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To Study Hematological Indices And Peripheral Smear Examination And Different Types Of Hemoglobin Variants In Microcytic Hypochromic Anemia

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Abstract:

To detect Hemoglobinopathies by Electrophoresis in Microcytic Hypochromic anemia in young population in the area covered by tertiary care centre at Index Medical College & Hospital. All young patients (10-30 years) who presented with pallor and were detected to have microcytic hypochromic picture on peripheral smear examination were included in the study. Microcytic hypochromic anemia is common problem in central India. The haemoglobinopathies (Structural and functional disorders of haemoglobin) are major World health problem. These are single gene, autosomal, recessive monogenic disorders that include thalassaemia and sickle cell anemia. Hemoglobinopathies presents as microcytic hypochromic anemia. They are misdiagnosed and treated as iron deficiency anemia in hemoglobinopathies iron is not required by the body. This causes burden to the patient economically as well as on health. The excess iron which is not required by body has a toxic effect on the body.Most common cause of microcytic hypochromic anemia was iron deficiency anemia and abnormal hemoglobin disorder . Differential diagnosis based on complete hemogram and peripheral smear is possible but special tests like serum iron profile and haemoglobin electrophoresis are a must for confirmation of the diagnosis.

Keywords: Haemoglobinopathies, Peripheral Blood Smear, Complete Blood Count (CBC), Haemoglobin electrophoresis,

I. INTRODUCTION

Hemoglobinopathies are serious genetic blood disorders requiring lifelong blood transfusions and treatment in its most severe, chronic form. If un - diagnosed or un-treated, these disorders ultimately lead to death. South Asia especially India, Pakistan and Bangladesh have high populations of hemoglobinopathies. **In India,** there are an estimated 100,000 thalassemia majors patients and nearly 3.5 to 4 million carriers of this genetic disorder. Tribal populations in India have a range of 5-40% sickle cell anemia sufferers. In Eastern India, variant hemoglobinopathies like HBE are as common as 3-50% of the population.

In India, Beta-Thalassemia is prevalent across the country, with an average frequency of carriers being 3-4% [1,2,3]. A higher frequency has been observed in certain communities, such as Sindhis, Punjabis, Gujaratis, Bengalis, Mahars, Kolis, Saraswats, Lohanas and Gaurs [3,4]. HbS is highly prevalent in the tribal populations of Southern, Central and Western states reaching as high as 48% in some communities8. HbE is common in the North Eastern states, and has a carrier frequency as high as 50%, in some areas. It is found in lower frequencies in the Eastern states of West Bengal, Bihar and Uttar Pradesh, while HbD is present in about 2% of people in Punjab.

Thalassemia:

The thalassemia are the commonest single-gene disorders. Thalassemia was first recognized by Cooley and Lee in 1925 as a form of severe anaemia associated with splenomegaly and bone changes in children. The term ‗thalassemia' is derived from the Greekθαλασσα- (meaning‗ thesea') since many of the early cases came from the Mediterranean region. However, it is now clear that the disorder is not just limited to the Mediterranean region, but occurs throughout the world, prevalent in the tropical and subtropical regions, including the Middle East, parts of Africa, Indian subcontinent and Southeast Asia.

The first case of β-thalassemia/Hb E disease in India was reported by Chatterjee et al[5] , and that of β-thalassemia/sickle cell disease by Naik and colleagues[6] Subsequently other variants haemoglobins were reported – C, D, F, G, H, J, K, L, M, Q (India), G, etc[7-11]. Haemoglobins S, D and E were observed to be quite common: Hb S has been found mostly in tribal communities, Hb D in Gujaratis and Punjabis and Hb E in Bengalis, Assamese and Nepalese.

Thalassemias are clinically divided into Thalassemia Major (TM), Thalassemia Intermedia (TI) and Thalassemia Minor or Trait according to severity. Thalassemia Major (TM) and the severe form of Thalassemia Intermedia (TI) constitute the major burden of disease as management of both requires lifelong blood transfusions and iron chelation. While Thalassemia minor is the carrier state in which the person is clinically normal and is commonly referred to as beta-Thalassemia Trait (BTT). The thalassemia syndromes (TM, TI) are caused by inheritance of abnormal beta thalassemia genes from both carrier parents, or abnormal beta - Thalassemia gene from one parent and an abnormal variant hemoglobin gene (HbE, HbS) from the other parent.

