## Original Research Article

# Hb estimation at point of care using cyanmethaemoglobin method

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### ABSTRACT

**Background:** Accurate estimation of haemoglobin (Hb) is an essential prerequisite for detection of anaemia and for assessing response to treatment. In India the gold standard cyanmethaemoglobin method is used for estimation of Hb in tertiary care institutions but accurate methods for Hb estimation are not being used in primary and secondary healthcare settings. Major national surveys have demonstrated the feasibility and accuracy of Hb estimation by indirect cyanmethaemoglobin method (ICM). However, ICM has not been used for Hb estimation in point of care because of reports that ICM underestimates Hb. A study was taken up to compare the accuracy of Hb estimation by direct (DCM) and indirect cyanmethaemoglobin (ICM) method.
**Methods:** ANMs and laboratory technicians pipetted out duplicate samples of blood for both direct and indirect Hb estimation. Another trained laboratory technician undertook Hb estimation using colorimeter in all samples. Hb values between duplicates were compared.
**Results:** There was excellent concordance between means and frequency distribution of Hb values between duplicates when Hb in both were estimated using DCM or ICM and also when Hb estimation was done by DCM in one and ICM in the other. Bland Altman Plots confirmed that across Hb levels there was excellent concordance between direct and indirect method of Hb estimation.
**Conclusions:** Indirect cyanmethaemoglobin method is accurate. It can be used at point of care for screening for anaemia and assessing improvement in Hb after treatment especially in primary and secondary health care settings.

**Keywords:** Hb estimation, Direct cyanmethaemoglobin method, Indirect cyanmethaemoglobin method, Accuracy, Anaemia, Point of care

## INTRODUCTION

Anaemia is the most common nutritional deficiency disorder in the world. Prevalence of anaemia in India is the highest in the world and the country is the home of largest number of anaemic persons.[1,2] Symptoms and signs associated with anaemia are non-specific.[3] Accurate estimation of Hb is therefore an essential pre-requisite for detection of anaemia and for assessing response to treatment.[3] India recognized that anaemia is a major public health problem especially in pregnant women and children and initiated National Anaemia Prophylaxis Programme in the 1970s.[4] The programme envisaged that all pregnant women and pre-school children will be given iron and folic acid supplementation. Review of the programme a decade later, showed that both coverage and compliance with the IFA supplementation was low. In the 1990s the programme was modified and renamed as National Anaemia Control Programme.[5] By then India's primary health care system had been established and the programme aimed at: a) screening of pregnant women by Hb estimation for detection and grading of anaemia, b) providing IFA tablets containing 100 mg of elemental iron and 500 µg of folic acid once a day for non-anaemic pregnant women, c)

providing IFA tablets containing 100mg of elemental iron and 500 µg of folic acid twice a day for anaemic pregnant women d) monitoring them for improvement in Hb.

Currently accurate Hb estimation by direct cyanmethaemoglobin method (DCM) is being used in research and haematology laboratories, speciality hospitals and medical college hospitals; but it is not being used in outpatient departments in secondary and primary health care institutions. To facilitate use of cyanmethaemoglobin method in community settings, the National Institute of Nutrition (NIN) had developed indirect cyanmethae-moglobin method (ICM) of Hb estimation. The accuracy and replicability of ICM has been documented.[6] Large scale surveys carried out by the National Nutrition Monitoring Bureau (NNMB), District Level Household Survey (DLHS) 2 and 4 Annual Health Survey Clinical Anthropometric and Biochemical component (AHS CAB) have demonstrated that well trained ANMs and laboratory technicians can accurately pipette 20 µlitres of blood from finger prick in community settings and Hb estimation from dried blood spot eluted in Drabkin's solution (ICM) provides accurate results.[7-11] Despite these experiences concerns have been expressed about the ability of frontline workers to pipette 20µlitres of blood accurately.[7-11] Publications indicating that ICM may underestimate Hb and overestimate prevalence of anaemia have also come in the way of wider use of ICM for Hb estimation in primary health care settings.[12] Despite reports of inaccuracies in Hb estimation with WHO colour scale, Sahli's method and Hemocue, these methods of Hb estimation are still being preferred and used because of convenience and ease of use.[13-19] Newer non-invasive methods of Hb estimation which are not accurate are entering the arena as methods for Hb estimation at point of care citing non-invasive nature as the advantage.

