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Comparison of haemoglobin estimation by Sahli's two - time average, Sahli's three - time average methods and automated analyzer method: A different approach in clinical pathology

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Abstract
Introduction: Haemoglobin is the iron containing oxygen carrying protein in the red blood cells in all the vertebrates. There are many methods for the estimation of haemoglobin with its pros and cons. Sahli's method is the traditional manual and low-cost effective method with a few disadvantages. Cyanmethemoglobin method is the alternative manual method for Sahli's method discovered to overcome the disadvantages of the Sahli's method. The Auto analyzer method is the newly advent commonly used method with a disadvantage of high instrumental charges. This study is to compare the manual Sahli's method with a few technical changes and the auto analyzer method of hemoglobin estimation.
Materials and Methods: A prospective study was conducted on forty blood samples which were collected from randomly selected patients attending routine blood examination, after obtaining their consent. With the samples, Haemoglobin was estimated by two methods, Sahli's Haemoglobin estimation method for three times and Electronic coulter counter method (Automated analyzer) for one time. The average of the first two times value [S-2] and the all three times value [S-3] of Sahli's method and the value obtained from the Autoanalyzer method [R] were entered using Microsoft Office Excel 2010 and were then exported to SPSS version 23 for statistical analysis.
Results and Conclusion: The results obtained were collected in spreadsheets and analysed statistically. Out of three different measurement methods for Hemoglobin estimation, we found Sahli's Three-time average has higher degree of agreement with auto analyzer method, has no significant difference with auto analyzer method and acceptable as a better alternative for auto analyzer method.

Keywords: Haemoglobin estimation, Sahli's method, electronic coulter method, auto analyzer method

Introduction

Haemoglobin is a two-way respiratory carrier, transporting oxygen from the lungs to the tissues and facilitating the return transport of carbon dioxide. In the circulation, haemoglobin has a high affinity for oxygen and a low affinity for carbon dioxide, organic phosphates, hydrogen and chloride ions. In the venous circulation, these relative affinities are reversed. To stress these remarkable properties, Jacques Monod conferred on haemoglobin the title of "Honorary enzyme" [1, 2]. If we call heme as its active site, oxygen is its substrate and hydrogen ions its inhibitors, then haemoglobin mimics the properties of the enzyme. Therefore, it became evident that unravelling the properties of haemoglobin was necessary to understand the mechanism of haemoglobin function as it pertains to respiratory physiology [3].

Haemoglobin consists of a polypeptide chain called globin related with protoheme IX – a complex planar of iron and protoporphyrin IX [4]. Haemoglobin constitutes of four subunits, each compromising of one polypeptide chain and one heme group. The adult haemoglobin polypeptide chain is of two kinds alpha and beta chains, identical in length but differ in the amino acid sequences. A polypeptide chain of 141 (alpha) and 146 (beta) amino residues constitute the iron protoporphyrin heme group. Apart from alpha and beta chains there are gamma and delta chains. N of the histidine is linked to the ferrous ion of the heme. Phenylalanine part of the polypeptide chain is linked to the porphyrin ring [5, 6].

Ferrous iron atom of the heme group reversible binds to the oxygen. Oxygen bonded heme group varies with that of partial pressure of oxygen.

When there is a cooperative interaction between the oxygen binding sites a sigmoid shape of the oxygen equilibrium curve is obtained. Red colour of the haemoglobin is obtained when it is fully saturated with oxygen at a partial pressure of 100mm Hg [7]. Haemoglobin within the erythrocyte forms carbamino compounds with carbon dioxide and buffers hydrogen ions which facilitates the carriage of carbon dioxide in blood [8].

There are many methods for the estimation of haemoglobin with its pros and cons. Some of the methods to estimate haemoglobin are listed below. In Drabkin haemoglobin estimation, 20 micro litre of blood is mixed with 5ml of Drabkin solution mixed well and kept at room temperature for 15 minutes. Absorbance was taken at 540 nm against Drabkin's solution. The hemoglobin level was estimated from the absorbance of cyanmethemoglobin standard solution containing known concentration of hemoglobin. The Drabkin haemoglobin estimation method was easy to perform and required only a small sample of blood. Drabkin solution should be stored in a dark and protected from light. The reagents required for the experimental procedure is expensive and need to be purchased separately. International Committee for Standardisation in Haematology has recommended Direct cyanmethemoglobin method for the quantitative estimation for haemoglobin. It is relatively a simple and cost-effective method. In the Drabkin's method of haemoglobin estimation, Oxidation of haemoglobin to methaemoglobin by potassium ferrocyanide. Cyanmethemoglobin is formed when methaemoglobin reacts with cyanide ions of potassium cyanide. Estimation of haemoglobin is done by cyanmethemoglobin curve [9, 10]. A stable compound cyanmethemoglobin is formed. During large scale surveys, transportation of the blood through vials to long distance laboratory was difficult and did not produce feasible results [11, 12].

