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**Original Research Article**

# High prevalence of alpha thalassemia in the tribal community of the western part of India! Reality or myth? Can simple hematology parameters; MCV and MCH act as screening tools at birth?

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## ABSTRACT

**Background:** The majority of adult tribal subjects in the western part of India, show microcytic hypochromic red cells, and borderline anemia with a normal iron profile, suggesting a high prevalence of thalassemia in this population.
**Methods:** The current study was designed to perform qualitative (to screen for Hb Bart's) and quantitative (to estimate percentage of Hb Bart's) hemoglobin electrophoresis with modification of the method, to evaluate the prevalence of α thalassemia and to determine gene frequency of α+ thal gene. Furthermore, the present study also aimed to evaluate common hematology parameters like MCV and MCH as screening tools to suspect α thalassemia at birth.
**Results:** Based on hemoglobin electrophoresis, the prevalence of α thalassemia in all its forms was found to be 66.66%. The estimated gene frequency for α+ thal was found to be 0.7453 and based on that, the extrapolated prevalence of α thalassemia was 93.52% (55.55% homozygous and 37.97% heterozygous). MCV<100 fl and MCH<31 pg were found to be reliable screening tools to predict α thalassemia at birth in full-term uncomplicated pregnancy.
**Conclusions:** Tribal community in the western part of India bears a very high prevalence of α thalassemia, it's a reality and not a myth. Simple hematological parameters like MCV (<100 fl) and MCH (<31 pg) measured at birth can prove to be cost-effective surrogate markers for α thalassemia. Large scale study using confirmatory genetic analysis is required to validate the findings.

**Keywords:** Alpha thalassemia, Hb Bart's, Cord blood, Hemoglobin electrophoresis, MCH, MCV

## INTRODUCTION

Human hemoglobin is described as a "show piece in the shop window of molecular genetics". Historically sickle cell disease (SCD) was the first human molecular disease reported in 1910.[1] More than 1420 human hemoglobin variants and 537 genetic mechanisms leading to various thalassemia have been detected since then. Fortunately, most of the variants do not produce significant morbidity and mortality and are restricted to a small geographic region or a few families.[2]

Better mutations are positively selected and undesirable mutations causing significant morbidity and mortality are eliminated by nature. However, mutations causing thalassemia and hemoglobinopathies have been

propagated over generations because the carriers of these autosomal recessive traits offer some survival advantage against deadly diseases like falciparum malaria over individuals who have two copies of the wild type allele; "balanced polymorphism". [3,4]

Thalassemias are a heterogenous group of hereditary hemoglobin disorders characterized by the reduction or total absence of synthesis of one or more than one globin chains, resulting in a wide spectrum of clinical manifestations, from asymptomatic to severe transfusion-dependent disease or even incompatible with life e.g. Hb Bart's hydrops fetalis. In thalassemia a relative excess of one chain over others, forms tetramers which precipitate on the cell membrane and cause hemolysis.[5,6]

The α thalassemia is one of the most prevalent isolated genetic disorders worldwide. Different genetic combinations of alpha thalassemia lead to an interesting phenomenon of trimodality. The combinations of three possible haplotypes: αα / (normal), α - / ($\alpha^+$ thal), and - -/ ($\alpha^0$ thal), produce 6 genotypes and 5 phenotypes.[7]

Observations from many clinical laboratories and research papers suggest that $\alpha^+$ thalassemia is very frequent in the tribal populations of India, to the extent that more than half of them are homozygous for the $\alpha^+$ thal gene, probably among the highest in the world. Moreover, $\alpha^+$ thalassemia even in homozygous form, does not produce significant disease apart from microcytic hypochromic red cells and high RBC count. Unfortunately, it is in a highly underprivileged population with poor socioeconomic status, and as this gene does not produce any ill effect on health, keeping the medical fraternity blissfully ignorant about a condition with such a high frequency.[8-10]

What is left for a curious student of molecular genetics is the survey of blood samples of the newborn of the tribal community. At birth 70 to 90 % of hemoglobin is Hb F ($\alpha_2\gamma_2$) and in case of shortage of α chains, the excess γ chains form Hb Bart's ($\gamma_4$) which can be detected qualitatively and quantitatively. Based on quantitative analysis of Hb Bart's, one can predict the genotype of α thalassemia.[11]

Moreover, the presence of α thalassemia in one form or the other lowers the hematological indices like MCV and MCH. These commonly performed and inexpensive hematological parameters can be used as screening tools for α thalassemia in neonates.[12,13]

The present study is an attempt to investigate newborn blood samples for a "genetic trait", suspected to have a disproportionately high prevalence of α thalassemia in the tribal community of the western part of India with the estimation of gene frequency.