Sickle Cell Anemia:

Sickle Cell Disease (SCD) is another haemoglobin disorder that requires lifelong management and contributes to infant and childhood morbidity and mortality. SCD is caused by inheritance of two abnormal HbS genes, one from each parent or Hb S gene from one parent and HbE or ù thalassemia gene from the other. Sickle cell syndromes include Sickle Cell Disease (SCD, HbSS), also called Sickle Cell Anemia (SCA), as well as disorders due to sickle cell gene combined with another hemoglobinopathy such as Hb C, E, or beta thalassemia.

The disorder we call ―Sickle Cell Disease‖ often abbreviated as SCD, had been present in Africa for at least five thousand years and has been known by many names in many tribal languages. What we call its ―discovery‖ in 1910 occurred, not in Africa, but in the United States. A young man named Walter Clement Noel from the island of Grenada, a dental student studying in Chicago, went to Dr. James B. Herrick with complaints of pain episodes, and symptoms of anemia. Herrick was a cardiologist and not too interested in Noel's case so he assigned a resident, Dr. Ernest Irons to the case. Irons examined Noel's blood under the microscope and saw red blood cells he described as ―having the shape of a sickle‖. When Herrick saw this in the chart, he became interested because he saw that this might be a new, unknown, disease. He subsequently published a paper in one of the medical journals in which he used the term ―sickle shaped cells‖.

In India - First described in the Nilgiri Hills of northern Tamil Nadu in 1952[12], the sickle cell gene is now known to be widespread among people of the Deccan plateau of central India with a smaller focus in the north of Kerala and Tamil Nadu[13]. Extensive studies performed by the Anthropological Survey of India[14] have documented the distribution and frequency of the sickle cell trait which reaches levels as high as 35 per cent in some communities.

II. MATERIALS & METHODS

A cross sectional study was carried out in a hospital in rural area of Indore on young patients (10- 30 years age group) who attended OPDs from May 2018 to June 2021.

Total 420 cases of moderate to severe anaemia were included in the study. **Inclusion Criteria**

All young patients (10-30 years) who presented with pallor and were detected to have microcytic hypochromic picture on peripheral smear examination were included in the study.

Exclusion Criteria:

1. Peripheral picture other than microcytic hypochromic anemia.
2. Those who are not willing.

All patients underwent following investigations:

1. Complete blood count.
2. Peripheral blood smear study.
3. Sickling Test.
4. Hemoglobin Electrophoresis.

Sample Collection:

Whole blood sample(10ml) was collected by venepuncture from all subject recruited in the study. For complete blood count and peripheral smear (7ml) of blood was transferred to a tube containing EDTA anticoagulant and another (3ml) was transferred to another EDTA containing tube for the determination of Cellulose Acetate Haemoglobin Electrophoresis.

Electrophoresis:

Cellulose acetate electrophoresis at alkaline pH:

Haemoglobin electrophoresis at pH 8.4–8.6 using cellulose acetate membrane is simple, reliable and rapid. It is satisfactory for the detection of most common, clinically important haemoglobin variants. [65.66.67]

Principle:

At alkaline pH, haemoglobin is a negatively charged protein, and when subjected to electrophoresis will migrate toward the anode (+). Structural variants that have a change in the charge on the surface of the molecule at alkaline pH will separate 42 from haemoglobin A. Haemoglobin variants that have an amino acid substitution that is internally sited may not separate, and those that have an amino acid substitution that has no effect on overall charge will not separate by electrophoresis.[68]

Equipment:

- **Electrophoresis tank and power pack**. Any horizontal electrophoresis tank that will allow a bridge gap of 7 cm. A direct current power supply capable of delivering 350 V at 50 mA is suitable for both cellulose acetate and acid agarose gels.
- Wicks of filter or chromatography paper.
- Blotting paper.
- Applicators. These are available from most manufacturers of electrophoresis equipment, but fine

micro capillaries are also satisfactory.

- Cellulose acetate membranes. Plastic-backed membranes (7.6 × 6.0 cm) are recommended for ease of use and storage. Staining equipment.

