Original Article

Comparative Study of Different Haemoglobin Estimation Methods

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Abstract

Introduction: Anaemia is one of the most common haematological findings encountered in clinical practice. Diagnosis of anaemia is mainly done by haemoglobinestimation. Among many methods available for hemoglobin estimation, for outpatient department we need fast and cheap method, for indoor patients we need more accurate methods for treatment direction and monitoring of the patient.

Methods: 500 EDTA blood samples were obtained randomly from various indoor and outdoor patient departments of a tertiary care hospital in Ahmedabad, India and their hemoglobin was measured by Sahli'sHaemoglobinometer, Cyanmethemoglobin Method &7-part haematology analyzer: Siemens ADVIA2120i.

Results: Repeatability standard deviationsof Sahli's method, Drabkin's method and cell counter respectively were 0.61 g/dl, 0.38 g/dland 0.15 g/dl. When comparing Sahli's method with Drabkin's method and cell counter, we found p value of <0.0001, suggesting significant difference between two methods. Whereas Drabkin's method was found to be comparable with cell counter with p value of >0.05.

Conclusion: Sahli's method although cheap and easy, is inaccurate and has subjective bias. So it can be used for screening purpose, but not for diagnosis and follow up of anaemia,Haemoglobin measurement by Drabkin's method is very cost effective and it is as efficient as cell counter. It is especially useful in fund deprived areas and where only haemoglobin value is required.Cell counter although highly accurate and versatile, requires good equipment, quality control, laboratory setup and trained personnel. So it should be preferably used when complete blood count is required.

Key Words: *Anaemia, Automated haematology analyser, Haemoglobin estimation, Drabkin's cyanmetheamoglobin method, Sahli's method.*

Introduction

Anaemia is more often encountered individually in third world countries, with most common cause being nutritional deficiencies. In developed countries it is more commonly seen as a consequence of other diseases like genetic causes, blood loss due to pregnancy or road traffic accidents, anaemia of chronic diseases, etc. First clue of presence of anaemia in any patient is mostly noticed at clinical examination by noticing presence of pallor, tachycardia, weakness, breathlessness etc. But most of the times it is the laboratory investigations that point us towards anaemic condition.

There are many methods available for hemoglobin (hb) estimation.In developing countries we are encountered with fund crunch and overcrowded

hospitals, so we must design the laboratory method in a way that it should be fast, cost effective and as accurate and reliable as possible. Sahli's method, $CuSo_4$ method and Drabkin's method are very cost effective. Hemocue method and Cell counter are still costly and require good equipment, quality control, laboratory setup and trained personnel for proper functioning. But in determining the treatment protocol when other RBC indices and complete blood picture are required, automated cell counter is more useful.

In this study, we conducted haemoglobin measurement of 500 random samples by three methods- Sahli's method, Drabkin's method and Automated Haematologyanalyser. We tried to find out comparison between results of these methods.

Methods

· This study was conducted on 500 blood samples obtained in 2 ml blood in K3 EDTA vacutainer from various indoor wards and outdoor patient departments in the central clinical laboratory of a tertiary care hospital in Ahmedabad.

· Samples were taken randomly and their Hb was measured by Sahli'sHaemoglobinometer, Cyanmethemoglobin Method &7-part haematology analyzer: Siemens ADVIA2120i.

· Hb estimation by Sahli'shaemoglobinometer (acid haematin method): Blood is mixed with N/10 HCL, resulting in the conversion of Hb to acid hematin, which is brown in colour. The solution is diluted till its colour matches with the brown coloured glass of the comparator box. The concentration of Hb is read directly.

· Hb estimation by Cyanmethemoglobin Method: Blood is diluted in a solution containing potassium cyanide and alkaline potassium ferricyanide. The latter converts Hb to methaemoglobin which is converted to cyanmethemoglobin (HiCN) by potassium cyanide. The absorbance of the solution is then measured in a spectrophotometer at a wavelength of 540 nm.

