Comparative Clinical Pathology (2020) 29:127–133
https://doi.org/10.1007/s00580-019-03037-3

ORIGINAL ARTICLE

Morphometric study of red blood cells in non-descriptive cattle with respect to age and sex

Ipsita Dash [1]

Received: 2 June 2019 / Accepted: 25 July 2019 / Published online: 6 August 2019

Abstract

The present study was conducted to investigate the influence of age (calf, young and adult) and sex (female and castrated male) on the morphometry (mean length and mean breadth) of red blood cells (RBCs) of non-descriptive cattle. Blood samples were collected from each non-descriptive cattle with respect to age and sex by jugular venipuncture and collected in EDTA vials. Then, blood smears were prepared on grease-free microscopic slides then air-dried, fixed on methanol, and stained with Giemsa stain for morphometric study. The morphometric study was done with the help of the ocular micrometer and stage micrometer under 40X objective. Since there was no previous report on comparative morphometrical analyses of castrated males and female ND cattle of Odisha, an attempt has been taken to undertake this particular study. For both mean length and breadth, no significant difference was there among the different age groups of female cattle. Highly significant differences ($p < 0.001$) were observed between the mean length of RBCs of male calves with young castrated males and adult males. For mean breadth of RBCs, a highly significant difference ($p < 0.01$) was there between the male calves and male adults. A highly significant difference ($p < 0.01$) was there between the mean length of RBCs of the male calves with the mean length of RBCs of the young females. Therefore, age and sex have a profound effect on the morphometry of RBCs in non-descriptive cattle. Therefore, careful attention must be done in studying and interpretation of anemic conditions on the basis of size.

Keywords Calves · Youngs · Adults · Length · Breadth

Introduction

Erythrocytes or red blood cells (RBCs) provide vital functions of oxygen transport, carbon dioxide transport, and buffering of hydrogen ions (Harvey 2008, 2010). The mature red blood cell (RBC) of the adult bovine is biconcave in shape (Kramer 2000; Harvey 2001; Barger 2010), has minimal central pallor, and has a width of 5–6 μm and a relatively long lifespan of approximately 130 days (Wood and Quiroz-Rocha 2010). Variation in sizes of erythrocytes is known as anisocytosis (Thrall 2012) which is mild to moderate in ruminants. The shape of a RBC is relatively uniform, but poikilocytosis is not unusual in blood smears of apparently healthy calves (Okabe et al. 1996). Morphology of cells has recently been indicated as a powerful indicator of cellular function (Lobo et al. 2015). The geometry of the cell has long fascinated biologists (Thompson 1917). Quantifying morphology of cell is fundamental to the statistical study of cell populations, and can help unravel mechanisms underlying cell and tissue morphogenesis (Sánchez-Corrales et al. 2018). Since there was no previous report on comparative morphometrical analyses of RBCs of castrated male and female ND cattle (Fig. 1) of Odisha, an attempt has been taken to undertake this particular study. Since comparative study on morphometrical parameters, i.e., RBCs of non-descriptive (ND) cattle with respect to age and sex are inadequate and to study the influence of age and sex on the morphometry of red blood as well as to interpret the anemic syndromes on the basis of size especially concerning the normocytic, macrocytic, and microcytic anemias; the present study is undertaken. Various authors have studied the influence of age (Adilli et al. 2013; Dash and Mohanty 2015), sex (Adilli et al. 2013), and breed (Adilli et al. 2014; Dash and Mohanty 2015) on the morphometry of RBCs.

✉ Ipsita Dash
ipsita.dash9@gmail.com

[1] P.G. Department of Zoology, Utkal University, Bhubaneswar, Odisha 751004, India

Fig. 1 Castrated non-descriptive cattle

Materials and methods

Blood samples collection

ND female and castrated male cattle each having three different age groups, that is, calves, young and adult were taken for this study. After disinfecting the sampling area, blood samples were taken from the jugular vein (Sastry 1983; Brar et al. 2002; Ledieu 2003) of each animal by a skilled veterinary expert. Dry and sterilized needles (Dispo Van Single Use Needle, Hindustan Syringes & Medical Devices Ltd., Faridabad, India) and dry syringes (Dispo Van Single Use Syringe, Hindustan Syringes & Medical Devices Ltd., Faridabad, India) were used for collection of blood (Sastry 1983) and collected in EDTA (ethylene diamine tetra acetic acid) vials. Slides were precisely identified according to their respective age and sex.

