Scientific Papers. Series D. Animal Science. Vol. LXVII, No. 2, 2024
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

VALIDITY OF HEMOGLOBIN ESTIMATION METHODS FOR CHOLISTANI CATTLE BLOOD: A METHOD-COMPARISON STUDY

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Abstract

The present study was conducted with objective to evaluate the validity of three hemoglobin (Hb) estimation methods, including the cyanmethemoglobin method (HbC), Sahli's method (HbS), and veterinary hematology analyzer (HbA) in Cholistani cattle (n=100). Blood samples were collected aseptically from apparently healthy cattle. The results regarding the overall data and the data for age, and sex-wise groups revealed that HbA was significantly ($p \leq 0.05$) different from HbC, whereas HbS was non-significantly ($p \leq 0.05$) different from the HbC. The Bland and Altman chart between HbS and HbC, and between HbA and HbC showed significantly higher level of agreement between HbS and HbC with no proportional bias on the distribution of data around the mean difference line (Mean= 0.39, 95% CI= 0.21 to 0.57). Cronbach's alpha and intraclass correlation coefficient between HbS and HbC, and between HbA and HbC for single and average values, on similar grounds, were also higher between HbS and HbC being 0.819 and 0.900 as compared to the values of 0.793, and 0.884 between HbA and HbC. Sahli's method (a three-time average) for Hb estimation in cattle blood is comparable to the gold standard technique of the cyanmethemoglobin method, endorsing its use as a point-of-care testing device in remote areas.

Key words: *Cholistani cattle, hemoglobin, point-of-care testing.*

INTRODUCTION

Hemoglobin (Hb) is a tetrameric structural and functional unit formed by the asymmetric pairing of two polypeptide chains, the alpha and beta globulins. Within the erythrocytes, it generates carbamino compounds with carbon dioxide and buffers hydrogen ions, facilitating carbon dioxide transport in blood (Brundha & Priyadharshini, 2019). Priorly, extensive reviews have emanated globally which have reviewed the merits and demerits of various Hb estimation methods mainly the Tallquist method, Copper sulphate method, Sahli's method, Lovibond comparator, cyanmethemoglobin method, Hb color scale, and HemoCue (Srivastava et al., 2014). Their precision, accuracy, sensitivity, specificity and repeatability for human blood has also been reported (Barduagni et al., 2003; Adam et al., 2012; Agnihotri et al., 2015). In comparison to these methods, lately the 3-part and 5-part automated veterinary hematology analyzers are frequently being used to determine Hb levels in human and veterinary hematology. These machines, though highly accurate and reliable, are yet costly, tedious and need trained personnel. Furthermore, the transfer of blood samples to the laboratory may delay treatment, resulting in disease aggravation (Adam et al., 2012). The use of such analyzers has resultnalty quite limited in resource-poor countries (including Pakistan) owing to aforementioned limitations.

The World Health Organization conducted research on Hb determination using the Hb color scale in which a color of the drop of blood was compared to specified red shades (Darshana & Uluwaduge, 2014). The scale comprises of a little card with six different red colors representing Hb levels of 4, 6, 8, 10, 12, and 14 g/dL. HemoCue portable photometer is another way for determining Hb. It comprises of disposable microcuvettes holding dry reagent and a single-purpose photometer. The precision of HemoCue for assessing Hb concentration in venous or capillary blood samples was inferior

and was not equivalent to that of an automated hematology analyzer (Kapoor et al., 2002).

The cyanmethemoglobin technique is widely regarded as the gold standard for Hb estimation. It can measure all forms of Hb except sulfhemoglobin. However, there are several drawbacks of using this method, as it may be toxic due to presence of cyanide in its reagent, presence of turbidity, and it requires skilled technician and presence of unique equipment (Kapoor et al., 2002; Srivastava et al., 2014).

Sahli's approach is doable by hand. It is less expensive, less time consuming, more convenient, and simpler to carry out. As a result, it is a superior choice for on-field investigations rightly coming under the definition of 'point-of-care testing device' (POCT) (Singh et al., 2015) However, visual mistake is likely when matching the brown hue of the comparator box in this procedure, and all types of hemoglobin cannot be measured (Balasubramaniam & Malathi, 1992).