· To calculate the repeatability (reliability) of each method[1]: A random sample was taken and its haemoglobin was measured 20 times by each method. Same sample was used for each method to remove any blood sample related variation.

Repeatability standard deviation of each method is calculated by:

$$s_r = \sqrt{\frac{\sum(x_i - \bar{x})^2}{(n-1)}}$$

S_r- is the repeatability standard deviation in g/dl.

x_i-is the ith measurement in g/dl.

x -is the average of the 20 measurements in g/dl.

n -is the number of measurements.

· Comparison of values obtained by two methods was done by Unpaired t-test for equal variances[2]:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2}{N_1} + \frac{S_2^2}{N_2}}}$$

$\mathbf{X_1}$ = Mean of one method

$\mathbf{X_2}$ = Mean of other method

$\mathbf{S_1}$ = Standard deviation of one method

$\mathbf{S_2}$ = Standard deviation of other method

$\mathbf{N_1}$ = Sample size of one method

$\mathbf{N_2}$ = Sample size of other method

If p-value obtained from t-test is >0.05, it means that there is no significant difference between values obtained from both methods and both methods are comparable. While if p value is <0.05, it shows that there is significant difference between results of both methods and they are not comparable.

Results

Repeatability of Sahli's method:-

Haemoglobin of a randomly selected sample was

measured 20 times by Sahli's acid hematin method. Minimum value obtained was 12.4 g/dl and maximum value: 14.3g/dl, with mean of 13.28 g/dl. Repeatability standard deviation (SD) was 0.61 g/dl.

It is known that 68% of the values lie within 1 SD of mean and 95% of the values lie within 2 SD of mean. Which means if we run the same sample 100 times; for 68 times, the values will lie between [13.28 ± 0.61] i.e, 12.67 to 13.89 g/dl, whereas for 95 times the values will lie between [(13.28 ± 2(0.61)] i.e. 12.06 to 14.5 g/dl. So we can say with 95% confidence that the Hb values will not vary more than 1.22 g/dl. Thus repeatability of Sahli's method is within acceptable limit of agreement.

Repeatability of Drabkin'smethod:-

When hb of selected sample was measured 20 times by Drabkin's method,minimum value obtained was 12.8 g/dl and maximum value was 14 g/dl, with mean of 13.42 g/dl. Repeatability standard deviation was 0.38 g/dl.

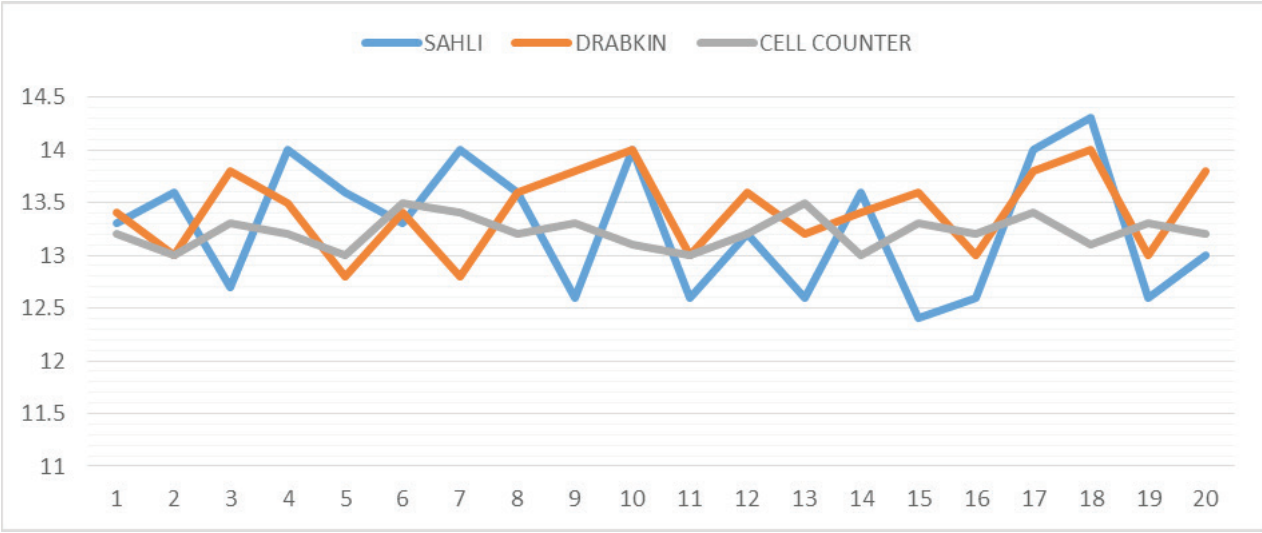
So if we run the same sample 100 times, for 68 times the values will lie between [13.42 ± 0.38] i.e, 13.04 to 13.8 g/dl, whereas for 95 times the values will lie between [(13.42 ± 2(0.38)] i.e. 12.66 to 14.18 g/dl. So we can say with 95% confidence that the Hb values will not vary more than 0.76 g/dl. Thus repeatability of Drabkin's method is within acceptable limit of agreement.

