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Dynamics of Physiological Poikilocytosis and Anisocytosis in Cholistani Cattle Blood

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ABSTRACT

The present study is the first account of physiological poikilocytosis and anisocytosis attained through stained peripheral blood smear in terms of prevalence, types, severity, and association with age, sex, and hemoglobin (Hb) levels in Cholistani cattle blood (n=64). The correlation of these red blood cells (RBC) forms has been made with certain RBC indices attained through a multi-species hematology analyzer. The total poikilocytes (24.7±0.2%) were significantly (p≤0.05) higher than the total anisocytes (8.4±0.1%) with echinocytes being the highest seen cells (5.4±0.5%). There was no statistical difference for poikilocytes and anisocytes between males and females. The spherocytes and schistocytes were significantly (p≤0.05) higher in young cattle as compared to adult cattle. All the animals in the study groups showed slight (13%-20%) to moderate (21%-30%) total poikilocytosis. Young (n=18; 28.1%) and females (n=7; 10.9%) revealed higher marked (>30%) total poikilocytosis. The severity of total poikilocytosis did not significantly (p≤0.05) affect any of the RBC attributes. Normal cells were significantly higher (p≤0.05) in low Hb group being 68.5±1.5% as compared to 65.09±0.8% for high Hb group. The highest values were attained for the relation between total anisocytes and Hb, and between total anisocytes and Mean Corpuscular Hemoglobin Concentration (r-square=0.159; 15.9% probability). In conclusion, the blood of healthy Cholistani cattle presents physiological peculiarities in terms of the presence of various poikilocytes and anisocytes. Furthermore, the multi-species hematology analyzer failed to predict the level of poikilocytosis and anisocytosis in the present study, whereas the stained PBS provided reliable results.

Keywords: anisocytes; Cholistani cattle; peripheral blood slide; poikilocytes

INTRODUCTION

In the last decade or so, veterinary hematology has seen immense advent both in predictive and confirmed diagnosis/prognosis of various systematic and blood-borne disorders in many species. Consequently, the 3-part and 5-part automated hematology analyzers are being used frequently to monitor complete blood counts (cbc) for ascertaining animal health. This is an accurate and reliable approach but is costly, and the transfer of blood samples to the laboratory may delay treatment, hence resulting in disease aggravation (Gul *et al.,* 2023; Von Konigslow *et al.,* 2019). The use of such analyzers is quite limited in resource-poor countries (including Pakistan) owing to their expensiveness, high maintenance, need for periodic validation, and expensive chemical reagents (Farooq *et al.,* 2023). Hence, examination of a peripheral blood slide (PBS) still remains an inexpensive, field-oriented, and quick method for determining blood cell morphology, and it cannot be considered as a 'lost art' especially for resource poor countries of the world (Chowdary *et al.,* 2018; Sandhya & Rashmi, 2017).

In the context of blood cell morphology, apart from morphometric measurements of red blood cells (RBC), poikilocytosis (abnormal RBC shape), and anisocytosis (abnormal RBC size) are considered vital diagnostic/prognostic markers (Dervišević *et al.,* 2022; Vasilatis & Christopher, 2023). They may be physiological or indicative of an underlying specific disease, dietary deficiency, biochemical alteration, and protein dysfunction. Furthermore, their quantification provides insight into the physiological functioning of various bodily organs such as liver, spleen, kidneys, *etc*. The mean corpuscular volume (MCV) and red cell distribution width (RDW) of the cbc report attained through hematology analyzers are considered indicative of macrocytosis and anisocytosis, respectively. However, visual assessment of a stained PBS is still a reliable tool for monitoring hematological alterations, either for clinical assessment or for comparing the results with those from hematology analyzers.

In young goats and calves, poikilocytes are normally higher in blood as compared to their counterpart adults, a phenomenon which has been attributed to hemoglobin class switching from fetal hemoglobin

(HbF) to neonatal hemoglobin (HbC) and ultimately to adult hemoglobin (HbA) (Dash, 2020; Signore *et al.*, 2023; Vasilatis & Christopher, 2023). The dynamics of physiological and pathological poikilocytosis and anisocytosis in dogs (Aniołek *et al.*, 2018; Athanasiou *et al.*, 2018), goats (Vasilatis & Christopher, 2023), rabbits (Christopher *et al.*, 2014), and humans (Hussain & Frayez, 2022) have been well documented. Similarly, extensive work has also been reported on morphometric attributes of RBCs in cattle blood (Dash, 2020; Dash & Mohanty, 2015; Parveen *et al.*, 2023). Standardization and grading of human blood cells have also been navigated thoroughly (Gulati, 2009). However, to the best of my knowledge, there is no such study for the blood of indigenous, humped Cholistani breed of cattle being reared under nomadic pastoralism of the Cholistan desert, Pakistan. The present study is the first account of assessing the physiological dynamics of poikilocytosis and anisocytosis in Cholistani cattle blood in terms of prevalence, types, severity, and association with age, sex, and hemoglobin (Hb) level. Furthermore, it assesses the prediction reliability of certain RBC attributes, *viz.* Hb, RBC count, MCV, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hematocrit (HCT), RDW-CV, and RDW-SD attained through hematology analyzer in predicting poikilocytosis and anisocytosis in Cholistani cattle blood attained through manual method of stained PBS microscopy. This preliminary data may assist in understanding the pathophysiology of various blood-borne disorders in cattle.

MATERIALS AND METHODS

Ethics Statement

The research study was approved by the 'Departmental Research Ethics Committee' of the Department of Physiology, the Islamia University of Bahawalpur (Approval No PHYSIO-77/2024-162).