Blood smears preparation, staining, and morphometric study

In the laboratory, smears were prepared on grease-free microscopic slides, air-dried, then fixed with methanol, and stained with Giemsa stain prepared from Giemsa powder (Qualigens CAS NO.51811-82-6 Product NO. 39382, scientific India Pvt. Ltd., Mumbai, Maharashtra, India) as protocol cited by Lillie (1977) . For several and even until the last years, morphometric studies of RBCs are essentially based on linear measures of erythrocyte size. For the morphometric study of erythrocytes, the ocular micrometer and an objective micrometer was used (Dash and Mohanty 2015). The entire data (30 observations) per age group of each sex were subjected for morphometrical analyses by using an ocular micrometer that was standardized against a stage micrometer (ERMA TOKYO, Japan made) using a standard light microscope (LABOSCOPE MICROSCOPES Research microscope M.No. BD-08 B, S. No. 21320 Mfg. by B.D. INSTRUMENTATION, Ambala Cantt, India) under 40X objective. Circumference and surface area of erythrocytes were calculated by using the formula $2\pi r$ and πr^2 respectively.

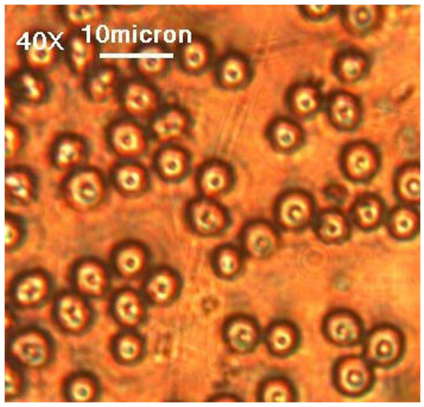

Fig. 2 Red blood cells

Photomicrography

Photomicrography of blood cells was done by a CC130-1.3 mega pixel microscopic camera (Mfg. by Catalyst Biotech, Maharastra, India) connected to a microscope (LABOSCOPE MICROSCOPES Research microscope M. No. BD-08 B, S. No. 21320 Mfg. By B.D. INSTRUMENTATION, Ambala Cantt, India) under 40X objective.

Statistical analyses

Each parameter is expressed as mean and standard error mean for all age groups and sex, and Microsoft Office Excel 2007 was used for statistical analyses. Data analyses for comparison were done with the help of Paleontological Statistics (PAST) version 2.17 (Natural History Museum, University of Oslo)

Table 1 Effect of age on morphometry of RBCs of male ND cattle

Parameters	Male calf ($n = 30$)	Male young ($n = 30$)	Male adult ($n = 30$)	F value
Length	5.69[a]	6.43[a]	6.52[a]	10.34**
SEM	0.15	0.10	0.11	
Breadth	5.46[a]	5.76	6.20[a]	8.053**
SEM	0.15	0.18	0.10	

Same lowercase superscript letters in the same row differ significantly at $p < 0.001$