Repeatability of Automated cell counter:-

When hb of selected sample was measured 20 times by automated haematology analyzer: Siemens ADVIA2120i,minimum value obtained was 13 g/dl and maximum value was 13.5 g/dl, with mean of 13.22 g/dl. Repeatability standard deviation was 0.15 g/dl.

So if we run the same sample 100 times, for 68 times the values will lie between [13.22 ± 0.15] i.e, 13.07 to 13.37 g/dl, whereas for 95 times the values will lie between [(13.22 ± 2(0.15)] i.e. 12.92 to 13.52 g/dl. So we can say with 95% confidence that the Hb values will not vary more than 0.30 g/dl. Thus repeatability of Cell counter is within acceptable limit of agreement.

Now let's compare distribution of repetitive measurements of the same sample by all three methods in single graph. (graph 1). Here we can see that the least variable method is Cell counter while the most variable one is Sahli's method.



Graph 1: X axis – No. of times test performed, Y axis – Haemoglobin value

Comparison of Sahli's method with Drabkin's method [Table 1]:

Ø Taking hemoglobin values of 500 samples obtained by Sahli's method as X_1 to X_{500} and hemoglobin values obtained using Drabkin's method as Y_1 to Y_{500}, we applied unpaired t-test assuming equal variances. The results were: t= 4.204 with corresponding p value <0.0001. This suggests that the difference between 2 methods is significant.

Ø Mean difference between both the methods was 0.6468 g/dl. Sahli's method underestimated Hb by 0.6468 g/dl as compared to Drabkin's method.

Comparison of Sahli's method with Cell Counter [Table 1]:

Ø Taking hemoglobin values of 500 samples obtained by Sahli's method as X_1 to X_{500} and hemoglobin values obtained using cell counter as Z_1 to Z_{500}, we applied unpaired t-test assuming equal variances. The results were t= 4.865 with corresponding p value <0.0001. This suggests that the difference between 2 methods is significant.

Ø Mean difference between both methods was 0.746 g/dl. Sahli's method underestimatedHb by 0.746 g/dl as compared to cell counter.

Comparison of Drabkin's method with Cell Counter [Table 1]:

Ø Taking hemoglobin values of 500 samples obtained by Drabkin's method as Y_1 to Y_{500} and hemoglobin values obtained by cell counter as Z_1 to Z_{500}, we applied unpaired t-test assuming equal variances. The result was t= 0.647 with corresponding p value = 0.5175, which is > 0.05. This suggests that the difference between 2 methods is insignificant.

Ø Mean difference between both methods was only 0.099 g/dl, i.e., Drabkin's method gave hb value of ±0.099g/dl as compared to cell counter.