Geolocation

The study was conducted in the Cholistan desert (for blood sampling) and in the post-graduate (PG) laboratory of the Department of Physiology (IUB), Pakistan (for lab work). This desert is located in Southern Punjab, a lower section of Punjab that includes the city of Bahawalpur, which has an annual rainfall of 90 to 200 mm. Cholistan desert is located in the city's south; this region's climate is desert, hot, subtropical, and monsoonal, with an annual rainfall of 180 mm. The average annual temperature is 28.33 °C, with June being the hottest month with daily maximum temperatures exceeding 45 °C (Farooq *et al.*, 2010; 2011).

Experimental Animals

The cattle of the Cholistani breed (n=64) being reared under nomadic pastoralism in the Cholistan desert, Pakistan, were incorporated in this study. Tobas (man-made and natural water reservoirs) were selected in the desert, which harbored Cholistani livestock. The Cholistani cattle were selected on the basis of their phenotype. All the animals were being reared under similar management and feeding conditions of pastoralism in which the young animals were kept in pens at the Tobas, whereas the adults were sent out in the desert for grazing (split-herding) (Farooq *et al.*, 2010; Saeed *et al.*, 2022). A detailed anamnesis provided by the livestock owners allied with visual and physical assessment was used to select healthy animals. The animals that displayed signs of lethargy or any underlying disease were not included in the study; only healthy and active animals were included.

Blood Collection and Analyses

The blood sampling was conducted from May to June 2023. Study animals were restrained in the field for blood sampling with the help of field staff. About 3mL of blood was collected aseptically in anticoagulant-added tubes from the higher neck jugular vein with the help of a disposable syringe. Each animal was bled once. The same restraining technique with the same personnel and time of collection were used to minimize the stress on the animal and normalize blood collection procedure. Samples were carried to the PG laboratory of the Department of Physiology, IUB, in an ice box. A drop of whole blood was used to make a thin smear which was appropriately stained using Field Stain (SDL Scientific Enterprise, Pakistan) (Lashari *et al.*, 2018).

Whole blood was analyzed for RBC attributes, *i.e.*, Hb, RBC Count, MCV, MCH and MCHC, HCT, RDW-CV, and RDW-SD through a pre-validated automated veterinary hematology analyzer (Rayto, RT-7600, China).

For red cell morphology, dried and stained slides were used to determine various morphological abnormalities of RBCs using a Camera-Equipped Microscope (Labo America, Lx 400, Inc. U.S.A) at a magnification of 100X. Three different field areas were selected, each area covering 100 RBCs, and hence, a total of 300 RBCs were examined on each slide. Different normal and abnormal forms of RBCs were thus identified, including poikilocytosis (sickle cells, spherocytes, stomatocytes, tear drop cells, bites cells, echinocytes, acanthocytes, schistocytes, elliptocytes, and rouleaux cells) and anisocytosis (macrocytes and microcytes) as described earlier (Kumiega *et al.*, 2020; Vasilatis & Christopher, 2023) (Figure 1). The percentages of each type of poikilocytes and anisocytes were determined accordingly.

Statistical Analysis

Statistical Package for Social Science (SPSS for Windows version 12, SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were inspected visually as well as through Shapiro-wilk test for normality. Overall mean (±SE) percentages for poikilocytes and anisocytes, and mean (±SE) values for RBC attributes were computed using prescribed formulae. As the percentages of poikilocytes and anisocytes lacked normality, hence the non-parametric Wilcoxon rank