The present study also aimed to evaluate whether simple hematological parameters like MCV and MCH can be utilized as screening tools to detect α thalassemia at birth. The results of this study may change our idea about what is "normal" for the tribal population.

## METHODS

This case-control study was conducted between April 2016 and November 2016 at Lok Samarpan Raktdan Kendra and research centre, Surat.

### *Inclusion and exclusion criteria*

The study group consisted of tribal females with full-term vaginal delivery. The samples from females with severe pregnancy-related complications or preterm delivery were rejected.

The control group consisted of nontribal pregnant females with full-term uncomplicated vaginal delivery.

### *Study design*

The cord blood samples were collected in K2 EDTA (Becton, Dickinson, and Company; NJ, US) from labor rooms of different obstetrics and gynecology hospitals of south Gujarat. Ethical approval was not required as the samples were collected from umblical cord after cutting.

All samples were analyzed on Sysmex KX 21 hematology analyzer which was calibrated with commercially available calibrators.

Qualitative hemoglobin electrophoresis was performed on all samples for the screening of α thalassemia (presence or absence of Hb Bart's). Fifty µl of well-mixed blood samples were mixed with 1.4 ml of normal saline (9 gm/l NaCl) and centrifuged at 10000 rpm for 5 minutes. The supernatant containing the plasma proteins was carefully removed. Fifty µl of alkaline water (0.4 ml liq. Ammonia in 1 litre of distilled water) was added to the red cell button and mixed well to ensure hemolysis and the tubes again were centrifuged at 10000 rpm for 10 minutes. Carefully 10 µl of the clear supernatant hemoglobin solution was transferred to the applicator well plate.

Mylar-backed cellulose acetate plates (Titan III, Helena laboratories, Beaumont, Texas) were slowly lowered in beakers filled with the electrophoresis buffer to avoid air bubble trapping inside and were allowed to soak for 10 minutes. The buffer contained Tris-10.2 gm, di-sodium EDTA 0.6 gm, and boric acid 3.2 gm dissolved in 1 litre of deionized water. Five ml of 50 gm/l thymol in 2-propanol was added as a preservative. Fifty ml of Glycerol per litre of buffer was added and mixed well to prevent drying; the pH of the buffer was 8.4. Then with the help of a multi-applicator (Super Z multi-applicator, Helena laboratories, Beaumont, Texas) the samples were applied lengthwise between one edge and the midpoint. Normally, 0.3 µl sample is enough for routine use, but for

the detection of very low values of Hb Bart's, it is preferable to make multiple (three) applications on the same spot. The strip was then inverted with the cellulose surface down (in contact with the filter paper wicks), keeping the charged edge towards the cathode. The current was switched on and the electrophoresis was conducted at 200 V DC. Since this was a heavy charging, good separation of close bands like Hb A and Hb F, was not possible but at the same time, it was possible to detect low quantities of Hb Bart's. Hb Bart's bands were noticed, moving faster than the rest of the material in form of thin lines within 30 minutes. After switching off the current, the strip was stained with ponceau S stain for better visualization.

The samples with detected Hb Bart's were subjected to quantitative hemoglobin electrophoresis.

The "cellogel" membrane was chosen for quantitative electrophoresis, which has smaller pore sizes than the dry membranes and can also absorb a larger amount of hemoglobin solution due to its thickness.

To get good separation of a very small amount of Hb Bart's from relatively large amounts of Hb A, F, and sometimes Hb S, buffers having pH between 6.5 and 7.2 were tried, of which, TEB buffer (pH- 8.4) neutralized to 6.7 with saturated citric acid was found to be satisfactory. Fifty ml of glycerol was added to 1 litre of buffer to prevent drying during any step of the process. Each sample was applied three times at the same spots in the centre with a 9 mm applicator to get the heavy application. During the electrophoretic run, Hb Bart's moved towards the anode, while the others stayed on midpoint or moved slightly towards the cathode, giving enough space to cut the bands and elute in water. Electrophoresis was performed at 250-300 volts DC till good separation of Hb Bart's bands was obtained. After switching off the current, each strip was lifted carefully and the band of Hb Bart's was cut and put in a labelled 12 x 75 mm glass tube containing 1 ml of alkaline water and closed with a tightly fitting rubber bung. The thick bands of Hb A and F were similarly cut, put in a glass bulb containing 10 ml of elution water, and tightly stoppered. Elution was achieved by putting the tubes and bulbs on a roller mixer for 15 minutes.