**Means highly significant at $p < 0.001$

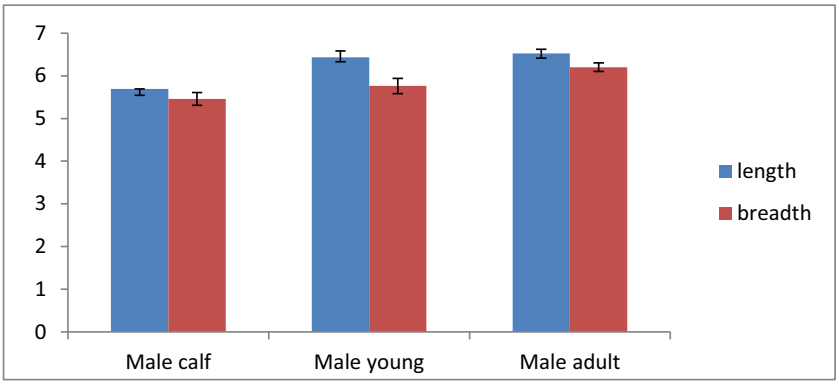

Fig. 3 Effect of age on the morphometry of RBCs of male ND cattle

for one-way analysis of variance (ANOVA) followed by Tukey's pair-wise comparison tests. Differences were classified as significant at $p < 0.05$ and highly significant at $p < 0.001$.

Results and discussion

The erythrocytes observed were biconcave in shape (Fig. 2) and the morphometry (length and breadth) were measured in micrometer (μm). The influence of age on the male ND cattle is shown (Table 1). Among the males, the highest mean length of RBCs was observed in adult males (6.52 ± 0.11) and the lowest was observed in male calves (5.69 ± 0.15); the highest mean breadth was observed in male adults (6.20 ± 0.10) and the lowest was observed in male calves (5.46 ± 0.15) (Fig. 3). The influence of age on the female ND cattle is shown (Table 2). Among the females, the highest mean length was observed in female calves (6.33 ± 0.14) and the lowest was observed in female youngs (6.05 ± 0.10); the highest mean breadth was observed in female adults (5.86 ± 0.12) and the lowest was observed in female calves (5.71 ± 0.10) (Fig. 4). The influence of both age and sex were shown (Table 3). Among the three age groups of both males and females, the highest mean length of RBCs was observed in adult males (6.52 ± 0.11) and the lowest was observed in male calves (5.69 ± 0.15); the highest mean breadth was observed in adult

Table 2 Effect of age on morphometry of RBCs of female ND cattle

Parameters	Female calves ($n = 30$)	Female youngs ($n = 30$)	Female adults ($n = 30$)	F value
Length	6.33	6.05	6.12	1.251 NS
SEM	0.14	0.10	0.12	
Breadth	5.71	5.78	5.86	0.3979 NS
SEM	0.10	0.13	0.12	

NS means not significant

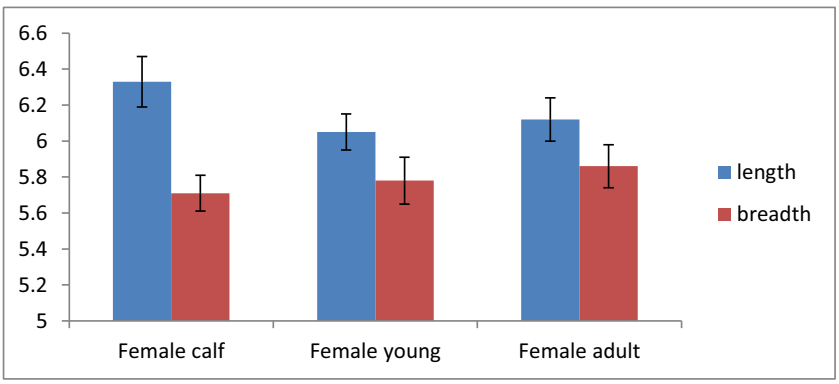

Fig. 4 Effect of age on the morphometry of RBCs of female ND cattle

Table 3 Effect of age and sex on the morphometry of RBCs of ND cattle

Parameters	Male calves ($n = 30$)	Male youngs ($n = 30$)	Male adults ($n = 30$)	Female calves ($n = 30$)	Female youngs ($n = 30$)	Female adults ($n = 30$)	F value
length	5.69[a]	6.43[a]	6.52[a]	6.33	6.05[a]	6.12	5.027**
SEM	0.15	0.10	0.11	0.14	0.10	0.12	
breadth	5.46[a]	5.76	6.20[a]	5.71	5.78	5.86	3.749**
SEM	0.15	0.18	0.10	0.10	0.13	0.12	

Same lowercase superscript letters in the same row differ significantly at $p < 0.001$