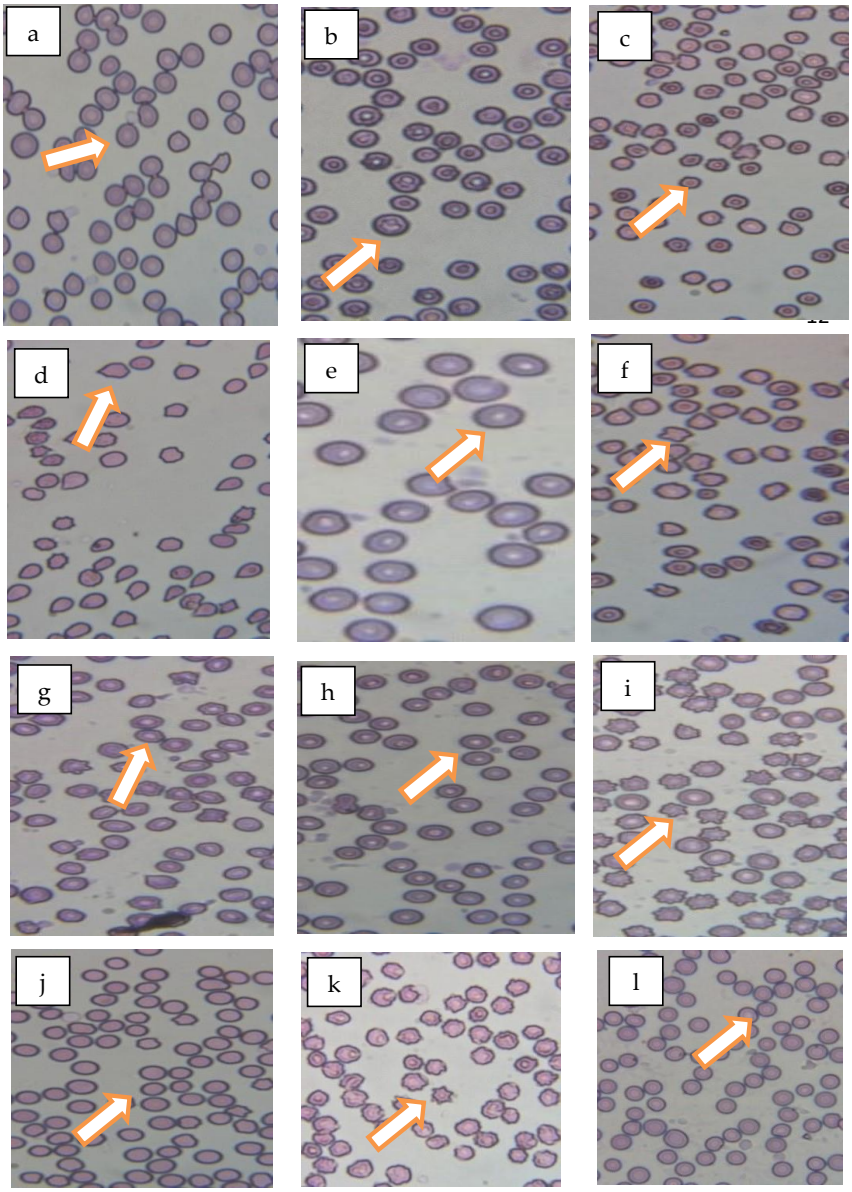

Figure 1. Various types of poikilocytes and anisocytes (a= normal cell; b= macrocyte; c= microcyte; d= tear drop cell; e= bite cell; f= schistocyte; g= elliptocyte; h= stomatocytes; i= echinocyte; j= spherocyte; k= acanthocyte; l= rouleaux) seen in the peripheral blood slide microscopy of Cholistani cattle (n=64).

sum test was implied to deduce the difference between age, sex, and Hb-based data. Using the k-clustering technique, the level of severity of total poikilocytosis was graded as slight (13%-20%), moderate (21%-30%), and severe (>30%). The level of severity was not graded for total anisocytosis as their values showed no substantial variation and were within a close range. The RBC attributes attained through the hematology analyzer, as per the level of severity of total poikilocytosis, were assessed through independent t-tests and ANOVA (post-hoc Duncan's). The cut-off value of 9.6g/dL was used to form two groups on the base of Hb level *viz*. low Hb (Hb1= ≤9.6g/dL, n=22) and high Hb (≥9.6g/dL, n=42) (Prodanović *et al.*, 2019). Pearson's correlation coefficient and linear regression were implied between total poikilocytes/total anisocytes of stained PBS microscopy and the RBC attributes attained through hematology analyzer. Regression prediction equations were thus attained and have been shown through scatterplots.

RESULTS

The results regarding normality testing revealed that the data for total poikilocytosis had highly skewed (1.5±0.7) with a higher coefficient of variance (CV) of 47.1% as compared to that for total anisocytes being less skewed (-0.06±0.7) and with less CV of 14.2%.

Overall mean (±SE) percentages for poikilocytosis and anisocytosis as per sex and age in Cholistani cattle blood are given in Table 1. Results revealed that the total poikilocytes (24.7±0.2%) were significantly (p≤0.05) higher than the total anisocytes (8.4±0.1%), whereas the normal cells had a mean (±SE) percentage of 66.7±0.9%. Amongst poikilocytes, echinocytes were the highest-seen cells (5.4±0.5%), followed by stomatocytes

(4.6±0.3%), spherocytes (2.4±0.3%), and acanthocytes (2.3±0.3%). On the contrary, sickle cells were the least noticed cells (0.1±0.03%) in the Cholistani cattle blood. Similarly, regarding anisocytes, macrocytes were higher (6.3±0.6%) as compared to microcytes (2.0±0.2%).

Results regarding the severity of total poikilocytosis as per sex and age are given in Figure 2. All the animals of the study groups showed slight to moderate total poikilocytosis with a higher frequency of moderate total poikilocytosis in females (n=17; 26.5%) followed by that in males (n=15; 23.4%). Males and adult cattle did not show marked total poikilocytosis, however, young (n=18; 28.1%) and females (n=7; 10.9%) revealed higher marked total poikilocytosis.

Regarding the sex-based results, echinocytes were significantly (p≤0.05) higher in females (5.7±0.5%) as compared to males (3.4±1.6%). However, no statistical difference (p≥0.05) was noticed for anisocytes within males and females. The spherocytes and schistocytes were significantly (p≤0.05) higher in young cattle as compared to adult cattle (Table 1).

Regarding the poikilocytes in Hb-based groups, normal cells were significantly higher (p≤0.05) in Hb1 group (low Hb≤9.6g/dL) being 68.5±1.5% as compared to 65.0±0.8% for Hb2 group (high Hb≥9.6g/dL), similarly schistocytes were higher (3.0±0.4%) in Hb1 group (low Hb≤9.6g/dL) as compared to that in Hb2 group (high Hb≥9.6g/dL) (1.8±0.1%) (Figure 3). However, no statistical difference (p≥0.05) was noticed for anisocytes in the two Hb-based groups.

Overall mean (±SE) values for various RBC attributes as affected by the severity of total poikilocytosis in sex and age-based groups are given in Table 2 and Table 3, respectively. None of the RBC attributes were affected significantly (p≤0.05) neither by the severity of total poikilocytosis nor by total anisocytosis in the study animals. Values of all attributes were within the physiological range reported earlier for similar species of cattle.