The absorbance of Hb Bart's and the rest (Hb A and Hb F) were taken in a chemistry analyzer with a 405 nm filter, against distilled water blank. The percentage of Hb Bart's was then calculated as follows:

$$\%\ Hb\ Bart's = \frac{Absorbance\ Hb\ Bart's \times 100}{Absorbance\ Hb\ Bart's + 10 \times Absorbance\ of\ Hb\ A\ and\ Hb\ F}$$

### *Statistical analysis*

Statistical analysis was carried out using Excel 2013, Microsoft, USA, and OpenEpi software tools.

## RESULTS

The results of qualitative Hb electrophoresis of 36 samples from tribal mothers resulted in a significant band in the region of Hb Bart's in 24 (66.66%) cases while other 12 (33.33%) samples from tribal mothers as well as all 42 samples from nontribal mothers did not show any band of Hb Bart's.

**Table 1: Result of hemoglobin electrophoresis in all 78 subjects.**

| Groups | % Hb Bart's | n |
|---|---|---|
| **Non-tribal** | 0 | 42 |
| **Tribal; normal** | 0 | 12 |
| **Tribal; suspected one gene deletion** | 0-4 | 4 |
| **Tribal; suspected two genes deletion** | >4 | 20 |

It is likely that due to technical limitations of hemoglobin electrophoresis, some heterozygous α+ thalassemia cases might not have been detected due to low concentration of Hb Bart's, but all the homozygotes were surely detected. On close observation of histogram frequency of Hb Bart's percentage (Figure 1), a small trough/separation could be observed at about 4%, above which, there were 20 cases and, in all probability, represent the homozygous cases of α+ thalassemia. So, a cut-off of 4% of Hb Bart's was decided to separate one gene deletion from two gene deletion cases.

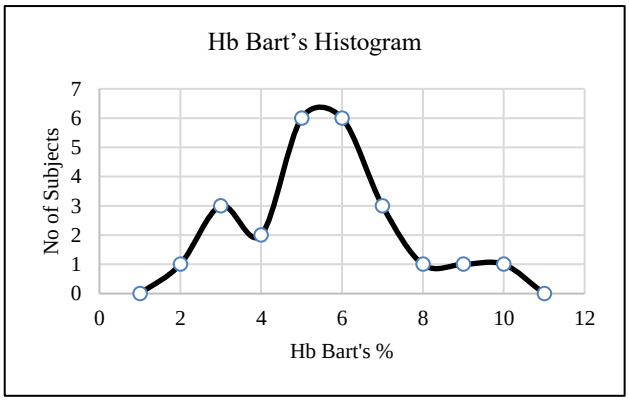


**Figure 1: Frequency Histogram of Hb Bart's%.**

**Table 2: Estimated gene frequency of α+thalassemia.**

| Genotype | Gene frequency in tribal |
|---|---|
| **α -** | 0.7453 |
| **αα** | 0.2547 |

Because the number of Hb Bart's was substantial in homozygous alpha thalassemia, technological limitations did not significantly interfere with quantitative estimation. Twenty cases where percent Hb Bart's were above 4% were considered homozygous α+ thalassemia

and the Hardy-Weinberg equation was used to calculate the gene frequency of the α+ thalassemia genotype as well as the percentages of other genotypes.

**Table 3: Projected percentage of α+thalassemia genotypes in tribal.**

| Genotype | Projected percentage in tribal |
|---|---|
| **α -/ α -** | 55.55 |
| **α - / αα** | 37.97 |
| **αα / αα** | 6.48 |

Based on extrapolated gene frequency, if the results of the current study were to be projected then those with normal genotype should be 2 out of 36, those with one gene deletion should be 14 out of 36, and those with two genes deletion should be of 36. However, only 4 cases with one gene deletion were detected. This could be due to low sample size as well as technical limitations which might have prevented the detection of heterozygous cases with one gene deletion due to low percentage of Hb Bart's.

When statistical comparison of MCV and MCH between different groups was done, a higher statistically significant difference with higher T statistics value (10.5793 for MCV and 16.75 for MCH) was observed while comparing MCV and MCH between cord blood samples of Hb Bart's positive tribal mother and nontribal mothers.