Reagents:

- Electrophoresis buffer- Tris/EDTA/borate (TEB), pH 8.5. Tris-(hydroxymethyl) amino methane (Tris), 10.2 g; EDTA (disodium salt), 0.6 g; boric acid, 3.2 g; water to 1 litre. The buffer should be stored at 4 °C and can be used up to 10 times without deterioration.
- Wetting agent. For example, Zip Zone Prep solution : 1 drop of Zip Zone Prep in 100 ml water.
- Fixative/stain solution. Ponceau S 5 g; trichloroacetic acid, 7.5 g; water to 1 litre Destaining solution. 3% (v/v) acetic acid, 30 ml; water to 1 litre.
- Haemolysing reagent. 0.5% (v/v) Triton X-100 in 100 mg/l potassium cyanide.

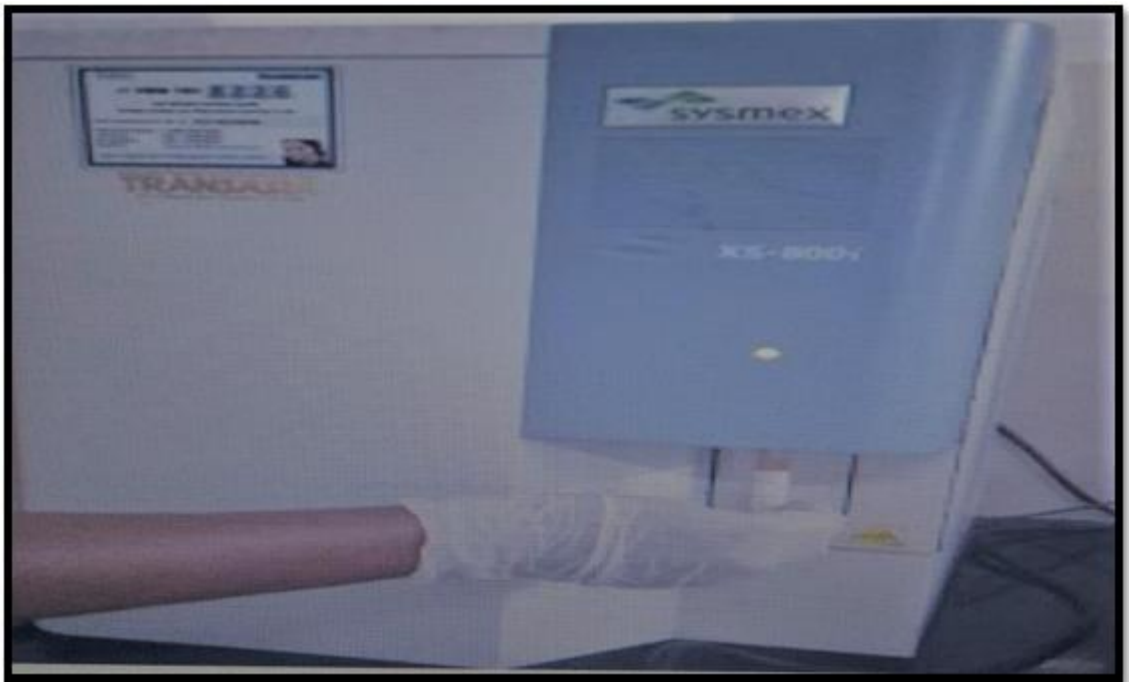
Interlab Genio S electrophoresis appratus and Interlab master kit of Reagents.