Table 1. Overall mean percentages (±SE) for poikilocytosis and anisocytosis as per sex and age in Cholistani cattle blood (n=64)

Poikilocytes (%)	Sex		Age		Overall (n=64)
	Females (n=39)	Males (n=25)	Young (n=36)	Adults (n=28)	
Normal cells	66.7±0.9	67.3±3.6	66.2±1.3	67.0±1.2	66.7±0.9
Poikilocytes					
Sickle cells	0.1±0.03	0	0.2±0.07	0.07±0.03	0.1±0.03
Spherocytes	2.3±0.3	3.1±0.6	2.6±0.5	2.3±0.4*	2.4±0.3
Stomatocytes	4.7±0.3	3.5±0.7	4.5±0.5	4.6±0.3	4.6±0.3
Tear drop cells	1.4±0.2	2.3±0.7	2.3±0.4	1.1±0.2	1.6±0.2
Bite cells	1.7±0.2	0.7±0.3	1.6±0.3	1.5±0.2	1.5±0.2
Echinocytes	5.7±0.5*	3.4±1.6	4.2±0.6	6.1±0.7	5.4±0.5
Acanthocytes	2.3±0.3	2.7±1.1	1.7±0.4	2.7±0.4	2.3±0.3
Schistocytes	2.6±0.3	1.2±0.3	3.0±0.6	2.1±0.2*	2.4±0.2
Elliptocytes	0.8±0.1	0.7±0.3	1.0±0.2	0.7±0.1	0.8±0.1
Rouleaux	3.1±0.2	3.7±0.5	2.7±0.3	3.4±0.2	3.1±0.2
Total	24.7±0.2	21.3±0.1	23.8±0.3	24.5±0.2	24.7±0.2
Anisocytes					
Macrocytes	6.1±0.6	7.7±2.6	6.3±1.5	6.3±0.4	6.3±0.6
Microcytes	1.9±0.2	2.9±1.3	2.9±0.6	1.5±0.2	2.0±0.2
Total	8.0±0.01	10.6±0.1	9.2±0.01	7.8±0.01	8.4±0.1

Note: *Significant at p≤0.05 within rows for sex and age groups.

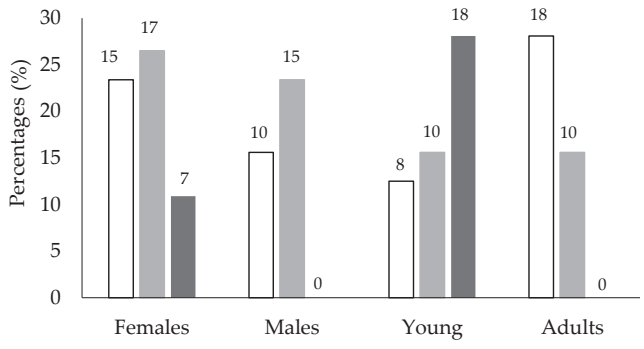

Figure 2. Severity of total poikilocytosis as per sex and age-based groups in Cholistani cattle (n=64) blood. Numbers on the bars indicate number (n) of animals. □= slight; ▒= moderate; ■= marked.

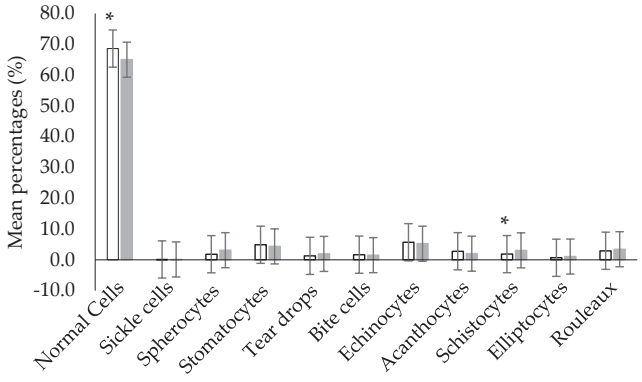

Figure 3. Physiological poikilocytosis in Cholistani cattle (n=64) blood in hemoglobin-based groups. Hb1= Low Hb ≤9.6g/dL, n=22; Hb2= high Hb ≥9.6g/dL, n=42. *on the error bars indicates significant difference (p≤0.05) for specific relevant RBC form, within two groups. □= Hb1; ▒= Hb2.

Table 2. Overall mean (±SE) values for various red blood cells (RBC) attributes as per sex, based upon the severity of total poikilocytosis in Cholistani cattle blood (n=64)

RBC attributes	Females (n=39)			Males (n=25)		Overall (n=64)
	Slight	Moderate	Marked	Slight	Moderate	
RBC count (10^{12}/µL)	6.7±0.6	6.5±0.2	7.0±0.3	6.9±0.4	7.3±0.7	6.7±0.1
Hemoglobin (g/L)	96.6±0.6	80.0±0.4	100.0±0.3	89±0.9	100.6±0.7	101.2±5.2
Hematocrit (%)	28.3±2.1	28.3±1.1	31.4±0.9	28.5±2.7	28.5±2.1	28.9±0.7
Mean corpuscular volume (fL)	42.5±2.1	43.8±1.4	46.2±2.1	41.4±2.8	38.3±2.0	43.6±0.9
Mean corpuscular hemoglobin (pg)	14.6±0.7	13.6±0.5	14.4±0.6	12.9±0.5	14.1±1.0	13.9±0.3
Mean corpuscular hemoglobin concentration (g/L)	34.7±2.0	31.0±0.6	31.3±0.4	31.1±0.4	36.4±0.8	31.0±9.5
Red blood cell distribution width-coefficient of variation (%)	13.4±0.9	14.8±0.3	14.4±0.4	14.8±0.8	12.8±0.8	14.4±0.2
Red blood cell distribution width-standard deviation (fL)	25.3±2.9	29.8±1.1	30.6±1.0	28.5±1.2	20.5±1.0	28.7±0.8

Table 3. Overall mean (±SE) values for various red blood cells (RBC) attributes as per age, based upon the severity of total poikilocytosis in Cholistani cattle blood (n=64)