Cholistani cattle are phenotypically a big, with a stumpy body, having short horns, long ears, and substantial dewlap both in males and females. Males possess a prominent hump. The body of this breed of cattle is speckled with red, black, or brown dots, and its tail features a black switch. The genetically superior Cholistani cows may produce 15 to 18L of milk each day (Farooq et al., 2010). This breed gained its fame since 2010 and extensive research work on its reproductive and productive attributes has since been reported from Pakistan (Tausif, 2008; Ali et al., 2009; Shahzad et al., 2010) Lately, our laboratory has published results regarding the reference intervals for various hematochemical profile of apparently healthy Cholistani cattle being reared under nomadic pastoralism in the Cholistan desert of Pakistan (Saeed et al., 2022). Furthermore, our laboratory has also initiated work on validation of various hematological attributes and deducing pen-side hematological formulae for various Cholistani livestock (Ahmad et al., 2022a; Ahmad et al., 2022b; Farooq et al., 2023). However, no work has yet been reported on assessing the diagnostic efficacy of various Hb estimation methods for this breed of cattle which may be used as point-of-care testing (POCT). The present study is therefore being devised with an objective to assess the validity of various Hb estimation methods (Sahli's method, cyanmethemoglobin method, automated veterinary hematology analyzer) for blood of Cholistani cattle being reared under pastoralism in Cholistan desert, Pakistan.

MATERIALS AND METHODS

The research work was conducted at the Cholistan desert (for blood sampling) and post-graduate laboratory of the Department of Physiology, Islamia University of Bahawalpur (for analyses). The cyanmethemoglobin technique is regarded as the gold standard for Hb detection. Pakistan throughout the time of June 2022 to May 2023. This desert has an area of 26,000 km^2 and is located in latitudes 27°42′ and 29°45′ North, longitudes 69°52′ and 75°24′ East, and at a height of roughly 112m above sea level (Farooq et al., 2017). It comes under the domain of semi-arid tropical climate with average temperature of 28.33°C. Month of June is considered as the warmest month with a temperature soaring beyond 45°C (Farooq et al., 2010). Cholistan has an annual rainfall of up to 180mm. November through January are coldest of the months having an average temperature of 13°C. Cholistani cattle (n = 100) being reared by the desert nomads of Cholistan were incorporated in this study. Detailed interviews were conducted with these nomads and clinical assessment of the animals, the overall health of the animals was assessed, and only apparently healthy animals were included in the research. According to the anamnesis provided by pastoralist herders, animals deemed to be listless, depressed, off-feed, and separated from the herd were excluded from the research. Demographic information was gathered through focal group discussion from the livestock herders.

As directed by protocol, a disposable syringe was used to take blood samples from the cattle's jugular vein. After being carefully inverted and placed in an icebox, the blood samples were moved into purple-topped EDTA-containing vacutainers (Becton Dickinson, USA) and examined for hematological analysis within eight hours. Three approaches were suggested in this study to estimate the Hb levels in the blood of Cholistani cattle; as given below:

a) Sahli's method: The Sahli's approach includes converting Hb to acid hematin and visually comparing the resultant color to that of conventional colored glass. The value of Hb

(HbS) was read straight from the graduated Hb tube (Balasubramaniam & Malathi, 1992). Three trained personnel took this reading separately in order to eliminate interpersonal errors and the reading was taken in broad daylight. Mean of these readings was taken into account.

b) Cyanmethemoglobin method: For the cyanmethemoglobin method, about 5mL of Drabkin's solution (SDL Company, Pakistan) was taken by adding 20 µL blood into it. It was mixed thoroughly and incubated for 5 minutes. After that, the reading was taken through spectrophotometer (Irmeco U2020, Germany) set at 540 nm wavelength (Sari et al., 2001) and Hb was deduced through standard curve (HbC). This HbC was considered as gold standard method for Hb determination.

c) Automated veterinary hematology analyzer: Blood was well mixed on a Roller Mixer (MixR-40, Daihan Scientific, Korea) and then subjected to an off-hand validated automated veterinary hematology analyzer (Rayto RT-7600, China) to determine the hemoglobin concentration (HbA).