There had not been any large scale laboratory studies documenting the accuracy of Hb estimation by direct and indirect cyanmethaemoglobin methods. A study was therefore taken up to assess accuracy of Hb estimation done by DCM and ICM by comparing Hb values in duplicate samples.

**METHODS**

The study design is shown in Figure 1. Between April 2019 and March 2020, Nutrition Foundation of India (NFI), was undertaking micronutrient supplementation studies in pregnant women attending Antenatal Clinic of Maternity Home, Badarpur and a community based randomised open trial on iron fortified iodised salt in South and South West Delhi ICDS blocks. Blood samples from consenting pregnant women participating in the micronutrient supplementation study in antenatal clinic and women in reproductive age participating in the community based studies on iron fortified iodised salt were being collected for estimation of Hb and biomarkers for haematinic nutrients. A small aliquot of blood from EDTA tube was

used for pipetting out duplicate samples of 20 µlitres of blood for assessing the concordance between DCM and ICM for Hb estimation. ANMs and laboratory technicians belonging to the research team trained in pipetting blood from finger prick or blood collected in EDTA tubes were requested to pipette out duplicate samples of 20 µlitres of blood and put the samples of blood collected: a) into two test tubes containing 5 ml of Drabkin's solution (for comparison between duplicates when Hb estimation was done using DCM), b) on two filter papers and dry the same; after drying elute into 5 ml Drabkin's solution (for comparison between duplicates when Hb estimation was done using ICM), c) put one sample onto a filter paper and dry the same and after drying elute into 5 ml Drabkin's solution; put the other sample into test tube containing 5 ml of Drabkin's solution (for comparison between duplicates when Hb estimation was done in one sample using DCM and in the other sample using ICM).
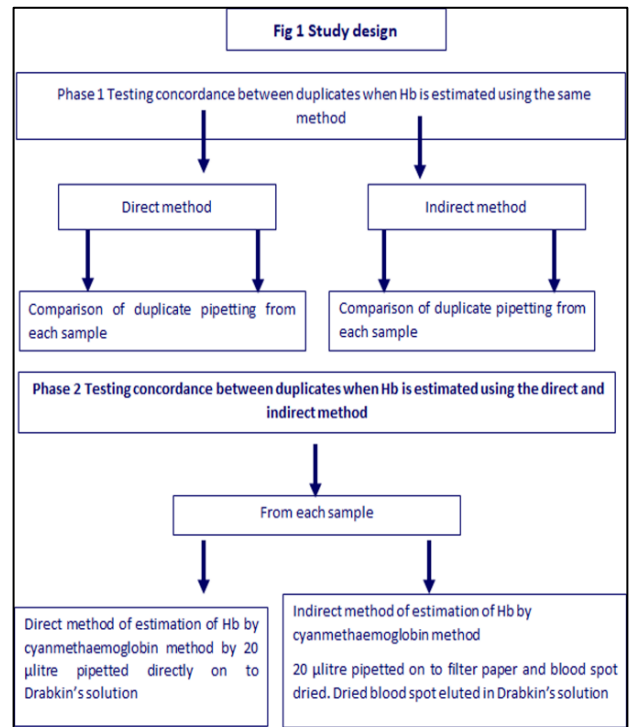


**Figure 1: Study design.**

Hb estimations in all samples was done by a separate laboratory technician trained in Hb estimation by cyanmethaemoglobin estimation method using colorimeter.

***Sample size***

It was not possible to estimate sample size because the lack of published data on differences between the DCM and ICM of estimation of Hb. Therefore, it was decided to initiate the study and compute the sample size based on the observed differences in Hb between DCM and ICM in the initial period.

*Data analysis*

The Hb data were tabulated in MS Excel. Data analysis was done using Statistical package for social sciences (SPSS) 26. Concordance in the Hb levels between duplicate samples were assessed by comparing means and standard deviations (SD), correlations, frequency distribution and Bland Altman Plot.

## RESULTS

The number of duplicate samples tested in the first and second phase is indicated in the Table 1.