Table 1: Comparison of Sahli's method, Drabkin's method & Cell counter

	Sahli's method	Drabkin's method	Cell counter
Range of Hb values	12.4-14.3 g/dl	12.8-14 g/dl	13-13.5 g/dl
Mean	13.28 g/dl	13.42 g/dl	13.22 g/dl
Repeatability standard deviation	0.61 g/dl	0.38 g/dl	0.15 g/dl
Method prediction range	12.6-13.9 g/dl	13.04-13.8 g/dl	13.07-13.37 g/dl

Discussion

Haemoglobin estimation is used as a screening test for detecting anaemia. This is a frequently identified abnormality in our population. Anaemia is not a diagnosis by itself and if detected, its underlying cause must be ascertained. Hence, accurate Hb estimation is essential so that further tests can be done to ascertain its cause and treat the patient accordingly. Few common methods for haemoglobin estimation are Sahli's acid haematin method, copper sulphate method, manual Drabkin's method, Hemocue method, automated haematology analysers, non-invasive Pulse co-oximetry method etc.

Sahli's acid haematin method is quick, easy to perform, inexpensive, does not require technical expertise and can be used as a bedside procedure. However it is less accurate, lacks a true standard, there is significant subjective variation in color matching and it does not measure all haemoglobins i.e. oxyhaemoglobin, sulphaemoglobin as they are not converted to acid hematin. In our study, Hb estimation by Sahli's method shows repeatability

standard deviation of 0.63, which is maximum as compared to 0.38 & 0.15 by Drabkin's method and Cell counter respectively. This means that Hb value of same sample on repeat testing will vary the most by Sahli's method.

On comparison of Sahli's method with Drabkin's method, we found that Sahli's method underestimated Hb by 0.6468 g/dl as compared to Drabkin's method. When compared to other studies,Prashant et al 2013[3]found that Sahli's method underestimated the hemoglobin by 1.12gm/dl in venous blood and p value <0.01 between Sahli's method andcyanmethemoglobin method. In a study by P Balasubramanian&A Malathi[4],1.13g/dl of difference was found between sahli's method and HiCN method. However a study done by MadhuraWasnik et al using 51 subjects did not find any significant difference between results obtained from Sahli's and HiCN methods (p= 0.954 i.e. >0.05)[5]

Table 2– Comparison with other studies

Criteria	Our study	Prashant et al, 2013, Dhule	Balasubramaniam et al, 1992, Bombay	Madhura et al, 2014
Mean difference in Hb value by Sahli's method &HiCN	Approx. -0.65 g/dl	Approx. -1.12g/dl	Approx. -1.13g/dl	Approx. +0.24 g/dl
P value significance	<0.0001	<0.05	<0.05	>0.05

On comparison of Sahli's method with cell counter, we found that Sahli's method underestimates Hb by 0.746 g/dl as compared to cell counter. When comparedwith other studies, Natarajan S et al(2010) had found results of lower haemoglobin by 0.37gm/dl using venous blood comparingSahli's method with the coulter auto analyzer[6]. Study by Bezarra da silva et al comparing Sahli's method with cell counter did not find any significant difference between the two methods.[7] They found mean difference of 0.2267g/dl. An interesting study done by Dr. MP Brundha and S Priyadharshini, 2019 compared Sahli's two time average and three time average methods with automated cell counter. In this study they found Sahli's three-time average method to be most comparable with autoanalyzer with mean difference of 0.9g/dl.[8]

Table 3: Comparison with other studies

Criteria	Our study	Bezerra Silva et al (2011)	Natarajan S et al (2010)	Dr. MP Brundha
Mean difference in Hb values by Sahli's method & cell counter	Approx. ±0.746 g/dl	Approx. ±0.2267g/dl	Approx. ±0.37g/dl	Approx. ±0.9g/dl
P value significance	<0.0001	---	---	---

Drabkin'scyanmethemoglobin method has the principle advantage of a stable Drabkin's reagent that reacts with all forms of Haemoglobin, except sulfhemoglobin, which normally occurs only in minute concentration in blood. It doesn't have visual bias of Sahli's method as color matching is not required and it has a reliable reference standard from WHO for direct comparison. However, Potassium cyanide is a poisonous substance and that is why Drabkin's solution must never be pipetted by mouth. Also diluted blood has to stand for a period of time to ensure complete conversion of Hb.