RBC attributes	Young (n=36)			Adults (n=28)			Overall (n=64)
	Slight	Moderate	Marked	Slight	Moderate	Marked	
Red blood cells count (10^{12}/µL)	6.9±0.4	6.8±0.5	8.0±0.6	6.6±0.7	6.4±0.2	6.6±0.3	6.7±0.1
Hemoglobin (g/L)	79.0±0.1	93.0±0.2	95.0±0.4	92.0±0.8	82.0±0.7	102.0±0.9	101.2±5.2
Hematocrit (%)	25.8±1.5	28.0±1.0	30.0±1.9	28.6±2.5	28.8±1.4	31.8±1.2	28.9±0.7
Mean corpuscular volume (fL)	37.5±2.0	41.2±2.1	37.5±2.1	43.0±2.8	45.3±1.5	48.9±1.8	43.6±0.9
Mean corpuscular hemoglobin (pg)	11.5±1.2	13.7±1.0	11.8±0.7	14.9±1.2	13.9±1.3	15.3±1.2	13.9±0.3
Mean corpuscular hemoglobin concentration (g/L)	30.5±1.4	33.4±1.9	31.4±1.4	33.2±1.7	30.7±0.5	31.3±1.8	31.0±9.5
Red blood cell distribution width-coefficient of variation (%)	15.1±0.4	14.3±0.4	16.2±0.3	13.9±0.7	14.7±0.7	13.8±0.9	14.4±0.2
Red blood cell distribution width-standard deviation (fL)	26.3±1.4	26.4±2.0	28.6±2.1	27.0±0.9	30.8±1.7	31.3±1.9	28.7±0.8

Pearson's correlation coefficient revealed that none of the types of poikilocytes had a significant ($p \leq 0.05$) relation with RBC attributes attained through the hematology analyzer. However, total anisocytosis had a positive relation with MCH ($r=0.300$; $p \leq 0.001$) and MCV ($r=0.309$; $p \leq 0.001$). Results for linear regression between the total poikilocytes (Figure 4) and total anisocytes (Figure 5) with the studied RBC attributes attained through hematology analyzer showed low relation with each other (as indicated through low r-square values). The highest values were attained for the relation between total anisocytes and Hb, and between total anisocytes and MCHC (r-square=0.159; 15.9% probability).

DISCUSSION

Poikilocytosis and anisocytosis are umbrella terms used for changes in RBC shape and RBC size, respectively. They have both been subclassified according to specific shape and size, respectively, some of which have unique diagnostic/prognostic value while others are non-specific. Physiological poikilocytosis is a species-specific phenomenon that is quite evident in dogs, pigs, and young goats (Lassen *et al.,* 2004; Vasilatis & Christopher, 2023). However, its pathological manifestation depends on the type of shape change in the RBCs. Extensive work has already been reported on RBC morphology of cattle; however, aspects of physiological poikilocytosis and anisocytosis, especially in context to reliability-correlation with RBC attributes attained through automated hematology analyzers, have not yet been navigated. This study is, hence, a novel one that has quantified physiological poikilocytosis (10 subclasses) and anisocytosis (02 subclasses) in cattle blood regarding prevalence, types, and severity as affected by age, sex, and Hb-level. Considering higher skewness and CV for the data of total poikilocytosis as compared to the data of total anisocytosis, three levels of severity were devised only for total poikilocytosis in the present study, *i.e.* slight, moderate, and marked. The study has also provided their level of predictability through various RBC attributes attained through a hematology analyzer. These preliminary results may assist in understanding etiopathological aspects of various blood-borne diseases in cattle in the long run. In case of lack of similar prior studies on cattle, the results of the present study have been discussed with similar studies conducted on species other than the cattle.

In the present study, the total poikilocytes were significantly higher than the total anisocytes in Cholistani cattle blood. Results similar to ours have been reported in a study conducted on Red Sindhi cattle, cross-bred, and an indigenous Indian cattle breed earlier (Dash, 2020). Yet another study from India has also reported similar results (Chowdary *et al.,* 2018). The higher percentage of poikilocytosis in Cholistani cattle blood in