**Table 1: Number of samples tested in phase 1 and 2.**

|  | Phase 1 | | Phase 2 |
|---|---|---|---|
|  | DCM | ICM | DCM versus ICM |
| Number of samples | 302 | 283 | 574 |

**Table 2: Comparison of Hb between duplicates.**

| Mean Hb (gms/dl) | |
|---|---|
| ICM | |
| Indirect method 1 | 10.7±1.49 |
| Indirect method 2 | 10.7±1.49 |
| **Mean Pair difference** | -0.04±0.0298 |
| **Correlation between 1 and 2: 0.98** | |
| **DCM** | |
| Direct method 1 | 10.8±1.51 |
| Direct method 2 | 10.8±1.48 |
| **Mean Pair difference** | -0.05 ±0.197 |
| **Correlation between 1 and 2: 0.99** | |
| **DCM versus ICM** | |
| Direct method | 10.8±1.49 |
| Indirect method | 10.7±1.46 |
| Mean Pair difference | -0.10±0.349 |
| Correlation between direct and indirect method: 0.97 | |
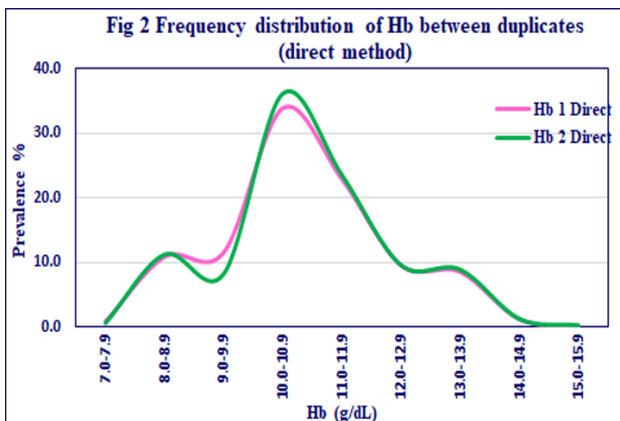


**Figure 2: Frequency distribution of Hb between duplicates (direct method).**

Means, SDs and correlation between duplicates when Hb was estimated using DCM or ICM or both is shown in Table 2. There was excellent concordance between duplicates irrespective of the method used for Hb estimation. Frequency distribution of Hb values in duplicates is shown in Figures 2, 3 and 4.
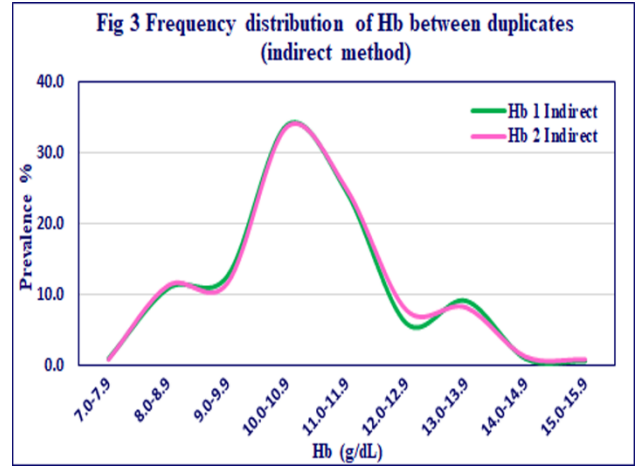


**Figure 3: Frequency distribution of Hb between duplicates (indirect method).**
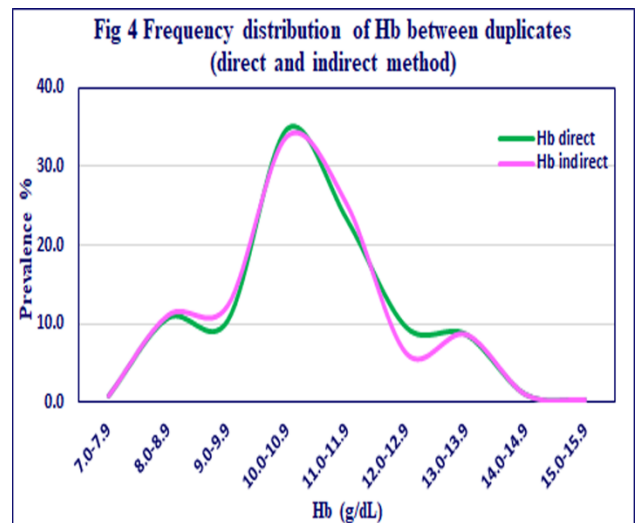