With the advent of automated blood cell counter by Wallace Coulter in 1956, the paradigm of hematological investigations shifted. A cell counter not only assesses Hemoglobin very accurately, but also measures all red cell indices such as total RBC count, hematocrit, MCV, MCH, MCHC, RDW, etc., Total and differential Leukocyte count and platelet count and other indices. A variety of fully automated instruments are now commercially available. They work on the principle of impedance measurement, high frequency measurement, light scatter at different angles, Fluorescence flow-through cytometryetc[12],[13].

Discrimination threshold:

- RBC: 30-250 fl
- WBC: RBCs are lysed with lytic reagent. The different WBC discriminator set at different levels ranges between 30-450 fl
- Platelets: 2-30 fl

In the ObGy OPD of our hospital, we use Drabkin's method to ascertain presence of anaemia as only Haemoglobin estimation is required most of the times. While for other OPDs as well as for indoor patients, where whole blood picture is required to assess the patient, automated cell counter is used. Both these methods have acceptable repeatability standard deviation of 0.38g/dl & 0.15 g/dl respectively, which is compared with a study by Prashant et al (2013)[3] in the table below.

Table 4: Comparison with other studies

Method	Repeatability standard deviation	
	Our study	Prashant et al 2013
Drabkin's method	0.38 g/dl	0.10g/dl
Cell counter	0.15 g/dl	0.019g/dl

Our comparison of Drabkin's method and cell counter was compared with other studies. Jacob Rosenbilt et al (2014) found variances of Drabkin's method and cell counter respectively 0.82% and 0.68% with mean difference of 0.64 g/d and p value significance >0.05[9]. In a study by WaqarAzim et al 2016, Drabkin's manual method only showed 2.67% variation as compared to Medonic CA 530 cell counter. Hb values obtained by manual Drabkin'smethod lies ±2.67% of cell counter[10]. In a study by Yufa et al,2013 correlation coefficient between HiCN method and cell counter was 0.97[11]. Variation of 3.8% was seen in HiCN method.

Table 5: Comparison with other studies

Criteria	Our study	Jacob Rosenbilt et al (2014)	Waqar et al, Multan, 2016
Difference in Hb values by HiCN& cell counter	Approx. ±0.1 g/dl	Approx. 0.64g/dl	Approx. ±2.67%
P value significance	>0.05	>0.05	---

As we can see that for Hb estimation by Drabkin's method and cell counter, our study is comparable to most of the studies. Drabkin's method shows very strong correlation with Cell counter values, which means Drabkin's method will access Hb of any sample almost as good as cell counter. Furthermore, Drabkin's method is much cheaper option than Cell counter. Cell counter although highly accurate and versatile requires good equipment, quality control, laboratory setup and trained personnel for proper functioning.

Conclusion

Ø Sahli's method although cheap and easy is inaccurate and has subjective bias. So it can be used for screening purpose. But it is not advisable to use this in clinical setup for diagnosis and follow up of anaemia,

Ø Hb measurement by Drabkin's method is very cost effective and it is as efficient as cell counter. It is especially useful in smaller setup and fund deprived areas where only haemoglobin value is required.

Ø Cell counter although highly accurate and versatile requires good equipment, quality control, laboratory setup and trained personnel. So it should be preferably used when other RBC indices and complete blood count is required.

Conflicts of Interest: None.

Ethical Considerations: All procedures performed were in accordance with the ethical standards of the institution.

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