar in newborn bovines (Orro et al. 2008, 2011) and newborn buffaloes described here (Table 3).

Orro et al. (2008), in a study with newborn bovines, observed a decrease of 75% in SAA concentrations between 3 and 59 days of life. Yamanaka et al. (2003) shown that serum concentrations of pro-inflammatory cytokines of calves increase immediately after colostrum intake and then decrease gradually, being almost undetectable 3 to 4 weeks of life. Since pro-inflammatory cytokines are the main inducers of hepatic production of APP, this could explain the gradual decrease and therefore differences between newborn and adult animals, highlighting the importance of considering the age of the animal when using this protein as a disease biomarker.

CRP, a moderate APP in bovines, has been poorly studied in buffaloes. In the present work, the RI of CRP in healthy buffalo calves ranged from 0.02 to 2.78 μg/mL (Table 3), with average concentrations of 0.84 μg/mL. Studies with adult buffaloes (El-Ashker et al. 2013) have found higher concentrations of serum CRP when compared with our study, with average of 25.0 μg/mL.

Fibrinogen is an APP in ruminants used as a reliable tool for the evaluation of the response of organism to inflammation, bacterial infections, and surgical trauma (Khan et al. 1997; Murata et al. 2004). In buffaloes, França et al. (2011b) comparing 6-, 12-, and 24-month-old animals observed that serum concentrations of fibrinogen increase with age. When comparing our work (Table 3) with 0–45-day-old buffaloes (Fagliari et al. 1998b), the first month of life buffaloes (Clemente et al. 2016), and 0–5-month-old bovine calves

(Nikunen et al. 2007), results were similar. Although França et al. (2011a) observed an increase in fibrinogen concentrations with age, when comparing to adult buffaloes, our results (Table 3) were similar to those of Fagliari et al. (1998a) but higher than the results of El-Ashker et al. (2013).

In conclusion, hematologic and acute phase protein RI for buffalo calves documented in this study can be used as a physiologic database and help the interpretation of laboratory results of newborn buffaloes during infection, inflammation, and trauma conditions. Also, when comparing results of this work with studies from the literature performed with adult animals, it is clear that the use of blood serum content of adult animals for determining the health of calves is not accurate, since many alterations occur with advancing age, highlighting the importance of undertaking further research related to hematology in newborn buffalo calves.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This research was approved by the Ethics Committee on Animal Use of Faculdade de Ciências Agrárias e Veterinárias, FCAV/UNESP (protocol number: 010885-08).

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