**Means highly significant at $p < 0.001$

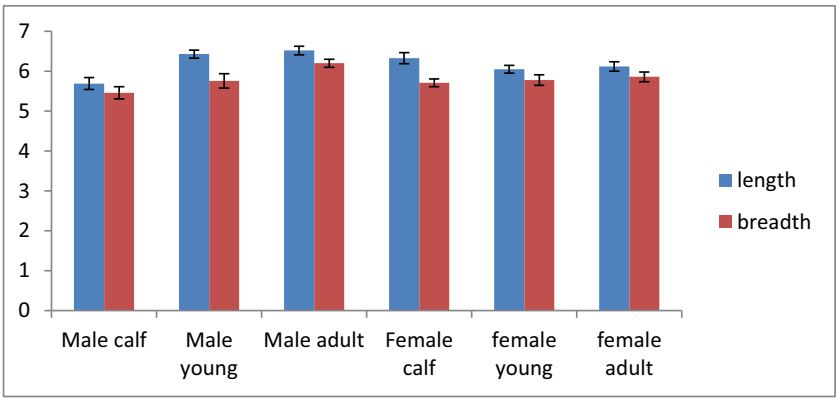

Fig. 5 Effect of both age and sex on the morphometry of RBCs of ND cattle

males (6.20 ± 0.10) and the lowest was observed in male calves (5.46 ± 0.15) (Fig. 5). For both the mean length and breadth, no significant difference was there among the different age groups of female cattle. A highly significant difference ($p < 0.001$) was observed between the mean length of RBCs of the male calves with young castrated males and adult males. For the mean breadth of RBCs, a highly significant difference ($p < 0.01$) was there between the male calves and male adults. A highly significant difference ($p < 0.01$) was there between the mean length of RBCs of the male calves with the mean length of the female youngs .

The surface area was measured in μm^2. The influence of age on the male ND cattle is shown (Table 4). Among the males, the highest mean circumference of RBCs was observed in adult males (20.48 ± 0.37) and the lowest was observed in the male calves (17.89 ± 0.47); the highest mean surface area was observed in the male calves (32.81 ± 1.13) and the lowest was observed in the male calves (26.01 ± 1.25). The influence of age on the female ND is shown (Table 5). Among the females, the highest mean circumference of RBCs was observed in the female calves (19.89 ± 0.44) and the lowest was observed in the female youngs (19.00 ± 0.33); the highest mean surface area was observed in the female calves (31.95 ± 1.66) and the lowest was observed in the female youngs (29.02 ± 0.92). The influence of both age and sex on the mean circumference and surface area were shown (Table 6). Among the three age groups of both males and females, the highest mean circumference of RBCs was observed in adult males (20.48 ± 0.37) and the lowest was observed in male the calves (17.89 ± 0.47); the highest mean surface area was observed in the young males (32.81 ± 1.13) and the lowest was observed in the male calf (26.01 ± 1.25). For both the mean

Table 4 Effect of age on circumference and surface area of RBCs of male ND cattle

Parameters	Male calves	Male youngs	Male adults	F value
Circumference	17.89[a]	20.22[a]	20.48[a]	10.34**
SEM	0.47	0.34	0.37	
Area	26.01[a]	32.81[a]	33.71[a]	11.28**
SEM	1.25	1.13	1.27	

Same lowercase superscript letters in the same row differ significantly at $p < 0.001$

**Means highly significant at $p < 0.01$

Table 5 Effect of age on circumference and surface area of RBCs of female ND cattle

Parameters	Female calves	Female youngs	Female adults	F value
Circumference	19.89	19.00	19.23	1.25 NS
SEM	0.44	0.33	0.38	
Surface area	31.95	29.02	29.80	1.71 NS
SEM	1.66	0.92	0.99	

NS means not significant

Table 6 Effect of age and sex on circumference and surface area of RBCs of ND cattle

Parameters	Male calves	Male youngs	Male adults	Female calves	Female youngs	Female adults	*F* value
Circumference	17.89	20.22[a]	20.48[a]	19.89[a]	19.00	19.23	5.02**
SEM	0.47	0.34	0.37	0.44	0.33	0.38	
Surface area	26.01	32.81[a]	33.71[a]	31.95[a]	29.02	29.80	5.19**
SEM	1.25	1.13	1.27	1.66	0.92	0.99	

Same lowercase superscript letters in the same row differ significantly at $p < 0.001$

**Means highly significant at $p < 0.001$

circumference and surface area, no significant difference was there among the different age groups of female cattle. Highly significant differences ($p < 0.001$) were observed between the mean circumference and surface of RBCs of the male youngs, male adults, and female calves (Figs. 6 and 7).