Data analysis was done using the Statistical Package for Social Science (SPSS for Windows version 12, SPSS Inc., Chicago, IL, USA). Using the Shapiro-Wilk test, the normality of the data was examined. Using the provided formulas, the means (± SE) and 95% CI for the three Hb values (HbS, HbC, and HbA) obtained in this study were calculated. ANOVA with Duncan's as a post-hoc test was suggested in order to determine the difference between the three Hb values for the total data as well as for the different study groups, namely age-wise (young, n = 45 and adults, n = 55) and sex-wise (males, n = 68 and females, n = 32). The degree of correlation between the three Hb levels was calculated using Pearson's correlation coefficient. Scatterplots were drawn and linear regression was carried out between these three Hb values and accordingly, regression prediction equations were computed. Considering cyanmethemoglobin as gold standard method of Hb estimation, level of agreement between HbS and HbC, and between HbA and HbC was assessed through Bland Altman agreement analysis (Bland and Altman, 1999). Likewise, as tests of agreement between HbS and HbC and between HbA and HbC, Cronbach alpha and intraclass correlation were also deduced from the whole data (Gerke, 2020; Shieh, 2020).

RESULTS AND DISCUSSIONS

The mean (± SE) values and 95% CI for the three Hb values attained in this study (HbS, HbC and HbA) in Cholistani cattle (n = 100) are given in Table 1.

Similarly, results for overall and group-wise (based on age and sex) data revealed that HbA was significantly ($p \leq 0.05$) different from the HbC, whereas HbS was non-significantly ($p \leq 0.05$) different from the HbC.

Table 2 displays the regression analysis results and the corresponding regression prediction equations for each age and sex group under consideration. A significant positive correlation coefficient ($p < 0.01$) was observed between HbA and HbC as well as between HbS and HbC. The greatest association between HbS and HbC, however, was found in adult stock (r = 0.866; adjusted r-square = 0.743).

Table 1. Mean (± SE) values and confidence intervals for hemoglobin determined through Sahli's, cyanmethemoglobin method and autoanalyzer

Groups		HbS (g/L)		HbC (g/L)		HbA (g/L)	
		x ± SE	CI	x ± SE	CI	x ± SE	CI
Sex	Females (*n* = 32)	100.0 ± 1.9a	96.3-103.8	102.1 ± 2.0a	98.0-106.2	105.0 ± 2.1b	100.7-109.4
	Males (*n* = 68)	98.0 ± 1.8a	94.3-101.7	105.9 ± 1.8a	102.1-109.6	99.6 ± 1.8b	95.8-103.4
Age	Young (*n* = 45)	96.9 ± 1.5a	93.9-99.9	103.0 ± 1.6a	99.7-106.2	99.7 ± 1.5b	96.6-102.7
	Adults (*n* = 55)	101.5 ± 2.2a	97.0-106.0	103.5 ± 2.4a	98.7-108.4	106.3 ± 2.6b	101.1-111.4
Overall (*n* = 100)		99.4 ± 1.4a	96.6-102.2	103.3 ± 1.5a	100.3-106.3	103.3 ± 1.6b	100.1-106.5

[a,b]Different letters within rows are different at $p \leq 0.05$ for the three hemoglobin values. HbS= Hemoglobin determined through Sahli's method; HbC= Hemoglobin determined through cyanmethemoglobin method, HbA= Hemoglobin determined through veterinary hematology analyzer; CI= Confidence interval.

Table 2. Linear regression between hemoglobin determined through Sahli's method, cyanmethemoglobin method and veterinary hematology analyzer in Cholistani cattle (n = 100)

Groups		HbS versus HbC	R	Adjusted r Square	HbA versus HbC	r	Adjusted r Square
Sex	Females (n = 32)	y=0.791(HbC)+1.9	0.860**	0.736	y=0.886(HbC)+1.4	0.848**	0.714
	Males (n = 68)	y=0.702(HbC)+2.3	0.708**	0.485	y=0.726(HbC)+2.2	0.711**	0.489
Age	Young (n = 45)	y=0.637(HbC)+3.1	0.707**	0.488	y=0.555(HbC)+4.2	0.597**	0.341
	Adults (n = 55)	y=0.795(HbC)+1.9	0.866**	0.744	y=0.910(HbC)+1.2	0.865**	0.743
Overall (n = 100)		y=0.761(HbC)+2.0	0.821**	0.671	y=0.832(HbC)+1.73	0.794**	0.626

**Significant correlation at $p \leq 0.01$. HbS= Hemoglobin determined through Sahli's method; HbC= Hemoglobin determined through cyanmethemoglobin method, HbA= Hemoglobin determined through veterinary hematology analyzer.

The Bland and Altman chart between HbA and HbC (Figures 1 and 2, respectively) and between HbS and HbC (Figure 1) indicated a better level of agreement with respect to the level of agreement.