**Figure 4: Frequency distribution of Hb between duplicates (direct and indirect method).**

Comparison between Hb values estimated in duplicate samples using DCM and ICM using Bland Altman Plot is given Figures 5, 6 and 7. Data from the Bland Altman Plot confirms that across Hb levels there was excellent concordance in Hb values between duplicates irrespective of the method used (Figures 5, 6 and 7). The observed differences in Hb values between duplicates when one sample was estimated using DCM and another using ICM cyanmethaemoglobin method was similar to the observed differences when both samples were estimated using either DCM or ICM for Hb estimation.
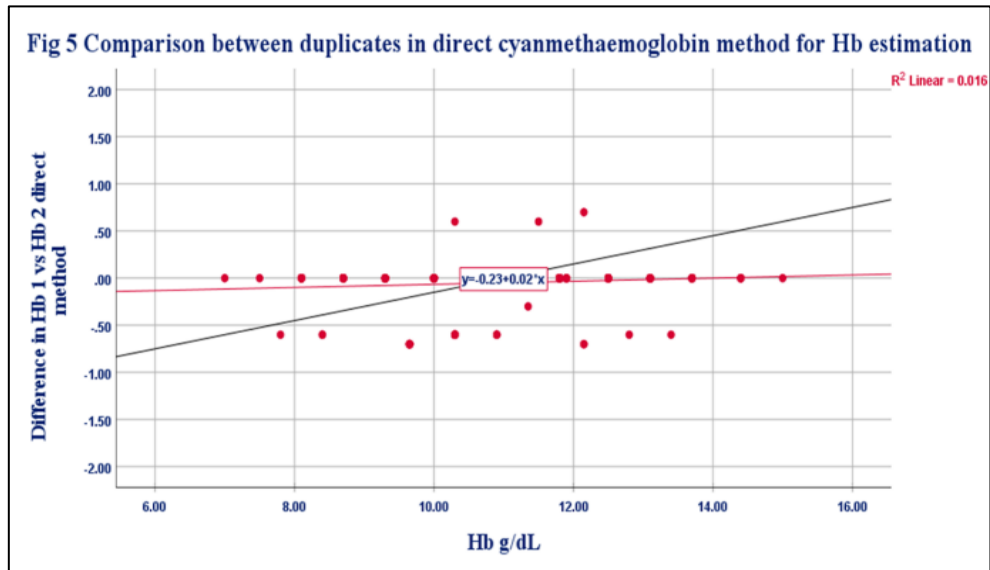
**Figure 5: Comparison between duplicates in direct cyanmethaemoglobin method for Hb estimation.**
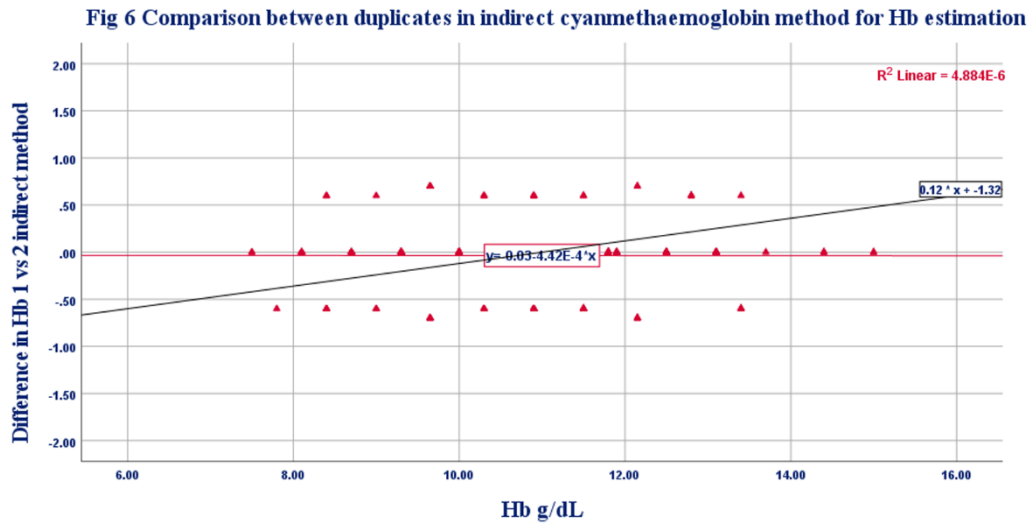