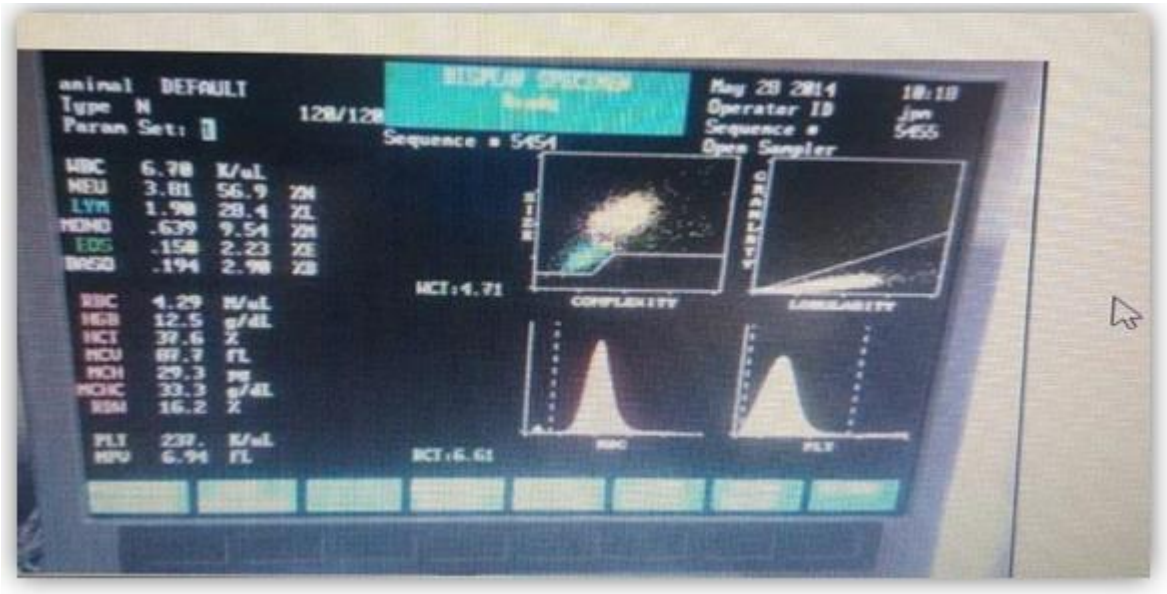
Complete Blood Count:

Complete blood count performed by an automated analyzer that counts the number and type of different cells within the blood. It aspirates a very small amount of the sample through the narrow tube. With this tube there are sensors that count the number of the cells going through it, and can identify the type of cell, this is called flowcytometry. For detection light detectors are used as well as for the measurement of the electrical impedances. Other instrument measure different characteristics of the cell to categorize them. Sysmex XS800i fully automated Cell counter was used for CBC. It is the 5-part differential instrument. It reports on all the five subpopulations namely, neutrophils, eosinophils, basophils, monocytes and lymphocytes. MCV is a key diagnostic indicator. An important factor viz., iron deficiency anemia(IDA) coexisting with beta thalassemia trait can have very low value for MCV. For all purposes MCV of <=65 fl, indicates possibility of coexistence of beta thalassemia trait and iron deficiency.

A CBC report provides:-

1. Leukocyte count with differential—total white blood cell count and a 5 part differential (lymphocytes, monocytes, neutrophils, basophils, and eosinophils).
2. Red blood cell count (also known as corpuscles).
3. Platelet count (also known as thrombocytes)—critical for blood clotting. 4. Platelet volume (measured in femtoliters).
4. Hemoglobin concentration (measured in grams per deciliter).
5. Hematocrit (also known as packed cell volume, expressed as a percentage measuring the total amount)





Automated cell counter Sysmex XS 800i

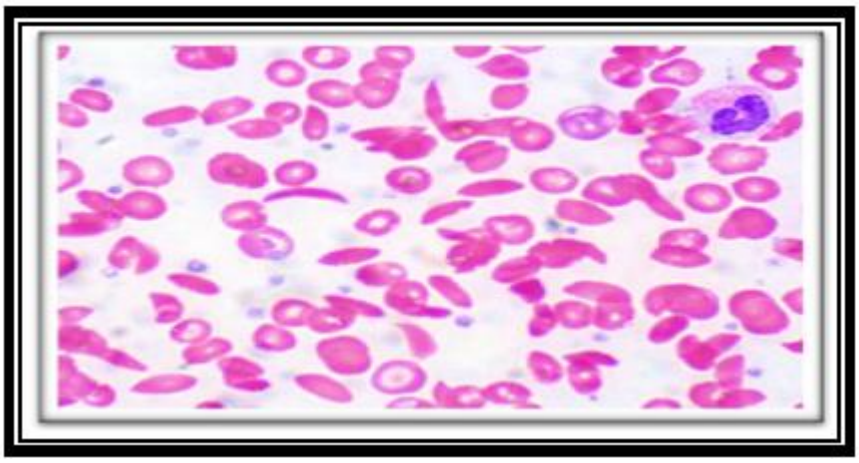
Peripheral Blood Smear:

A blood smear is a blood test used to look for abnormalities in blood cells. The three main blood cells that the test focuses on are:

- Red cells,
- White cells,
- Platelets.