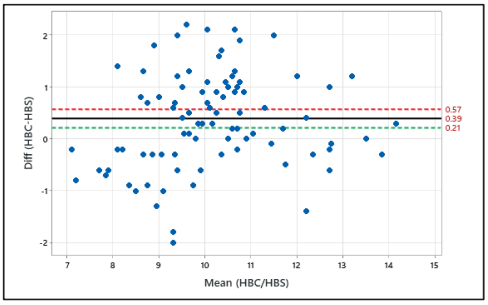

Figure 1: Scatterplot of Bland and Altman Test between Difference of Hemoglobin Determined through Cyanmethemoglobin (HbC) and through Sahli's Method (HbS) (HbC-HbS) and Average of Both Hemoglobins (HbC+HbS/2). Black line indicates mean difference (0.39) whereas the upper red and lower green lines indicate upper (0.57) and lower (0.21) values for 95% CI, respectively

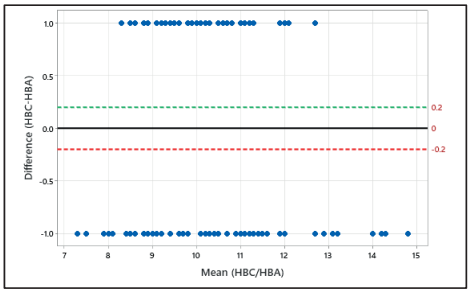

Figure 2: Scatterplot of Bland and Altman Test between Difference of Hemoglobin Determined through Cyanmethemoglobin (HbC) and through Veterinary Automated Hematology Analyzer (HbA) (HbC-HbA) and Average of Both Hemoglobins (HbC+HbA/2). Black line indicates mean difference (0.0) whereas the upper green and lower red lines indicate upper (0.2) and lower (-0.2) values for 95% CI, respectively

Additionally, there was no discernible proportional bias in the data distribution along the mean difference line between HbS and HbC (Mean = 0.39, 95% CI = 0.21 to 0.57).

Table 3 presents the findings for the intraclass correlation coefficient and Cronbach alpha between HbA and HbC and between HbS and HbC. The average and single measure values between HbA and HbC were 0.793 and 0.884, respectively, while the values between HbS and HbC were higher at 0.819 and 0.900.

Table 3. Cronbach alpha and intraclass correlation between hemoglobin determined through Sahli's method, cyanmethemoglobin method and autoanalyzer

HbS and HbC			
Intraclass Correlation		95% CI	Cronbach Alpha
Single Measure	0.819	0.74-0.87	0.900
Average Measures	0.900	0.85-0.93	
HbA and HbC			
Single Measure	0.793	0.70-0.85	0.884
Average Measures	0.884	0.82-0.92	

HbS= Hemoglobin determined through Sahli's method; HbC= Hemoglobin determined through cyanmethemoglobin method, HbA= Hemoglobin determined through veterinary hematology analyzer

Blood analysis is one of the vital and precise tools being used widely in medical practice. Therefore, globally, clinical hematology both for human and veterinary medical sciences has gathered considerable footing. And it has laid precise foundations of diagnosis/prognosis of blood-borne disorders. The development of 3-part and 5-part automated veterinary hematology analyzers has replaced the manual hematology methods such as measuring RBC and WBC using hemocytometers, packing cell volume through microcentrifugation (Organization, 2000), differential leukocyte count through stained blood smears (Hu et al.,

1993), and measuring Hb levels through cyanmethemoglobin (Kapoor et al., 2002; Srivastava et al., 2014). However, the expensiveness, periodic maintenance, need for trained personnel, continued validation, and expensive chemical reagents for these analyzers deem these analyzers unfit to be utilized as a POCT devices. Being portable, easy to use, and provision of quick results, the POCTs are in high demand especially in developing/under-developed parts of the world which either have restricted or limited excess to standard laboratory analyses (Chevalier et al., 2003; Abuelo & Alves-Nores, 2016) The present study assessed the validity and diagnostic accuracy of three Hb estimation methods (Sahli's method, cyanmethemoglobin method, automated veterinary hematology analyzer) for Cholistani cattle blood. The Sahli's hemoglobinometer, which is a POCT device for Hb estimation in humans, gave results for Hb which were similar to those attained through the automated veterinary hematology analyzer in the present study. This endorses its vitality as a POCT device for Hb estimation in cattle blood. There is almost no work conducted on the study of diagnostic accuracy and efficacy of Sahli's hemoglobinometer for cattle blood, hence, as per need, the results of the present study have been compared with prior studies conducted on human blood.