**Table 4: Statistical comparison of MCV and MCH between different groups.**

| Category | n | MCV fl | | MCH pg | |
|---|---|---|---|---|---|
| | | **Mean±SD** | **AUC; p; T-statistics** | **Mean±SD** | **AUC; p; T-statistics** |
| **Non-tribal versus tribal: total** | 42 | 109.5±4.94 | 0.915; <0.001; -8.58 | 34.9±1.83 | 0.956; <0.001; -11.82 |
| | 36 | 98.5±6.37 | | 28.9±2.63 | |
| **Non-tribal versus tribal: Hb Bart's positive** | 42 | 109.5±4.94 | 0.973; <0.001; -10.58 | 34.9±1.83 | 1.00; <0.001; -16.75 |
| | 24 | 95.9±5.17 | | 27.7±1.37 | |
| **Non-tribal versus tribal: Hb Bart's negative** | 42 | 109.5±4.94 | 0.803; <0.001; -3.55 | 34.9±1.83 | 0.863; <0.001; -4.74 |
| | 12 | 103.6±5.52 | | 31.7±2.75 | |
| **Tribal: Bart's positive versus tribal: Hb Bart's negative** | 24 | 95.9±5.17 | 0.867; <0.001; -4.12 | 27.7±1.37 | 0.877; <0.001; -5.86 |
| | 12 | 103.6±5.52 | | 31.7±2.75 | |

While comparing red cell indices between cord blood samples of Hb Bart's negative tribal mother and non-tribal mothers, the difference was significant but with a lower T statistics value (3.55 for MCV and 4.74 for MCH).

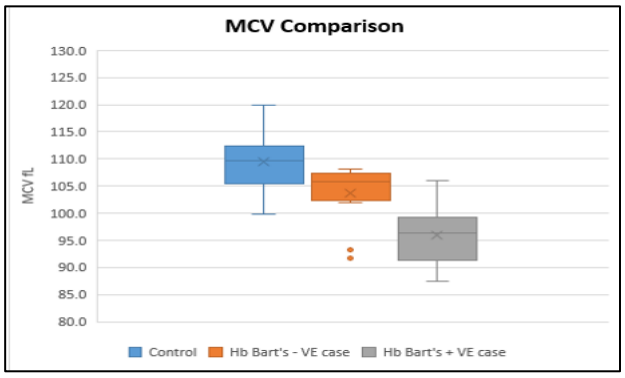


**Figure 2 (a): Box and Whisker plot comparing MCV in three groups.**

The same can be seen in the Box and Whisker plot of MCV (Figure 2a) and MCH (Figure 2b) showing the comparison between the three groups.

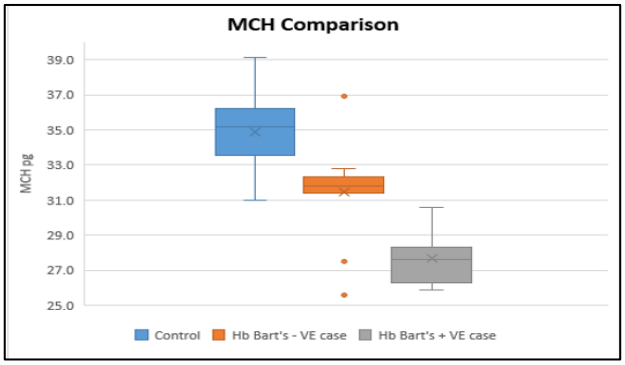


**Figure 2 (b): Box and Whisker plot comparing MCH in three groups.**

In an attempt to evaluate common red cell parameters; MCV and MCH as screening tools to predict α Thalassemia at birth, ROC analysis was performed yielding AUC in MCV curve (Figure 3a) of 0.95 and in MCH (Figure 3b) curve of 0.973. Based on statistical analyses, the cut-off MCV<100 fl and MCH<31 pg were kept, and sensitivity, specificity, PPV, NPV, and accuracy were calculated (Tables 5 and 6). As shown in Table 6, MCH at 31 pg was found highly sensitive as a screening tool.