Blood films are made by placing a drop of blood on one end of a slide, and using a spreader slide to disperse the blood over the slide's length. The aim is to get a region, called a monolayer, where the cells are spaced far enough apart to be counted and differentiated. The slide is left to air dry, after which the blood is fixed to the slide by immersing it briefly in methanol. The fixative is essential for good staining and presentation of cellular detail. After fixation, the slide is stained to distinguish the cells from each other. Routine analysis of blood in medical laboratories is usually performed on blood films stained with Romanowsky stains such as Wright's stain, Giemsa stain.

Microscopic examination of the shape, size, and coloration of red blood cells is useful for determining the cause of anemia. Disorders such as iron deficiency anemia, sickle cell anemia, megaloblastic anemia and microangiopathic hemolytic anemia result in characteristic abnormalities on the blood film.[60]



Reticulocyte Count:

Principle: Brilliant cresyl blue is an isotonic stain selectively stains nucleic material of erythrocytes called reticulocyte , which can be seen under a microscope directly or with a counter stain.[61,62,63]
The reaction takes place only in supra-vitally stained unfixed preparations.
a) Staining Solution: Manufactured by Biolab diagnostics (I) PVT. LTD is used. In 25 ml reticulocyte stain, brilliant cresyl blue 20 mMol/L,Citric acid 180mMol/L, Sodium chloride 1.55mMol/L & phosphate buffer 0.1 mMol/L are present which are polychromatic materials, preservatives and stabilizers.
Method Equal volume of the patient's EDTA – anticoagulated blood was added to the dye solution and mixed. Mixture was kept at 37oC for 20 min. Film was made on the glass slides in the usual way. When dried , examined the films without fixing or counterstaining.
Counting Reticulocytes: To count the cell, 100x oil immersion objective is used. When the count is < 10% , a convenient method is to survey successive fields until at least 100 reticulocytes have been counted and to count the total red cells in at least 10 fields to determine the average number of red cells per field.

Normal range :	
Adult :	0.5- 2.5%
Infants (full term cord blood)	2-5 %

Sickling Test:
When red cells containing Hb S are subjected to deoxygenation, they become sickleshaped while cells that do not contain Hb S remain normal. Certain reducing chemical agents such as 2% sodium metabisulfite or sodium dithionate can deprive red cells of oxygen.

Procedure:-
Blood and 2% sodium metabisulphite reducing agent in equal proportion was mixed on a glass slide and a cover slip was placed over it then it was sealed with petroleum jelly or paraffin was mixture. Amount of HbS in red cells and degree of deoxygenation influence the speed and extent of sickling. Sickling is usually evident after 30 minutes; if it is not then the slide is re examined after allowing it to stand overnight. The sickled cells have minimum of two pointed projections , If more than 25% show sickling , the test is positive.[64]

III. RESULTS & DISCUSSION
Out of 420 cases, there were 308 (73.4%) were negative on electrophoresis (Group I) and 112 (26.7%) patients were positive on electrophoresis (Group II). Majority of the patients were negative on electrophoresis. Table 1