Another method for the estimation of haemoglobin is indirect cyanmethemoglobin method works on the same principle except that it involves spotting of blood on filter paper. Due to the more advantages like minimal invasiveness, easy packing and transportation of long distance this method is used for large scale surveys [13].

In the traditional Sahli's haemoglobin estimation method compromises of simple instruments and does not involve any complex method for estimation. Sahli's haemoglobin estimation consists of a Sahli's haemoglobin tube which is graduated with gram percentage 2 to 24. A comparator with a brown glass standard is present. For uniform illumination an opaque white glass is present behind the comparator. Sahli's pipette or haemoglobin pipette marked till 20 micro litres without a bulb is seen. A stirrer made up of thin glass rod is also present. In Sahli's method conversion of haemoglobin to acid haematin and visually comparing the colour developed with that of standard tinted glass which were standardised with a value of 14 gm/dl. The graduated Sahli's haemoglobin tube gives the haemoglobin value [14].

The very latest method that is the Electric coulter counter method works on the Coulter principle. The Coulter principle states that particles pulled through an orifice, concurrent with an electric current, produce a change in impedance that is proportional to the volume of the particle traversing the orifice. This pulse in impedance originates from the displacement of electrolyte caused by the particle [15, 16]. Through a narrow aperture a precise volume of blood is allowed to pass and impedes an electrically charged field. 'Blip' sound is produced which is counted as a cell. Greater the electrical displacement, larger the Red blood cell. In a separate chamber, the same volume is hemolyzed and colorimetrically analyzed to determine the hemoglobin concentration. There are certain values which are measured from the electric coulter method they are Red blood cell count, Mean corpuscular volume, Haemoglobin concentration. Using the measured value obtained certain values like the Mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, haematocrit and Red blood cell width can be calculated [17, 18]. This study is carried out to compare the manual Sahli's method with a few technical changes i.e. Two time average [S-2] and Three – time average [S-3] and the auto analyzer method [R] of hemoglobin estimation, to know which method is compatible in a detrimental state where we could not use auto analyzer method.

Materials and methods

A prospective study was conducted on forty dipotassium EDTA blood samples which were collected from randomly selected patients attending routine blood examination at the from Clinical Laboratory of a tertiary care dental hospital in South India, after obtaining their consent. With the samples, Haemoglobin was estimated by two methods, Sahli's Haemoglobin estimation method and Electronic coulter counter method (Automated analyzer). The testing for Sahli's method was done without any delay while the samples were run on the automated analyzer immediately or within 30 minutes. To rule out the interobserver variability blood sampling and analysis of the hemoglobin was done by a single trained laboratory technician who underwent training in the clinical laboratory given by expert pathologist. The technician, first allowed to estimate the hemoglobin values by the two methods (Sahli's method and Autoanalyzer method) on pilot samples then the study was commenced. Sahli's method of hemoglobin estimation was done three times for each sample as per the Standard Operating Procedure of the laboratory. The same samples were run in Mindray electronic coulter counter which was regularly calibrated and checked for quality control as per Standard Operating Procedure using manufacturer provided stabilized control reagents. The hemoglobin value was taken down in a separate laboratory record. The average of the first two times value [S-2]and the all three times value [S-3] of Sahli's method and the value obtained from the Autoanalyzer method [R] were entered using Microsoft Office Excel 2010 and were then exported to SPSS version 23 for statistical analysis. The reference [R] value was fixed as the cut off for pass or fail for the Sahli's methods S-2 and S-3.

Statistical analysis

Statistical analysis was performed using SPSS 23 for Windows (Microsoft, Seattle, WA, USA). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of two times average [S-2] and three times average [S-3] of Sahli's method was calculated. The values then compared with the results of automated analyzer[R] (gold standard).

The statistical analysis, one-way Anova was done to compare the experimental methods whether any difference

between the three [S-2, S-3 and R].

Results

The gender distribution of the forty-sample population consisted of 23(57.5%) males with 17(42.5%) females' representation [Table 1]. A comparison of two time average [S-2] and three time average [S-3] of Sahli's method used in the current study against the reference autoanalyzer values [R] are summarised in Table 2. By assessing the descriptive statistics of the Hemoglobin values for 40 samples tested with Sahli's method and Autoanalyzer method [Table 3]. The mean of the hemoglobin values of two time average [S-2] and three time average [S-3] of the Sahli's method showed quite similar results. However, the mean Hemoglobin value of S-2 (13.2 ±2.45 gm/dl) and mean value of S-3(13±2.22 gm/dl) were slightly higher by 1.1 and 1respectively when compared with the mean of R, the reference method (12.1± 1.86 gm/dl). The S-3 method (three time average) was found to be most sensitive technique (Sensitivity 96.15%; specificity 53.33%). The sensitivity of the S-2 method was 95.45% with 55% specificity. Figure 1 and 2 show the graphical representation of Bland- Altman analysis for comparison between the two methods i.e. Two-time average of Sahli's method and Autoanalyzer [Figure 1] and Three-time average of Sahli's method and Autoanalyzer [Figure 2] with differences in means and upper and lower 95% limits of agreement [LOA]. One-way Anova was used to find out the statistical difference between the methods. The results showed the P value of 0.068. Hence, there is no significant difference between the three methods which were compared in this study.