**Figure 6: Comparison between duplicates in indirect cyanmethaemoglobin method for Hb estimation.**
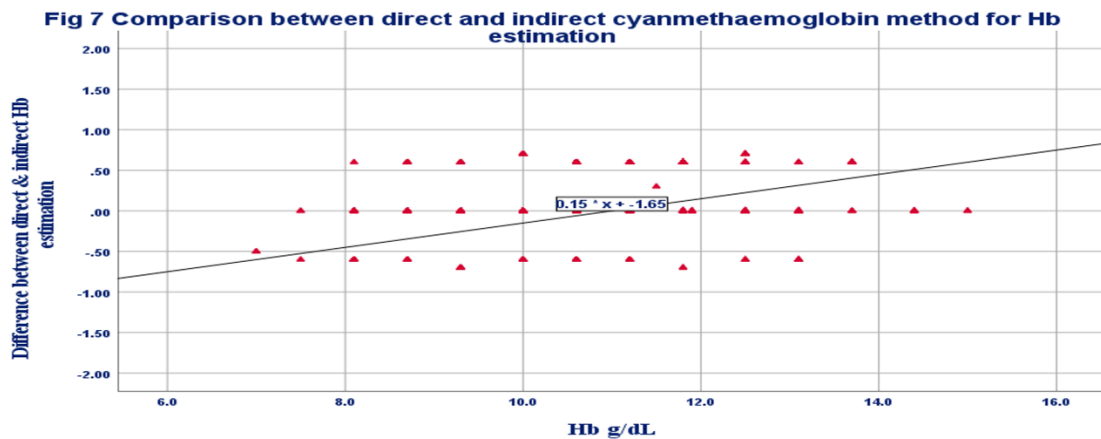


**Figure 7: Comparison between direct and indirect cyanmethaemoglobin method for Hb estimation.**

Fig 8 Hb estimation by indirect cyammethaemoglobin method



20 μl blood from finger prick deposited on filter paper and dried

Dried blood on filter paper fully eluted in Drabkin's solution

Filter paper after complete elution in Drabkin's solution which has been decanted

Non-elution of dried blood from filter paper in one sample collected after use of spirit to clean finger tip
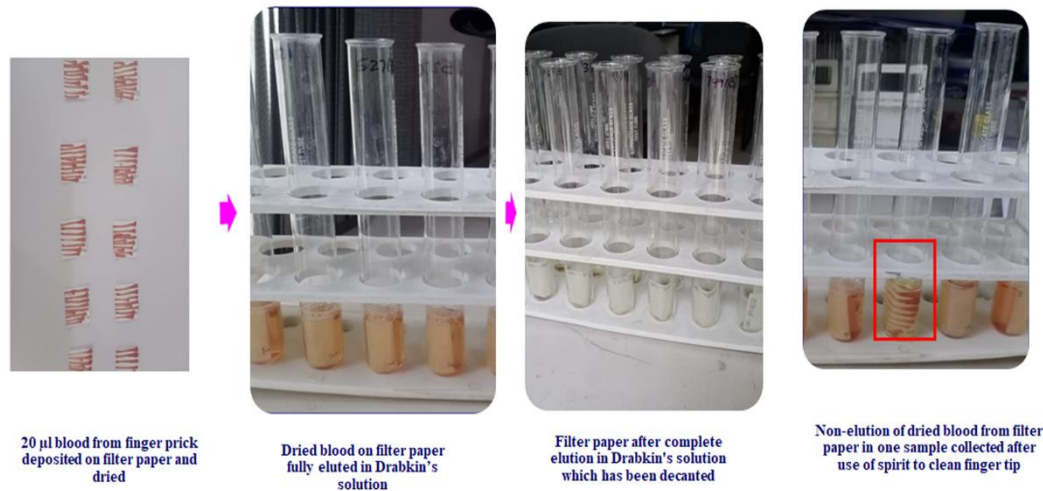
**Figure 8: Hb estimation by indirect cyanmethaemoglobin method.**