Table No.1: Distribution of patients according to group

Group	Number	Percentage
Cases negative on Electrophoresis (Group-I)	308	73.30%

Cases positive on Electrophoresis (Group-II)	112	26.60%
Total	**420**	**100.0%**

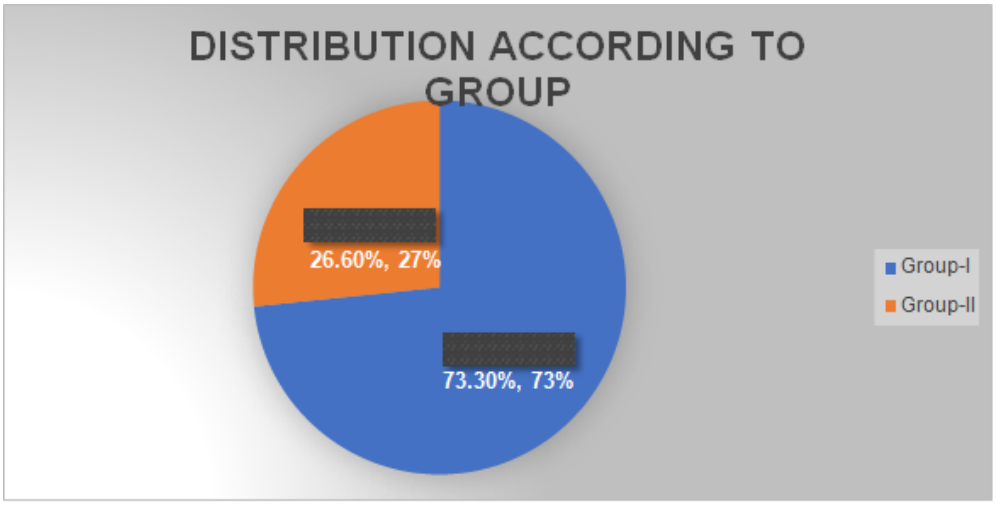


Fig 1: Pie diagram showing distribution according to group

The above table shows the distribution according to hemoglobinopathies in relation to gender positive on electrophoresis. Of the 42 females, 12 (28.5%) were having beta thalassemia trait, 05 (11.9%) were having sickle beta thalassemia, 14 (33.4%) were having sickle cell anemia and 11 (26.2%) were having sickle cell trait. Of the 70 males, 20 (28.6%) were having beta thalassemia trait, 03 (04.3%) were having sickle beta thalassemia, 29 (41.5%) were having sickle cell anemia and 18 (25.8%) were having sickle cell trait.

Table No. 2: Distribution according to hemoglobinopathies in relation to gender

	Female		**Male**	
	No.	**%**	**No.**	**%**
Sickle cell Trait	11	26.2	18	25.8
Sickle cell anemia	14	33.4	29	41.5
Sickle beta thalassemia	05	11.9	03	04.3
Beta thalassemia trait	12	28.5	20	28.6
Total	**42**	**100.0**	**70**	**100.0**

The association between hemoglobinopathies and the gender was found to be statistically not significant (p=0.068), showing that hemoglobinopathies are independent of the gender. Sickle cell anemia and beta thalassemia trait were commonly seen in both the genders.

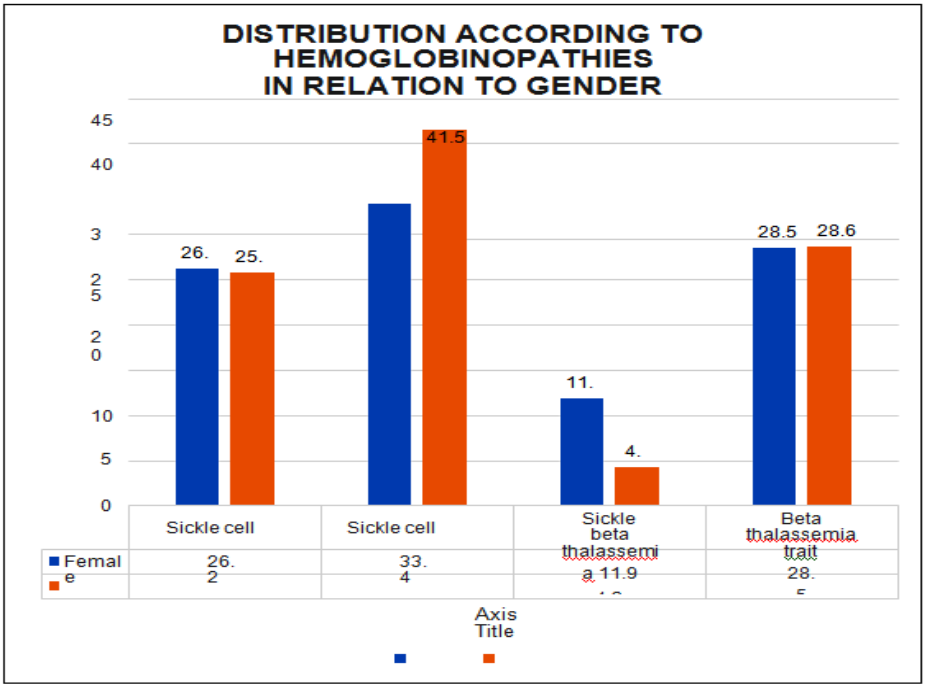


Fig 2: Bar diagram showing distribution of patients according to hemoglobinopathies in relation to gender

The above table shows the distribution according to hemoglobinopathies in relation to gender and age in cases positive on electrophoresis (Group II)

<=13 years:

Of the 15 females, 3 (2.0%) were having beta thalassemia trait, 2 (13.4%) were having sickle beta thalassemia, 8 (53.4%) were having sickle cell anemia and 2 (13.4%) were having sickle cell trait.