A process which stops the functions of the testes leading to sterilization is known as castration (Carlson 1996). Several non-genetic factors including castration, i.e., removal of testis affecting hematological parameters of farm animals have been observed (Svoboda et al. 2005; Fisher et al. 2001). The castrated animals showed clinical macrocytic anemia, i.e., increased MCV which will make them be more susceptible to severe anemia than intact goats which might prove fatal thereafter (Olaifa 2018). Castration has been shown to elicit physiological stress, inflammatory reactions (indicated by acute-phase proteins), behavior associated with pain, suppression of

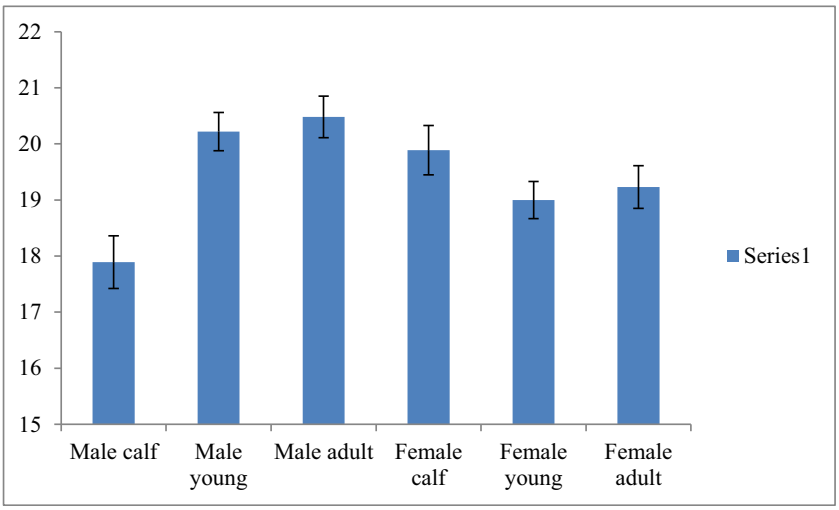

Fig. 6 Effect of both age and sex on the circumference of RBCs of ND cattle

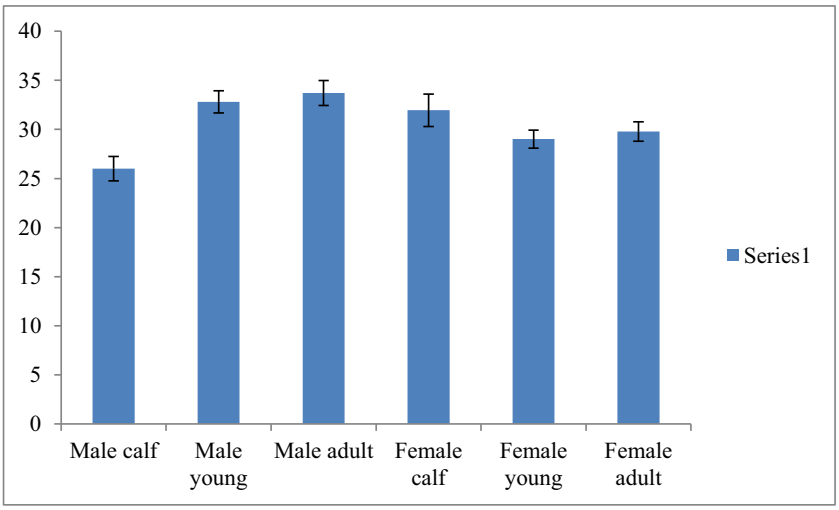

Fig. 7 Effect of both age and sex on the surface area of RBCs of ND cattle

immune function, and a reduction in performance (Molony et al. 1995; Fisher et al. 1996, 1997; Ahmed and Ahmed 2011) to varying degrees. Various authors have studied the effects of surgical castration on serum enzymes and plasma proteins (Robertson et al. 1994; Oyeyemi et al. 2000; Mohammad et al. 2008)