## DISCUSSION

All technical documents on anaemia start with the statement that symptoms reported by anaemic persons such as inability for sustained manual work, easy fatiguability and shortness of breath are seen in many other conditions and are non-specific.[6,13,14] Clinical signs of anaemia such as pallor are difficult to detect and quantify; koilonychia is a specific sign of anaemia but there is a large time gap between onset of anaemia and development of the sign. Koilonychia disappears only when new nail grows months after correction of anaemia. Therefore, anaemia should be diagnosed and response to treatment monitored only by Hb estimation in circulating blood.[6,12,13] Accurate Hb estimation will ensure: at individual level, early detection of anaemia and enable monitoring the impact of treatment in anaemic person; in surveys, accurate assessment of prevalence of anaemia in different groups and changes in prevalence over time.

Ideally scientists prefer the measurement of circulating Hb mass; undoubtedly when this indicator is used, it is clear that in pregnancy there is an increase in Hb mass, though there is a reduction in circulating Hb g/dL because of haemodilution. However, measurement of circulating Hb mass is not feasible and hence globally use of Hb g/dL in circulating blood is accepted for diagnosis of anaemia. It is well known that Hb values are higher if estimation is done from venous blood as compared to finger prick blood. As we move towards universal screening for anaemia by Hb estimation, majority of the Hb estimations will be done in primary health care settings and during community-based surveys. In these settings most of the blood collection will be from finger prick and therefore finger prick collection of blood for Hb estimation is accepted.

Accuracy at sample collection and use of accurate method of Hb estimation are critical for ensuring accuracy in reported Hb. Finger prick collection of blood is taught to laboratory technicians and ANMs and collection of blood for Hb estimation is one of their assigned tasks. But there has been worries about accuracy with which they can pipette out 20 μlitres of blood from finger prick, day after day. In the present study ANMs and laboratory technicians trained in finger prick blood collection and pipetting took up the task of making duplicates in over eleven hundred samples. Data from the study indicate that they were able to accurately pipette 20 μl of blood in a sustained manner. Experience gained in collection of 20 μlitres of blood in community setting in national surveys (NNMB surveys, DLHS 2 & 4 and AHS CAB) have also clearly documented that it is possible to ensure accuracy in pipetting out 20 μlitres of blood from finger prick: if persons were given appropriate training, accuracy in pipetting assessed, only those who were accurate in pipetting for collection of blood were given the task of collection of blood and quality of pipetting is monitored by ensuring duplicate sample collection in 10% of samples.[7-11]

Choosing the right method for Hb estimation is another critical requirement for ensuring accurate Hb results. Cyanmethaemoglobin method for Hb estimation was described nearly nine decades ago and remains the gold standard for Hb estimation even today.[14] This is because: a) RBCs get lysed and evenly distributed in the Drabkin's reagent, b) almost all forms of Hb combine with the Drabkin's reagent to form stable cyanmethaemoglobin, c) the method is precise and the samples can be directly compared with the standard, d) Hb estimation by cyanmethaemoglobin method can be done using a wide

range of equipment ranging from colorimeter, spectrophotometer and auto-analyser.

In community settings it is not possible to perform Hb estimation using DCM. In the 1970s, National Institute of Nutrition, standardised Hb estimation by cyanmethaemoglobin using indirect method and demonstrated that it is an accurate, feasible and inexpensive method for Hb estimation in community setting.[6] However, there have been reports indicating that there was incomplete elution of the dried blood spot in Drabkin's solution resulting in under estimation of Hb in ICM.[15] The NIN guidelines specifically stated that while collecting the blood samples on filter paper for ICM, fingertip should not be cleaned with spirit; spirit denatures the protein and denaturation of proteins prevents complete elution of the blood from filter paper. Figure 8 shows complete elution of dried blood on the filter paper in Drabkin's solution in two samples collected when ether was used to clean the fingertip and non-elution of dried blood from filter paper in one sample collected after use of spirit to clean fingertip. Some of the articles reporting under estimation of Hb with ICM clearly state that the fingertip was cleaned with spirit.[15] It is imperative that the instruction that alcohol preparations (methyl, ethyl or isopropyl) should not be used for cleaning the fingertip if filter paper collection of blood is to be done and only ether should be used has to be reinforced repeatedly so that this mistake is not committed. This precaution is essential to ensure that accuracy of Hb estimation is not compromised due to incomplete elution of dried blood from filter paper.