Of the 27 males, 8 (29.7%) were having beta thalassemia trait, 1 (3.8%) were having sickle beta thalassemia, 11 (40.8%) were having sickle cell anemia and 7 (25.9%) were having sickle cell trait**.**

Table No.3: Distribution of hemoglobinopathies in relation to gender and age

	<=13 years				**>13 years**			
	Female		**Male**		**Female**		**Male**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Sickle cell trait	2	13.4	7	25.9	9	33.4	11	25.6
Sickle cell anemia	8	53.4	11	40.8	6	22.3	18	41.9
Sickle beta thalassemia	2	13.4	1	3.8	3	11.2	2	4.7
Beta thalassemia trait	3	2.0	8	29.7	9	33.4	12	27.9
Total	**15**	**100.0**	**27**	**100.0**	**27**	**100.0**	**43**	**100.0**

>13 years:

Of the 27 females, 9 (33.4%) were having beta thalassemia trait, 3 (11.2%) were having sickle beta thalassemia, 6 (22.3%) were having sickle cell anemia and 9 (33.4%) were having sickle cell trait.
Of the 43 males,12 (27.9%) were having beta thalassemia trait, 2 (4.7.2%) were having sickle beta thalassemia, 18 (41.9%) were having sickle cell anemia and 11(25.6%) were having sickle cell trait.

Table No.4: Distribution of final diagnosis in relation to age in both the groups

	<=13 years				>13 years			
	Group I		Group II		Group I		Group II	
	No.	%	No.	%	No.	%	No.	%
Sickle cell trait	-	-	9	21.5	-	-	20	28.6
Sickle cell anemia	-	-	19	45.3	-	-	24	34.3
Sickle beta thalassemia	-	-	3	7.2	-	-	5	7.1
Beta thalassemia trait	-	-	11	26.2	-	-	21	3.0
Anemia of Chronic disease & other causes	19	16.3	-	-	37	19.4	-	-
Iron deficiency	98	83.8	-	-	154	80.7	-	-
Total	**117**	**100.0**	**42**	**100.0**	**191**	**100.0**	**70**	**100.0**

The above table shows the distribution according to final diagnosis in relation to age in both the groups.
In <=13 years:
In Group I (cases negative on electrophoresis), 19 (16.3%) patients were having anemia of chronic disease & other causes and 98 (83.8%) patients were having iron deficiency.
In Group II (cases on electrophoresis), 11 (26.2%) patients were having beta thalassemia trait, 3 (7.2%) patients were having sickle beta thalassemia, 19 (45.3%) patients were having sickle cells anemia and 9 (21.5%) patients were having sickle cell trait.

Table No. 5: Severity of Anemia in Various Hemoglobinopathies

	Mild Anemia (10-11gm/dl)	Moderate Anemia (7-10gm/dl)	Severe Anemia (4-7gm/dl)	Very Severe Anemia (≤ 4gm/dl)
Beta thalassemia trait	01	12	13	06
Sickle beta thalassemia	00	04	03	01
Sickle cell anemia	01	17	21	04

Sickle cell trait	01	12	11	05
Total	**03**	**45**	**48**	**16**

In >13 years:

In Group I, 37(19.4%) patients were having anemia of chronic disease & others and 154 (80.7%) patients were having iron deficiency.

In Group II, 21 (3.0%) patients were having beta thalassemia trait, 5 (7.1%) patients were having sickle beta thalassemia, 24 (34.3.2%) patients were having sickle cells anemia and 20 (28.6%) patients were having sickle cell trait.

In Group I, in both the age groups, iron deficiency was more commonly seen and in Group II, in both the age groups, sickle cell anemia was the commonest.