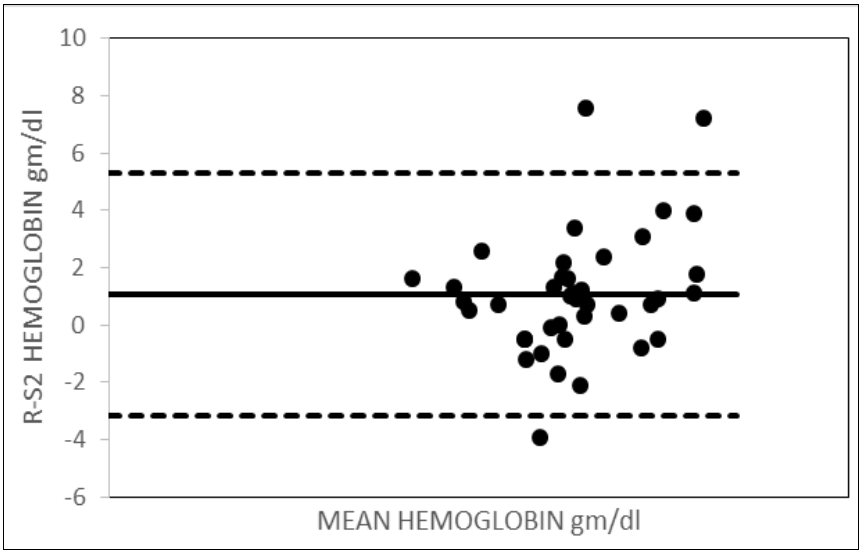

Fig 1: Bland- Altman plot for Sahli's Two time average (S-2) with the reference values

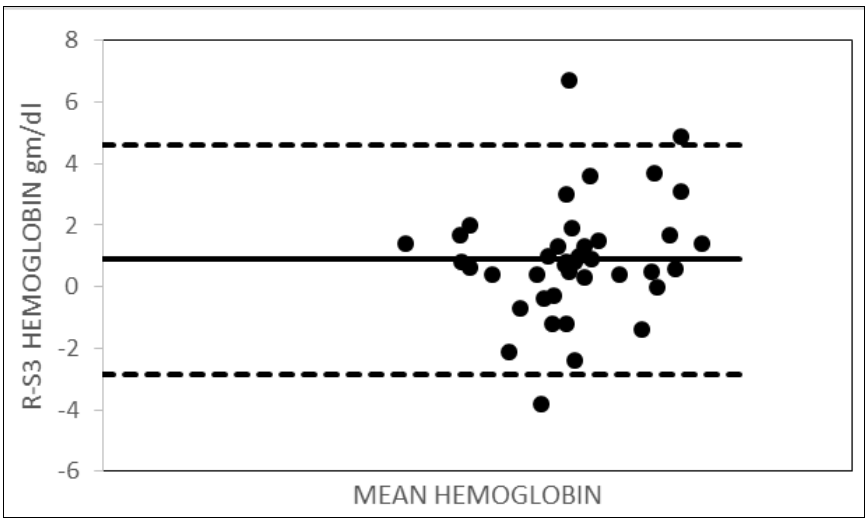

Fig 2: Bland-Altman plots for Sahli's Three time average (S-3) with the reference values

Table 1: Sex distribution of the study population

Gender	N	Percentage
Males	23	57.5%
Females	17	42.5%
Total number	40	100%

Table 2: Comparison of results obtained by the two methods (S-2 and S-3) with the reference method (R).