Anisocytosis are seen in different age groups. According to some authors (Harvey et al. 1984; Brun-Hansen et al. 2006; Aoki and Ishii 2012), age can be considered when establishing the reference values in domestic animals. According to Schlam and Carlson (1982), Harvey et al. (1984), Meinkoth and Clinkenbeard (2000), and Harvey (2008), fetal erythrocytes are larger than those of adults. During gestation and at birth, the erythron compartment increases; at birth, 9 % of the RBCs are reticulocytes (McGrath and Palis 2008). Fetal calf RBCs are less fragile and larger than adult bovine RBCs (Harvey et al. 1984). The increasing of erythrocyte diameter with an increase in age in castrated male ND cattle observed in our study could be interpreted by the persistence of RBCs after parturition formed during embryonic life and decreasing of the diameter or length by the stem cell adaptation to new conditions of life after parturition (Adilli et al. 2013). Age (Harvey et al. 1984; Brun-Hansen et al. 2006; Aoki and Ishii 2012; Dash and Mohanty 2015; Dash et al. 2015), sex (Shaikat et al. 2013), breed (Dash and Mohanty 2015; Dash et al. 2015), exercise (Aceña et al. 1995; Zobra et al. 2011), pregnancy and lactation (Masoni et al. 1985; Roy et al. 2010; Mariella et al. 2014), and emotional states are variables to be taken in account when establishing reference values in domestic animals. Age, sex, exercise, and emotional states are variables to be considered when establishing reference values in domestic cattle (Adams et al. 1992; Aceña et al. 1995; Brun-Hansen et al. 2006; Mohri et al. 2007; George et al. 2008; Kapale et al. 2008). The breed can affect the diameter of erythrocytes in cattle (Adilli et al. 2014).

Conclusion

Age and sex have a profound effect on the morphometry of red blood cells of non-descriptive cattle, and this study can provide a baseline reference to which further studies may be compared. By this study, interpretation of anemic syndromes in non-descriptive cattle can be well understood which will helpful for a veterinarian for proper diagnosis and interpretation of diseases as blood plays a vital role for the regulation of the physiological condition of an organism.

Compliance with ethical standards

Ethical approval The investigation followed guidelines for the care and use of animals.

Conflict of interest The author declares that she has no conflict of interest.

References

Aceña MC, Garcia-Belenguer S, Garson M, Purroy A (1995) Modifications hematologiques at musculaires pendant la corrida chez le taureau de combat. Rev Méd Vét 146(4):277–282

Adams R, Garry FB, Aldridge BM, Holland MD, Odde KG (1992) Hematologic values in newborn beef calves. Am J Vet Res 53(6): 944–950

Adilli N, Mohamed M, Omar B (2013) The influence of age, sex and altitude on the morphometry of red blood cells in bovine. Vet World 6(8):476–478. https://doi.org/10.5455/vetworld.2013.476-478

Adilli N, Mohamed M, Belabbas H, Achouri A (2014) Preliminary study of the influence of red blood cells size on the determinism of the breed in cattle. Vet Med Int 2014:1–4. https://doi.org/10.1155/2014/429495

Ahmed SA, Ahmed EA (2011) Behavioral responses of castrated buck kids at different ages by using different methods of castration. J Am Sci 7:200–209

Aoki T, Ishii H (2012) Hematological and biochemical profiles in peripartum mares and neonatal foals (heavy draft horse). J Equine Vet Sci 32:170–176

Barger AM (2010) Erythrocyte morphology. In: Weiss DJ, Wardrop KJ (eds) Schalm's veterinary Hematology. Wiley-Blackwell Publishing Ltd, Ames, pp 144–151