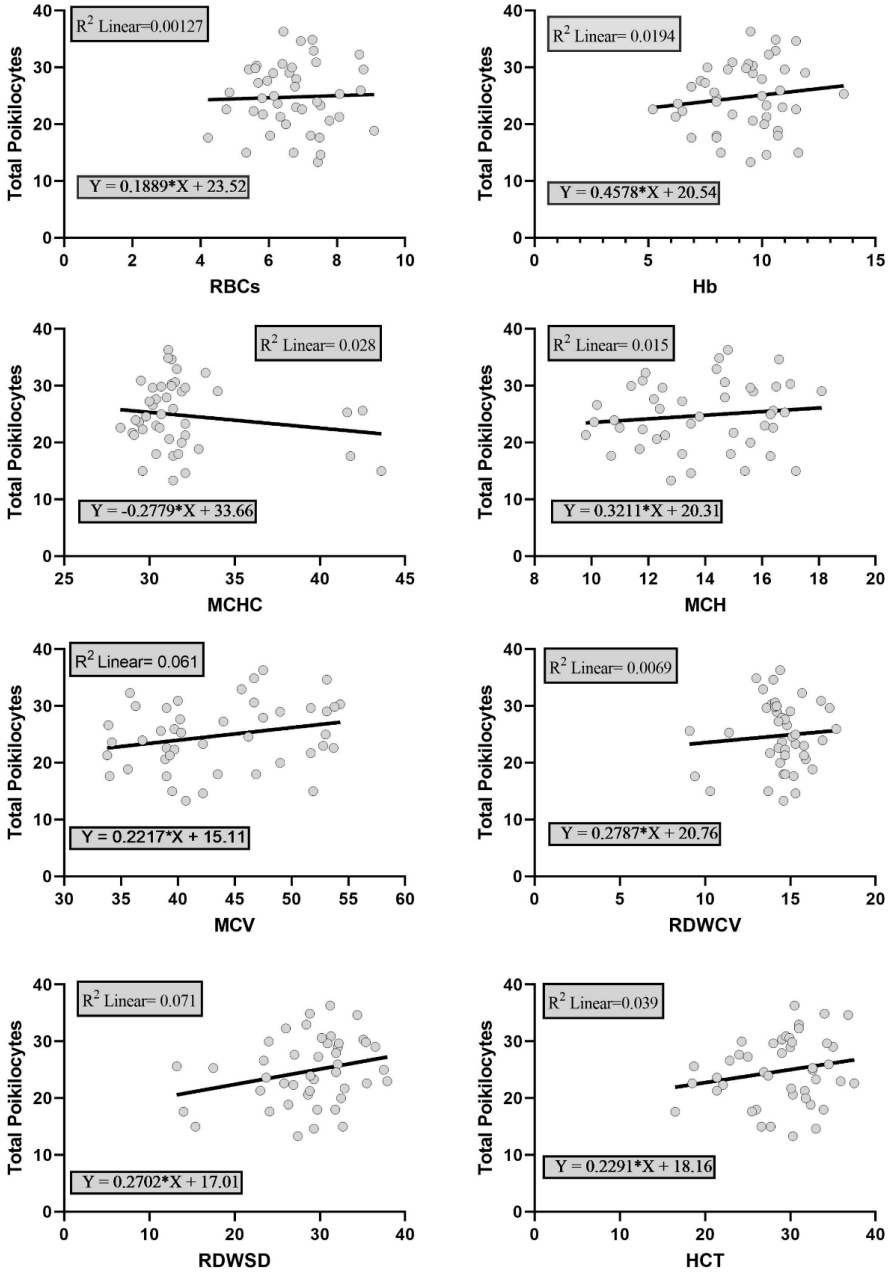

Figure 4. Scatterplot for loglinear regression between total poikilocytes and various red blood cells indices in Cholistani cattle (n=64) blood. RBC= RBC count, Hb= Hemoglobin, MCH= Mean corpuscular volume, MCV= Mean corpuscular volume, MCHC= Mean corpuscular hemoglobin concentration, RDWCV= Red cell distribution width-coefficient of variation, RDWSD= Red cell distribution width-standard deviation, HCT= Hematocrit.

our study seems to be an inherent characteristic of this breed, which may be attributed to its adaptability to a harsh desert climate.

Amongst the poikilocytes, echinocytes were the most seen cells in the stained PBS, followed by stomatocytes, spherocytes, and acanthocytes. Echinocytes, acanthocytes, and schistocytes are mostly considered to be physiologic in nature, in young cattle, and young goats. These three forms are also indications of aging/old blood (Vasilatis & Christopher, 2023). Echinocytes (crenated cells) are spiculated RBCs with evenly-spaced and evenly-distributed spicules on the surface of the RBCs. They are formed due to the increase in surface area of outer lipid layer as compared to the inner lipid layer of the RBC. On the contrary, the acanthocytes (spur cells) have irregularly spaced and variably-sized spicules. An increased cholesterol in the RBC membrane initiates the production of these cells. In a stained PBS, the differentiation of these two spiculated cells is quite difficult. A prior study conducted on three cattle breeds has reported echinocytes in the stained PBS of Red Sindhi cattle, cross-bred, and an indigenous Indian cattle breed (Dash, 2020). Similarly, 5.0% of echinocytes have been reported for black-mottled young cows of Russia in the stained PBS (Derkho *et al.*, 2019). It has been established that echinocytosis is an artefactive alteration mostly either due to *in vitro* cell aging, which results in the decreased ATP or due to less EDTA in the blood (Senior, 2010).