The results for overall data as well as for age and sex-wise groups in the present study showed that the Hb values for Cholistani cattle blood attained through automated veterinary hematology analyzer (HbA) were significantly different from those attained through cyanmethemoglobin method (HbC) and through Sahli's hemoglobinometer (HbS). However, the values for HbS and HbC were non-significantly different within each other, indicating a substantial diagnostic accuracy and efficacy of Sahli's method for this breed of cattle. Our results are not in line with most of the research work conducted on humans (Sari et al., 2001) and animals (DeNicola, 2011) which have shown better efficacy and diagnostic accuracy of auto-analyzers as compared to Sahli's method. Visual impairment of the observer, less light, and fading out of the comparator-color-block of the Sahli's hemoglobinometer are few of the drawbacks which decrease its efficacy for Hb estimation (Critchley & Bates, 2005). Sahli's method is a subjective test based on visual comparison and has a lower sensitivity, specificity, positive predictive value and negative predictive value as compared to other Hb estimation methods such as cyanmethemoglobin method and auto-analyzers (Barduagni et al., 2003; Brundha & Priyadharshini, 2019). A digital hemoglobinometer, in a study, was found to have lower sensitivity and specificity of 89.4% and 63.6% for pregnant women in India, respectively as compared to 98.7% and 90.2% for autoanalyzer (Toppo et al., 2019) Similarly, while comparing Sahli's method with cyanmethemoglobin method for human adults, a lower sensitivity of 86.2% has been reported as compared to 96.5% for copper sulphate method (Agnihotri et al., 2015) Apart from all this, Sahli's method is still being used as a POCT device for clinical and research purposes, especially in resource-poor settings such as Asian countries, both for human (Sari et al., 2001; Wasnik et al., 2014) and veterinary (Pathan et al., 2011; Ibrhim, 2014; Osman et al., 2017) medical sciences.

An interesting study conducted in India compared the efficacy of Sahli's method (two-time and three-time average) and autoanalyzer for Hb determination in human blood. It was reported that the three-time average of Sahli's method was statistically not different from that attained through autoanalyzer (Brundha & Priyadharshini, 2019). These results coincide with the results of the present study. In our study, average reading from three trained personnel was taken for Hb using Sahli's method which is not different from the gold standard technique of cyanmethemoglobin.

Regarding the studies on the efficacy of Sahli's method in livestock blood, the only study conducted in Iraq has compared Sahli's method against autoanalyzer for bovine, caprine and sheep blood (Ibrhim, 2014). This study has also reported that the values attained through Sahli's method are lower and statistically different than those attained through auto-analyzers for all three studied species. Another study has compared the efficacy of Mission Plus (MP) human device for Hb estimation with gold standard technique for healthy cattle blood using Passing Bablok regression analyses and Bland

Altman chart. They have reported that the human MP device is equally effective for Hb estimation as the gold standard technique for cattle blood (Heller et al., 2021).

In the present study, gold standard method of Hb estimation *i.e.* cyanmethemoglobin (HbC) was compared both with the HbS and HbA. Three main statistical tests *viz*. Bland and Altman, Cronbach alpha and Intraclass coefficient were implied for assessing the level of agreement within these three Hb values. No proportional bias on the distribution of data around the mean difference line was noticed between HbS and HbC, and there was a strong level of agreement between HbS and HbC, as compared to that between HbA and HbC. Hematology analyzers, these days, are extensively being used both by human and veterinary medical practitioners and researchers. These machines are highly sensitive machines and are validated by the manufacturers as per the (inter)national standards to be sent out into the market. However, their performance has been marred by their need of periodic validation and continued quality control measures. The difference in Hb values attained through veterinary hematology analyzer and through gold standard method in the present study may be indicative of the fact that the machines need periodic validation through manual hematological methods as reported earlier (DeNicola, 2011; Vis and Huisman, 2016; Kratz et al., 2019).

CONCLUSIONS

In a nutshell, the present study reveals that the value of Hb for cattle blood attained through Sahli's method is comparable to that attained through gold standard technique of cyanmethemoglobin method. This endorses the on-field use of this POCT device for Hb estimation in cattle being reared in far-flung areas for quicker, cheaper and reliable results. However, we recommend three-time average of the values taken through this device for better results. It is further recommended that other POCT devices for Hb estimation being used in human practice such as HemoCue, MissionPlus, and Hb color charts/scales, may also be validated for livestock blood.

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