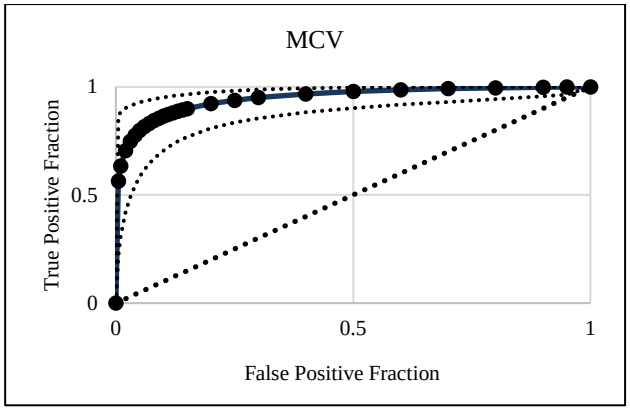


**Figure 3 (a): ROC curve of MCV.**

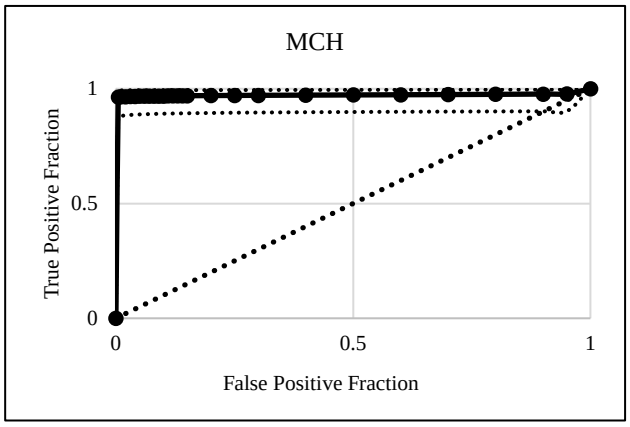


**Figure 3 (b): ROC curve of MCH.**

**Table 5: Evaluation of MCV as a screening tool at 100 fl cut-off.**

| Para/group | Hb Bart's +ve | Hb Bart's -ve | Total |
|---|---|---|---|
| **MCV<100** | 19 | 3 | 22 |
| **MCV>100** | 5 | 51 | 56 |
| **Total** | 24 | 54 | 78 |

Sensitivity: 79.17%, specificity: 94.44%, positive predictive value: 86.36%, negative predictive value: 91.07%, accuracy: 89.74%

**Table 6: Evaluation of MCH as a screening tool at 31 pg cut-off.**

| Para/group | Hb Bart's +ve | Hb Bart's -ve | Total |
|---|---|---|---|
| **MCH<31** | 24 | 2 | 26 |
| **MCH>31** | 0 | 52 | 52 |
| **Total** | 24 | 54 | 78 |

Sensitivity: 100.00%, specificity: 96.30%, positive predictive value: 92.31%, negative predictive value: 100.00%, accuracy: 97.44%

## DISCUSSION

The tribal population of India is unique since they have two inherited genetic disorders; sickle cell (β chain qualitative disorder) and α thalassemia (α chain quantitative disorder) together. Since sickle cell disease produces significant morbidity and mortality, extensive work has been done on it to understand the disease. While on the other hand α thalassemia is largely neglected as even in the homozygous state, does not produce significant disease, other than mild anemia. The prevalence of α thalassemia in tribal communities of Gujarat is found to be very high in the present study. The estimated gene frequency for $\alpha^+$ thal was 0.7453 and the extrapolated prevalence of α thalassemia was 93.52% (55.55% homozygous and 37.97% heterozygous). Similar observations were found in a few studies conducted in India.[9,14] The reason for such a high degree of selection of $\alpha^+$ thal gene is not known. It must have some beneficial role to play in the clinical severity and morbidity of sickle cell anemia patients.[15] It is also believed that $\alpha^+$ thalassemia in homozygous and heterozygous state offer protection against severe and complicated falciparum malaria otherwise having high mortality.[3,4]

The present study also evaluated the utility of cord blood MCV and MCH as screening tools for α thalassemia. The advantage of utilizing MCV and MCH as screening tools is that they are routinely prescribed, easily available, and cost-effective. The genetic study is required for the confirmation but it is costly and requires a sophisticated setup. Many laboratories carry out molecular tests designed to detect only those deletions or mutations common to the population to avoid high costs, which may miss many uncommon mutations.[16,17] In parts of the world where there is a high frequency of $\alpha^+$ thal, the cost would be enormous if one wants to diagnose all carriers. It should also be remembered that most countries harboring a high frequency of the gene are also poor in resources.[18] So, the role of cost-effective and widely available screening tools like MCV and MCH become much more important if the correlation is well established by large-scale studies.

Considering the technical limitations and small scale of the present study, these preliminary findings must follow with a large-scale, comprehensive study involving more sophisticated confirmatory investigations like α gene mapping by sequencing to validate the findings.

## CONCLUSION

Mild to moderate anemia with microcytic hypochromic red cells in the tribal population of Gujarat is largely due to α+ thalassemia and that should be considered "normal" for them. These subjects should not be treated with iron therapy unless diagnosed to have iron deficiency.

At birth, simple hematological parameters like MCV (<100 fl) and MCH (<31 pg) can be the reliable screening tools to suspect α thalassemia.

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