Brar RS, Sandhu HS, Singh A (2002) Veterinary clinical diagnosis by laboratory methods, 1st edn. Kalyani Publisher, Ludhiana, p 10

Brun-Hansen HE, Kampen AH, Lund A (2006) Hematologic values in calves during the first six months of life. Vet Clin Pathol 35(2):182–187

Carlson GP (1996) Clinical chemistry tests. In: Smith BP (ed) Large animal internal medicine, 2nd edn. Mosby Publisher, St. Louis

Dash I, Mohanty PK (2015) Morphological features and influence of age and breed on the morphometry of red blood cells of female cattle. IJSR 4(2):259–264

Dash I, Bhattacherjee A, Mohanty PK (2015) Hematological analysis of three breeds of cows. IJB 2(1):31–39

Fisher AD, Crowe MA, Alonso de la varga ME, Enright WJ (1996) Effect of castration method and the provision of local anaesthesia on plasma cortisol, scrotal circumference, growth and feed intake of bull calves. J Anim Sci 74:2336–2343

Fisher AD, Crowe MA, O'Nualláin EM, Monaghan ML, Prendiville DJ, O'Kiely P, Enright WJ (1997) Effects of suppressing cortisol following castration of bull calves on adrenocorticotropic hormone, *in vitro* interferon-γ production, leukocytes, acute-phase proteins, growth, and feed intake. J Anim Sci 75:1899–1908

Fisher AD, Knight TW, Cosgrove GP, Death AF, Anderson CB, Duganzich DM et al (2001) Effects of surgical or banding castration on stress responses and behaviour of bulls. Aust Vet J 79(4):279–284

George JW, Lane VM, Snipes JN (2008) Changes in bovine hematology reference intervals from 1965 to 2001. Vet Clin Pathol 36:313–315

Harvey JW (2001) Atlas of veterinary hematology: blood and bone marrow of domestic animals. WB Saunders Company, Philadelphia, pp 1–40

Harvey JW (2008) The erythrocyte: physiology metabolism and biochemical disorders. In: Kareko JJ, Harvey JW, Bruss ML (eds) Clinical biochemistry of domestic animals, 6th edn. Academic Press, San Diego, pp 173–240

Harvey JW (2010) Erythrocyte biochemistry. In: Weiss DJ, Wardrop KJ (eds) Schlam's Veterinary Hematology, 6th edn. Wiley-Blackwell Publishing Ltd., Ames, pp 131–135

Harvey JW, Asquith RL, Mcnulty PK, Kivipelto J, Bauer JE (1984) Hematology of the foals up to one year old. Equine Vet J 16(4): 347–353

Kapale PM, Jagtap DG, Badukale DM, Sahatpure SK (2008) Haematological constituents of blood of Gaolao cattle. Vet World 1(4):113–114

Kramer JW (2000) Normal hematology of cattle, sheep and goats. In: Feldman BF, Zinkl JG, Jain NC (eds) Schlam's veterinary hematology, 5th edn. Lippincott Williams and Wilkins, Philadelphia, pp 1075–1084

Ledieu D (2003) Prélèvements en cytologie. Dans: Encyclopédie vétérinarie. Edns Scientifiques et Médicales Elsevier. Biologie Clinique, pp 0030

Lillie RD (1977) HJ Conn's biological stains, 9th edn. The Williams and Wilkins Company, Baltimore, pp 606–607

Lobo J, Youg-Shun See E, Biggs M, Pandit A (2015) An insight into morphometric descriptors of cell shape that pertain to regenerative medicine. J Tissue Eng Regn Med. https://doi.org/10.1002/term.1994

Mariella J, Pirrone A, Gentilini F, Castagnetti C (2014) Hematologic and biochemical profiles in Standardbred mares during peripartum. Theriogenology 81(4):526–534

Masoni F, Lagadic M, Plassiocrt G, Guigand Le, Wyers M (1985) Paramèters Hématologiques de la chèvre laitière Variations physiologiques chez l' animal Sain autour de la mise-bas. Rec Méd Vét 161(1):41–49