Results	Sahli's Two time average (S-2)	Sahli's Three time average (S-3)
True positive	21	25
True negative	11	8
False positive	9	7
False negative	1	1
Sensitivity (%)	95.45%	96.15%
Specificity (%)	55%	53.33%
Likelihood Ratio	2.12	2.06
Positive predictive value	70%	78%
Negative predictive value	91.67	88.89%

Table 3: Descriptive statistics of different methods

Group Name	N	Mean	Std deviation	SEM
Sahli's Two average	40	13.2	2.45	0.388
Sahli's Three average	40	13	2.22	0.352
Autoanalyzer	40	12.1	1.86	0.295

Discussion

For the estimation of hemoglobin, practically we use autoanalyzer of various types from three part to five-part analyzers. The electronic autoanalyzer use venous blood of small amount and works with the principle of spectrophotometric method. It is acceptable and suitable for many laboratories for the routine analysis with proper quality control method. A proper and regular calibration is also needed for the continuous use of Autoanalyzer. The major requirements of the autoanalyzer method are good laboratory with regular power supply and electronic data handing services. The cost calculations for the instrumentations are crude and various other factors like consumables, electricity charges and services are applicable to the Autoanalyzer method. The disadvantages of the autoanalyzer usually reflected in small scale laboratories where the cost-effective manual methods like Sahli's method and Cyanmethemoglobin method are being practiced [19]. The Sahli's method is usually practiced for the practical purpose of the undergraduate and postgraduate students of dental and medical institutions.

This study focused on the Sahli's method with two different approaches i.e. Two time average and three time average, against the autoanalyzer method because of its cost effectiveness, easy to perform and the reagents are readily available. To maintain the near true values only venous blood sample were used in this study. The study done by Hema Anand *et al.* [20], aimed to compare the efficacy of Hemoglobin Colour Scale method with Sahli's method and auto-analyzer for the hemoglobin estimation. The observed values of that study showed a systemically higher result for HCS method and Sahli's method than the autoanalyzer values. Our study also showed the similar finding as the mean value of the Sahli's method using Two time average (13.2gm/dl) and Three time average (13gm/dl) were slightly higher than the mean value of autoanalyzer method (12.1gm/dl). The study also showed that the Sahli's method was in high agreement with the auto analyzer which is well correlating with our study. Regarding the sensitivity and specificity aspects of the methods used in that study, the sensitivity was 98.2% and specificity was 66.2%. In our study, the sensitivity of the Sahli's Two time average and Three-time average is 95.45% and 96.15% with the 55% and 53.33% respective specificity. By comparing with the Hema *et al* study, the sensitivity and specificity were relatively lower in our study but in an acceptable range. Ganesh Mohan *et al.* [21] conducted a study on predonation hemoglobin screening methods, aimed to compare CuSO4 method and HemoCue against a standard haematology analyzer. The study stated that the mean value of the CuSO4 method and HemoCue method were higher than the mean value of the Autoanalyzer. In this way, as per our study and other related studies, the methods other than the auto analyzer method showed a higher mean value than the auto analyzer method.

The study by Bland JM *et al.* [22] have used correlation to compare two measurement methods, but we decided not to use correlation as it is misleading. Bland- Altman analysis defines, "if two methods are to agree then the mean of the difference between every paired determination will not be statistically different from zero and a limit of agreement can be established". The study by Paddle *et al.* [23] used the Bland-Altman analysis method to compare the HCS method with auto analyzer method. As it is an acceptable method of statistical evaluation to compare two different measurement methods, in this study we followed the same for the identification of limits of agreement. Our results showed limits of agreement for Sahli's Two-time average [S-2] method as 3.16-5.32gm/dl below and above the reference values, for the Sahli's Three-time average [S-3] method as 2.86 – 4.63gm/dl below and above the reference values which reflected the data around the mean of the difference.

In this study, we statistically compared the Sahli's Two time average [S-2], Three time average [S-3] with the autoanalyzer method [R] by using One-way Anova test. It showed the p value of 0.068 and there is no significant difference between the three methods. The study by P Balasubramaniam *et al.* [24] showed a p value of less than 0.001 for the Sahli's single time method and the Drabkin's method of hemoglobin estimation. This study proved the use of various technical changes in the same Sahli's method by taking Two time and Three time average and overcame the disadvantages of the Sahli's method and made the methods as acceptable one.

By considering the results of this study, the Sahli's S-2 method and S-3 method has no statistically significant difference with the auto analyzer [R] method. As the Sahli's S-3 method has a higher degree of agreement than the S-2 method, S-3 method i.e. the Three - time average of the Sahli's method is considered to be a better alternative than

the S-2 method i.e. Two -time average of Sahli's method for the auto analyzer method. In case of detrimental state like electricity issues, instrumentation issues and calibration issues if one could not use the auto analyzer, as a back up one can use the Sahli's Three time average [S-3] method with a limit of agreement 2.86 – 4.63gm/dl below and above the reference values which reflected the data around the mean of the difference.

Conclusion

Out of three different measurement methods for Hemoglobin estimation, we found Sahli's Three-time average has higher degree of agreement with auto analyzer method and has no significant difference with auto analyzer method. By taking the average of three times Sahli's value [S-3] would be a best alternative for autoanalyzer in a detrimental state where we could not use automated method, instead of using a single time Sahli's value.

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