There had been numerous reviews of the large number of methods currently being used for Hb estimation.[16-19] The perceived advantages and the problems in using some of the methods for accurate estimation of Hb are summarised below:

### Haemoglobin colour scale

It has been advocated in the past by WHO for use in setting where no laboratory facility is available. The method is inaccurate because Hb marking are in 2 g/dL interval and optical colour matching from dried blood is difficult.[20-22]

### Sahli's haemoglobinometer

It has been supplied to primary health centres in India for the last four decades because it was inexpensive and did not require colorimeter for Hb estimation. ANMs, nurses and doctors are familiar with the method. This method is time consuming. Hb estimation is not accurate because acid haematin is not as stable as cyanmethaemoglobin, optical colour matching is inaccurate and the colour of the standard panel fades with time.[22]

### Hemocue

It has been used in the Global Demographic and Health Surveys because of ease of use, elimination of the need to pipette accurate quantity of blood and results being available on the spot of blood collection within minutes. There have been several studies which have documented that Hemocue over estimates Hb, and does not have a linear relationship to Hb estimated by cyanmethaemoglobin.[23-25]

Despite reports of inaccuracies in Hb estimation with WHO colour scale, Sahli's method and Hemocue are still being preferred in many settings and used because of convenience and ease of use. Newer non-invasive methods of Hb estimation which are not accurate are entering the arena as methods for Hb estimation at point of care citing non-invasive nature as the advantage.[26] All reviewers agree that cyanmethaemoglobin method is the best but also state that due to practical considerations the other methods may have to be considered for use.[16-19] They also recommend that when a less accurate method eg Hemocue is used to get immediate results this should be followed by a laboratory work up next day using accurate methods of Hb estimation. In India Hb estimations are not done under emergency conditions but is done in primary and secondary health care settings to detect persons with anaemia and provide appropriate treatment (eg 30 million pregnant women/year twice during pregnancy). In India, majority of persons screened in community settings will not be able to get a repeat Hb examination in a hospital setting. The programme implementors have to realise that the vulnerable groups cannot afford the opportunity, time and cost of repeat confirmatory Hb estimation and therefore, should be screened with the best and the most accurate method for Hb estimation near home, both for diagnosis and for monitoring the improvement with treatment.

Almost all of the studies and reviews on Hb estimation by various methods use sensitivity and specificity of the method against Hb cut off points used to define anaemic and non-anaemic persons.[16-19] Hb is estimated not only to find out whether a person is anaemic or not, but also to assess the severity of anaemia; in anaemic persons repeat Hb estimation will have to be done to assess impact of treatment on Hb levels. Sensitivity and specificity analysis will not be of much use under these circumstances. It has been shown that Hemocue over estimates Hb as compared to cyanmethaemoglobin method but the relationship between the two methods is not linear.[24] It is important to assess how the method under investigation performs across the whole range of Hb levels using Bland Altman Plot. Data from the present study indicate that results of Hb estimation by direct and indirect cyanmethaemoglobin method from duplicate samples are comparable across the whole range of Hb levels.

### Limitations of the study

The study was conducted using blood collected by venepuncture and put in EDTA tubes. Pipetting was done comfortably in the in the laboratory by trained ANMs and technicians using blood containing anticoagulant. The

study did not collect duplicate blood samples from finger prick in primary health care settings to assess accuracy of ICM in comparison with DCM.

**CONCLUSION**

Data from the present study has shown that indirect cyanmethaemoglobin method is as accurate as direct cyanmethaemoglobin method for Hb estimation. The feasibility of use of ICM for accurate Hb estimation in community setting has been well demonstrated in national surveys. Laboratory technicians and colorimeters are available in primary and secondary health care centres; dried blood spot collected in community setting can be sent to the laboratories for Hb estimation. ICM can therefore be used at point of care for screening for anaemia and assessing improvement in Hb after treatment especially in community and primary health care settings. It is essential that the tried and tested gold standard cyanmethaemoglobin method is used for Hb estimation at all levels of health care to enable early detection and effective treatment of anaemia. This may help to achieve rapid decline in prevalence of anaemia and enable India to achieve the National and SDG targets for reduction in anaemia within the time frame.

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