McGrath K, Palis J (2008) Ontogeny of erythropoiesis in the mammalian embryo. Curr Top Dev Biol 82:1–22

Meinkoth JH, Clinkenbeard KD (2000) Normal hematology of the dog. In: Feldman BF, Zinkl JG, Jain NC (eds) Schalm's veterinary hematology, 5th edn. Lippincott Williams and Wilkins, Philadelphia, pp 1057–1063

Mohammad BF, Zghoul AL, Raidal AK, Abdelsalam R, Talafha Q, Omar A, Baniismail ZA (2008) Cellular and some biochemical changes in blood and peritoneal fluid constituents in Awassi lambs following elective castration. Am J Anim Vet Sci 3:23–27

Mohri M, Sharifi K, Eidi S (2007) Hematology and serum biochemistry of Holstein dairy calves: age related changes and comparison with blood composition in adults. Res Vet Sci 83(1):30–39

Molony V, Kent JE, Robertson IS (1995) Assessment of acute and chronic pain after different methods of castration of calves. Appl Anim Behav Sci 46:33–48

Okabe J, Tajmla S, Yamato O et al (1996) Hemoglobin types, erythrocyte membrane skeleton and plasma iron concentration in calves with poikilocytosis. J Vet Med Sci 58:629–634

Olaifa AK (2018) Comparison in haematological and biochemical changes in normal, acute and chronically castrated West African Dwarf goats. Int J Res Med Sci 6(5):1623–1627

Oyeyemi MO, Olaifa AK, Onwuka SK, Akinloye AK, Utho OA (2000) The effects of bilateral orchidectomy on some serum enzymes and proteins in the West African Dwarf buck. Afr J Biomed Res 3:105–108

Robertson IS, Kent JE, Molony V (1994) Effect of different methods of castration on behaviour and plasma cortisol in calves of three ages. Res Vet Sci 56:8–17

Roy S, Roy M, Mishra S (2010) Hematological and biochemical profile during gestation period in Sahiwal cows. Vet World 3(1):26–28

Sánchez-Corrales YE, Hartley M, Jv R, Marée AFM, Grieneisen V (2018) Morphometrics of complex cell shapes: lobe contribution elliptic Fourier analysis (LOCO-EFA). Educ Technol Res Dev 145:156778. https://doi.org/10.1242/dev.156778

Sastry GA (1983) Veterinary clinical pathology, 3rd edn. CBS Publishers and Distributors, Delhi, pp 1–30

Schlam OW, Carlson GP (1982) Equine medicine and surgery: the blood and the blood forming organs, 3rd edn. American veterinary publication, Bhubaneswar

Shaikat AH, Hassan MM, Khan SH, Islam MN, Hoque MA, Bari MS, Hossain ME (2013) Hemato-biochemical profiles of indigenous goats (*Capra hircus*) at Chittagong, Bangladesh. Vet World 6(10): 789–793

Svoboda M, Eichlerova K, Horak V, Hradecký J (2005) Development of haematological indices in melanoma-bearing Liběchov minipigs. Acta Vet Brno 74(4):603–611

Thompson DW (1917) On growth and form. Cambridge University Press, Cambridge

Thrall MA (2012) Erythrocyte morphology. In: Thrall MA, Weiser G, Allison RW, Campbell TW (eds) Veterinary hematology and clinical chemistry, 2nd edn. Wiley-Blackwell A John Wiley and Sons, Inc., Publication, Ames, p 63

Wood D, Quiroz-Rocha GF (2010) Normal hematology of cattle. In: Weiss DJ, Wordrop KJ (eds) Schalm's veterinary hematology, 6th. Wiley-Blackwell Publishing Ltd, Ames, pp 829–835

Zobra R, Ardu M, Niccolini S, Cubeddu F, Dimauro C, Bonelli P, Dedola C, Visco S, Parpaglia MLP (2011) Physical, hematological and biochemical responses to acute intense exercise in polo horses. J Equine Vet Sci 31:542–548