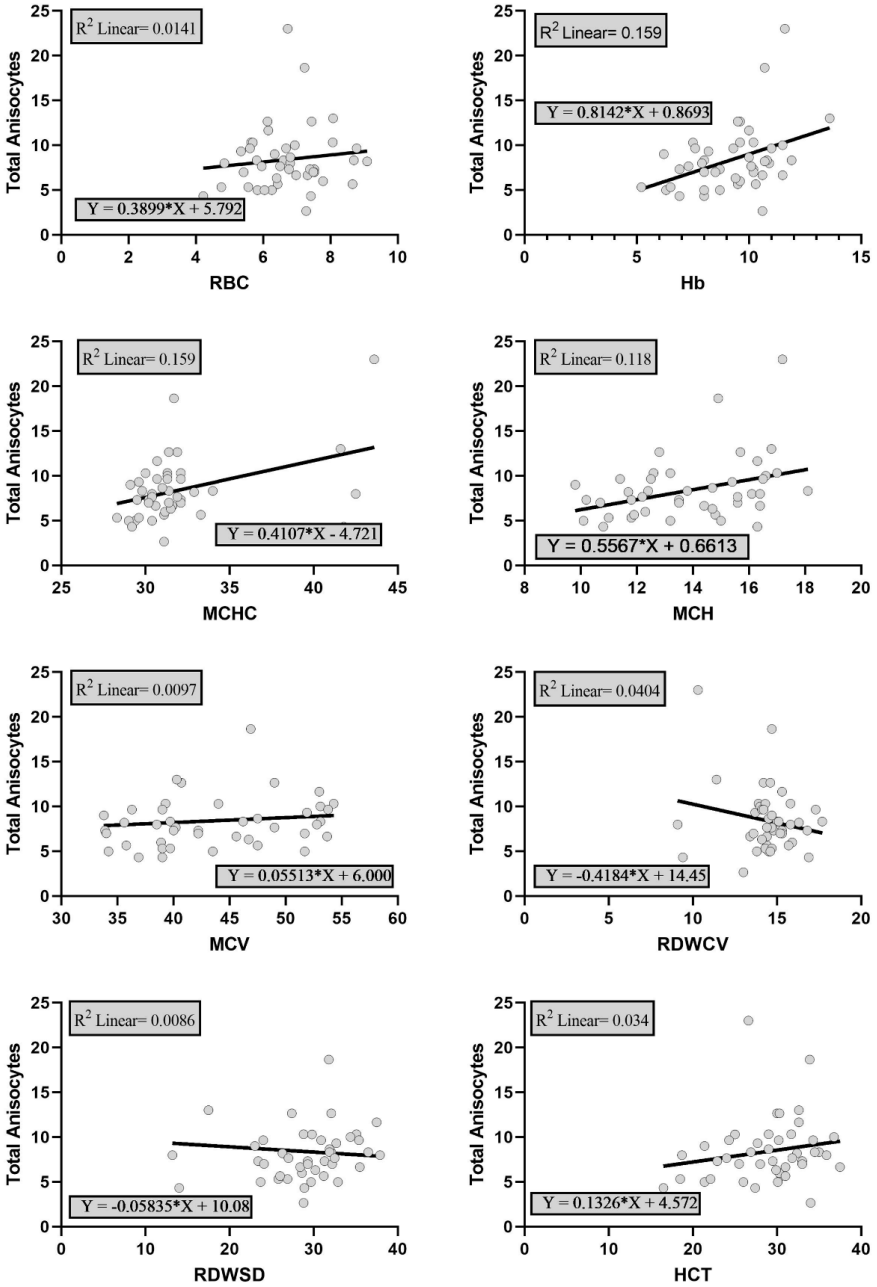

Figure 5. Scatterplot for loglinear regression between total anisocytes and various red blood cells indices in Cholistani cattle (n=64) blood. RBC= RBC count, Hb= Hemoglobin, MCH= Mean corpuscular volume, MCV= Mean corpuscular volume, MCHC= Mean corpuscular hemoglobin concentration, RDWCV= Red cell distribution width-coefficient of variation, RDWSD= Red cell distribution width-Standard deviation, HCT= Hematocrit.

Drugs such as non-steroidal anti-inflammatory drugs also induce echinocytosis. In dogs (Kumiega *et al.*, 2020; Miglio *et al.*, 2023), cats, and exotic pets (Christopher *et al.*, 2014; Dervišević *et al.*, 2022; Poljičak-Milas *et al.*, 2009), the hepatic and renal disorders result in higher number of echinocytes. However, as they are majorly considered artefactive, their pathogenic importance is of little value, especially in cattle. The higher number of echinocytes and acanthocytes in our study could, hence, be plausibly attributed to artefacts of the blood slide.

Stomatocytes are cup-shaped RBCs with elongated central pallor. The prevalence of these cells in the present study (4.6±0.3%) is in line with the values of stomatocytes reported for Indian indigenous (Dash & Mohanty, 2015) and black-mottled Russian cattle breeds (Derkho *et al.*, 2019). Their clinical significance has been reported for dogs but not for cattle (Castillo & Williams, 2021). In cattle, they have been considered an artefact of a thick blood smear, which may be the cause of a higher number in our study as well.

Results regarding the severity of total poikilocytosis as per sex and age in the present study revealed that all the animals showed slight (13%-20%) to moderate (21%-30%) total poikilocytosis. However, a higher frequency of marked (>30%) total poikilocytosis was noticed for young, followed by that in female Cholistani cattle. A study conducted on three cattle breeds (Indian indigenous, Red Sindhi, and crossbred) has reported that the

appearance of slight to marked poikilocytosis in young cattle is a physiological characteristic of cattle (Dash & Mohanty, 2015). Further studies have also reported that healthy cattle, at any age and sex, may show mild to marked poikilocytosis (Dash, 2020). None of the cattle in our study showed total poikilocytosis less than 13%. However, a higher value of 37% has been reported from anemic cattle in India (Chowdary *et al.,* 2018). A prior study on apparently healthy goats has reported a higher prevalence of poikilocytosis in goat kids (45.2%) as compared to adult goats (19.3%). Hemoglobin class switching from fetal hemoglobin (HbF) to neonatal hemoglobin (HbC) and ultimately to adult hemoglobin (HbA) in young ruminants has been given as a plausible physiological justification (Dash, 2020; Signore *et al.,* 2023; Vasilatis & Christopher, 2023). Furthermore, as presented above, echinocytes and acanthocytes were the cells most commonly seen among poikilocytes in the present study. Hence, a higher value for total poikilocytosis in the present study could be an artifactual appearance. This feature could also be a physiological peculiarity of the blood of this adaptable and thermotolerant breed of cattle (Farooq *et al.,* 2010; Saeed *et al.,* 2022).

Age-based results of the present study revealed that schistocytes and spherocytes were higher in young cattle (less than one year old) than in adult cattle (above one year old). Both these forms of poikilocytosis are, in fact, RBC fragmentation. Schistocytes are formed due to vascular abnormalities, which result in damage to blood/RBCs or turbulent blood flow in the vessels. Disseminated Intravascular Coagulation (DIC) in young calves and dogs is typically indicative of higher schistocytes (Kumiega *et al.,* 2020). In female cattle, bacterial sepsis due to mastitis may induce DIC, which enhances schistocytes in the blood smear (Reshetnikova & Krylova, 2023; Suryawanshi *et al.,* 2019). Theileriosis-positive cattle have also been reported to have a higher number of schistocytes (Happi *et al.,* 2020). Spherocytes, on the other hand, lose their biconcavity, and become spheres. Their prevalence is majorly correlated with immune-mediated hemolytic anemia (IMHA) in dogs. However, as the RBCs of cats, cattle, and horses have smaller central pallor, identification of spherocytes is difficult in these species as compared to dogs. The higher number of these two poikilocytes in young cattle of our study could be attributed to an underlying asymptomatic blood-borne disorder (such as theileria). It is noteworthy that the present study was conducted on apparently healthy Cholistani cattle, which are indigenous cattle breeds in Pakistan. This breed is a thermotolerant, tick-resistant breed. Substantial prevalence of theileria has been reported for this breed; however, its self-adaptability to climate and potential for disease-resistance may deem it asymptomatic for the disease(s) (Saeed *et al.,* 2016).