Table No. 6: Comparison of blood parameters between the two groups

Parameter	Group I [Mean±SD] (n=308)	Group II [Mean±SD] (n=112)
Hb	6.49 ± 2.20	6.61 ± 2.22
MCV	63.89 ± 8.64	64.70 ± 8.09
MCH	19.25 ± 3.41	20.18 ± 3.03
MCHC	29.53 ± 3.46	30.50 ± 2.82

Table No.7: Comparison of other parameters between the two groups

Parameter	Group I [Mean±SD] (n=308)	Group II [Mean±SD] (n=178)
Hb A	96.55 ± 2.23	48.19 ± 66.12
Hb F	0.75 ± 0.44	13.77 ± 15.23
Hb S	-	54.34 ± 19.52
Hb A2	2.39 ± 0.68	3.95 ± 2.01
Serum iron	41.79 ± 8.96	
TIBC	434.73 ± 103.51	
Saturation	10.36 ± 4.34	

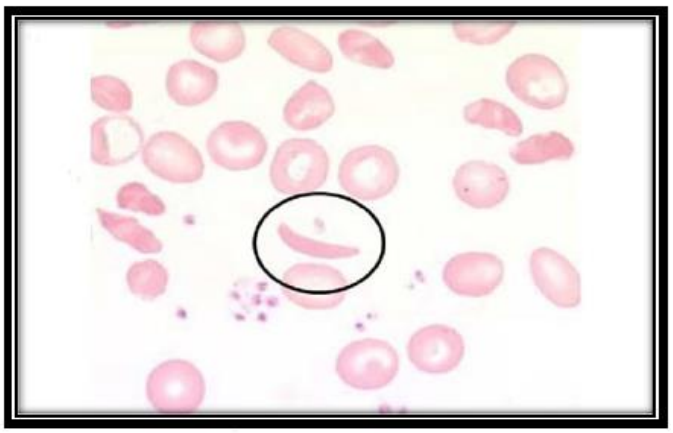


Fig. 3 : Peripheral Smear of hemolytic anemia showing sickle cell, anisopoikilocytosis and target cells.

Fig. 4: Wet Preparation of Sickling test with Sodium Metabisulphite showing sickle cells after 24 hours.

VI. DISCUSSION

Microcytic Hypochromic is a very common finding amongst Indian rural population and has a varied etiology. A wide segment of these is due to hemoglobinopathies. The definitive diagnosis of which is possible only by using electrophoresis. Without this tool the cases of microcytic hypochromic anemia due to hemoglobinopathies would remain undiagnosed. The present study was undertaken to assess the utility of electrophoresis in establishing with certainty the etiology behind microcytic hypochromic anemia. The study was conducted on 420 individuals attending the OPD of Index Medical College (tertiary care centre) between May 2018 to June 2021. The initial diagnosis was made on Hemogram & Peripheral smear which showed reduced hemoglobin and MCV with microcytic hypochromic cells respectively. Subsequently, all of these cases were subjected to electrophoretic study. 112 out of the total of 420 cases were found to have one or the other form of hemoglobinopathy. Rest i.e 308 (73.30%) were subjected to further investigations like iron studies and revealed a mix of Iron Deficiency Anemia 252 and anemia of chronic disease 56 . Out of 112 cases 43 cases were of Sickle cell anemia, 32 cases of Beta thalassemia, 29 cases of sickle cell Trait and 8 cases of sickle – beta thalassemia.

Other studies conducted from time to time showing burden of hemoglobinopathies in various parts of India, are in consistence with our study. In a study by Narang et al 78 (39%) cases of one or the other form of haemoglobinopathies were 75 documented. [57] A study conducted in Gujarat from 2010 to 2011, 2022 individuals were screened out of which 788(38.97%) were having haemoglobinopathies (Patel et al., 2012). [69]

V. CONCLUSION

Many times complete blood picture may not reveal the exact morphology needed to identify the hemoglobinopathy, hence in such type of cases Hb electrophoresis is done. Hemoglobin electrophoresis is a rapid, reliable and cost effective method to detect hemoglobinopathies. To reduce the burden of hemoglobinopathies screening of all anemic patients should be done in all areas and in all communities where the socioeconomic condition indicate frequent occurrence of genetic mutations. General counselling and screening of the adolescents should be done to avoid marriage between carriers. Andatory rules for Neonatal screening for hemoglobinopathies after six months should be taken by Government. The ANMs, ASHA and Anganwadis should be taught about the importance of screening of Hemoglobinopathies. So that they can explain the purpose of screening to the female during pregnancy and to the families in the rural areas. At primary level, the CHCs &PHCs should be equipped with instruments for basic testing of hemoglobinopathies

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