In the present study, 3.1±0.2% of rouleaux was observed in the stained PBS for Cholistani cattle blood. Higher values of 10.0±1.0% and 7.0±0.5% for Russian black-mottled cows after the first calving and for cows after the second calving, have been reported elsewhere, respectively (Derkho *et al.,* 2019). Similarly, rouleaux formation in blood slides of Indian native cattle blood has also been reported (Dash & Mohanty, 2015). All these results are contradictory to the general notion that the bovine blood is rouleaux-resistant unless there are increased inflammatory proteins. This is due to a characteristic feature of the phospholipid matrix of the cell membrane of bovine (and other ruminants) RBC, according to which there is more sphingomyelin instead of phosphatidylcholine (Derkho *et al.,* 2019). The RBCs of pigs, dogs, and cats, on the contrary, have the tendency of rouleaux formation both in physiological and pathological conditions. The presence of rouleaux in our study may be due to an adaptable mechanism of Cholistani breed, which renders them to have higher plasma proteins, which in turn, causes the RBCs to form rouleaux.

Regarding the Hb-based groups, the normal cells were significantly higher in the low Hb group (Hb1) (≤9.6g/dL) as compared to that for the high Hb group (Hb2) (≥9.6g/dL) in the present study, similarly, schistocytes were also higher in low Hb group as compared to those in high Hb group. All the Cholistani cattle (n=64) included in the present study were clinically or visually healthy, and no obvious signs of anemia were evident. However, considering the cut-off value of 9.6g/dL, from the total cattle, few (n=22) had low Hb indicative of anemia. The present results cannot be discussed as no such prior studies are available. However, an intricate study on Hb variants of this breed in correlation to RBC morphology could provide a better picture.

For anisocytosis as observed in stained PBS, macrocytes were higher (6.3±0.6%) in the present study as compared to microcytes (2.0±0.2%). However, these values are lower than those of 26.8±1.0% reported for Russian black-mottled cattle, which tended to decrease with age (Derkho *et al.,* 2019). The reticulocytes (immature RBCs) larger than the normal RBCs are referred to as macrocytes. It has been elaborated that the basophilic stipplings in the blood of ruminants cause regenerative anemia, increasing erythropoiesis, and ultimately increased macrocytes (Jain, 1986; Stirn & Freeman, 2022). Mild to moderate anisocytosis is considered physiological in cattle, whereas marked anisocytosis may be due to the presence of large polychromatophilic RBCs showing a regenerative bone marrow response (Signore *et al.,* 2023). In young cattle, mild to marked anisocytosis is an indication of larger RBCs than those of adult animals (Brun-Hansen *et al.,* 2020). Pathological and physiological macrocytosis has been reported for horses and dogs; however, its clinical significance in cattle is questionable (Freeman & Klenner, 2015; Harvey, 2011).

In the present study, no RBC attributes were affected significantly by the severity of total poikilocytosis nor by total anisocytosis in the study animals. Values of all attributes were within the physiological range reported earlier for similar species of cattle presented elsewhere (Saeed *et al.,* 2022). This physiological peculiarity could be assigned to the justification that the Cholistani cattle breed is a thermotolerant breed and tends to maintain its hematochemical profile within close range as an adaptive physiological mechanism, as shown through various previous studies conducted on reference values of this breed (Farooq *et al.,* 2017; Saeed *et al.,* 2016; 2022).

The reliability of predicting total poikilocytosis/ total anisocytosis through stained PBS from the RBC attributes attained through an automated hematology analyzer in the present study revealed that both the total poikilocytes and total anisocytes had a very low relation with each other, as indicated through r-values and low r-square values. The highest level of relationship was attained between total anisocytes and Hb, and between total anisocytes and MCHC in the present study (r-square=0.159; 15.9% probability). Higher correlation has been presented through Pearson's correlation and regression analyses between poikilocytes/anisocytes and hematology analyzer-deduced indices for human blood (Kumar *et al.,* 2013). However, there is a paucity of such literature for ruminant species. The automated hematology analyzers provide rapid cell counts and vital RBC indices such as MCV, MCH, MCHC, RDW-SD, and RDW-CV. The MCV and RDW are considered indicative of macrocytosis and anisocytosis, respectively (Caporal & Comar, 2013). They normally fail to detect any intra-erythrocytic bodies and hemoparasitism. The multi-species hematology analyzers, in vogue also give questionable results regarding poikilocytosis and anisocytosis in the form of RBC indices and RDWs.

CONCLUSION

In conclusion, the blood of healthy Cholistani cattle presents a wide range of physiological poikilocytosis (13%-36%) and anisocytosis (2%-23%), which could be a physiological peculiarity of this breed. The young and female cattle showed marked total poikilocytosis as compared to their counterpart male and adult groups. The severity of alteration in RBC shape and size does not affect the hematological attributes in Cholistani cattle breed. Furthermore, the multi-species hematology analyzer failed to predict the level of poikilocytosis and anisocytosis in the present study, whereas the stained PBS provided reliable results. The data of this preliminary study may assist in understanding etiopathologic aspects of various blood-borne disorders in cattle. We recommend PBS in adjunct to the results of hematology analyzers for assessing reliable health status in cattle. As this work was conducted on apparently healthy animals, future research direction could include incorporation of diseased cattle. In addition, the use of artificial intelligence in differentiating and counting various forms of RBCs and cell counting could be navigated.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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