TO DETECT HEMOGLOBINOPATHIES BY DOING HEMOGLOBIN ELECTROPHORESIS IN MICROCYTIC HYPOCHROMIC ANEMIA

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ABSTRACT:The aim of the study was analyze the types of haemoglobinopathies in patients of Anemia using hemoglobin electrophoresis. Haemoglobinopathies like thalassaemia and sickle cell anemia etc are increasing due to unawareness of rural population. 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 anemiainhemoglobinopathies 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. By doing electrophoresis in microcytic hypochromic anemia, we can categorize anemia into different groups. Electrophoresis helps in giving correct diagnosis. 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:Hemoglobinopathies,MicrocyticHypochromic   Anemia,,   Haemoglobin electrophoresis, Sickle cell anemia, Thalassaemia.

I.INTRODUCTION

Anemia is defined as decrease in hemoglobin concentration below lower limit of normal with reference to age and gender anemia is not a diagnosis but a finding that requires further investigation.While investigating anemia, apart from low hemoglobin levels certain other parameters need consideration. Red cell indices, red cell count, and morphological examination of peripheral blood film are the basic tests in line of investigations for thesepatients.The most recent reports showed an increase in its frequency among low income group and more in underdeveloped countries. The causes of anemia vary by age in general anemia can be caused by either decreased production (as seen with nutritional deficiencies, bone marrow failure, pure red cell aplasia, sideroblastic anemia, congenital dyserythropoieticanemiasetc) or it can be due

to increased destruction (as seen with congenital hemolytic anemias, autoimmune hemolytic animas, drugs or microangiopathies).

Hemoglobinopathies consist of thalassemias and variant haemoglobin a major health problem in the Indian subcontinent. Betathalassemias being the commonest monogenic disorder across India. In India the gene frequency of haemoglobinopathies is 4.2%, having a population over 1 billion and over 12000 infants born each year with a clinically significant hemoglobinopathies. According to world health organization (WHO) 5% of the world population is a carrier for hemoglobin disorders.Hemoglobinopathy patients can benefit temporarily with nutritional supplementation and blood transfusions but long term outcome can be better if specific diagnosis is made and specific therapy or precautions are undertaken.Prevalence of haemoglobinopathies the frequency of beta-thalassaemias is high in the Mediterranean area, the Middle East, the Indian Subcontinent, and the Far East. The incidence of the alpha-thalassaemias is particularly high in the Far East, but the condition is not rare in the Mediterranean area, the Middle East, and the Indian Subcontinent. Sickle cell anaemia shows the highest occurrence in tropical Africa (Cao et al., 1993). The inherited haemoglobinopathies are large groups of autosomal recessive disorders that include thalassaemias and sickle cell anemia.Thethalassaemias are caused by the defective or nonproduction of one of the globin chains of the haemoglobintetramer.Thalassaemias is differentiated into alpha, beta and delta-thalassaemia as per type of globin chain involved. While sickle disorder results from homozygosity for the S mutation, an A• T substitution at codon 6 of the beta-globin gene leading to the replacement of valine for glutamic acid.Haemoglobinopathies like thalassaemia, sickle cell anemia etc are increasing trend due to unawareness of the population. The General incidence of thalassaemia trait & sickle cell Haemoglobinopathy in India varies between 3-17% & 1-44% respectively.All case of anemia subjected to be investigated by CBC & peripheral smear and then all these cases are subjected to special investigations. Affected person and their families with haemoglobinopathies must be offered genetic counseling. The aim of the study was analyze the types of haemoglobinopathies in patients of Anemia using hemoglobin electrophoresis from 3 years and 2 months in Madhya Pradesh.

II.MATERIALS & METHODS

Material for study obtained from the clinical cases suspected of anemia from index medical hospital and department of medicine in Indore. Study was conducted from 2017 oct to 2020Dec.A total of 100 cases were included in this studymicrocytic hypochromic anemiaon peripheral blood film (PBF)All patients who presented with pallor and were detected to have microcytic hypochromic anemia on peripheral examination were included in the study. Anemic patients having cause other than microcytic hypochromic anemia and confirmed cases of Iron deficiency anemia wereexcluded. Investigations were done to confirm that Anemia is microcytic hypochromic anemia and to find out hemoglobinopathies as a cause MHA. Complete hemogram was performed and Hb electrophoresis was done after studying the iron profile and ruling out iron deficiency anemia as cause of Microcytic Hypochromic anemia. Other tests which were performed included serum Iron level by performing serum Iron level, Ferritin and TIBC.

Hemoglobin electrophoresis was performed using Inter Lab Genio S electrophoresis apparatus and commercially available Interlab Master Kit at PH 8.6

Figure 1: Showing InterlabGenio S electrophoretic apparatus

Inclusion criteria

All young Population patients who presented with pallor and were detected to have microcytic hypochromic anemia on peripheral examination.

Exclusion criteria

1. Those who are not willing.
2. Peripheral picture other than microcytic hypochromic anemia.
3. Confirmed cases of iron deficiency anemia.

The following investigation were done to diagnose microcytic hypochromic anemia using complete hemogram by counter and peripheral blood smear examination .

1. Hb%
2. Hematocrit
3. MCV
4. MCH
5. MCHC
6. RDW
7. TC
8. Platelet count

Comprehension of the whole picture with peripheral smear was done. Peripheral smear was air dried and stained with Leishman stain. Other investigation to differentiate between the different types of microcytic hypochromic anemia were done according to the following flow chart.

All patients underwent following investigations .

➢ Complete blood count.
➢ Peripheral blood smear study.
➢ Red cell indices
➢ Reticulocyte Count.
➢ Sickling Test.
➢ Hb F and haemoglobin electrophoresis with quantification of bands are done in all these cases.

Electrophoresis

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

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 from haemoglobin A. haemoglobin variant 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.

Material Required

- ❖ 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 used

- ➢ Electrophoresis buffer – Tris/EDTA/borate (TEB),pH 8.6 Tris (hydroxymethy) amino methane(Tris),10.2 g ; EDTA (disodium salt) 0.6g; boric acid3.2 g ; water to 1 lit . The buffer should be stored at 4° Cand can be used up to 10 times without deterioration.
- ➢ Wetting reagent - for example zip zone prep solution : 1 drop of zip zone prep in 100 water.
- ➢ Fixative / stain solution – ponceau S 5 g ; trichloroacetic acid 7.5 g; water to 1 litre.
- ➢ Destainingsolution . 3 % (v/v) acetic acid , 30 ml ; water to 1 litre
- ➢ Haemolysingreagent . 0.5% (v/v) Triton X-100 in 100 mg/l potassium cyanide.
- ➢

III. METHODOLOGY

Direct peripheral smears were prepared. Under aseptic precaution 5ml of blood was drawn in K2EDTA vacutaners. Patients who received blood transfusion within 4 weeks were deferred till 4 weeks after transfusion. Haemolysate with a concentration of 1.6g/dl (in ratio of 1:5) was prepared for haemoglobin (Hb) electrophoresis.4 One part of blood was fed into cell count analyzer and readings were analysed. Other part of blood was used to prepare hemolysate. Hemolysate was fed into Haemoglobin electrophoretic machine and readings were analyzed using .Study design: Cross sectional Study.Hemoglobin electrophoresis was performed to identify variant and abnormal haemoglobin including hemoglobin A1 (HbA1), hemoglobin A2 (HbA2), hemoglobin S (HbS), hemoglobin F (HbF), and hemoglobin C (HbC).

The following references range was taken.

❖ HbA1 : 95%-98%
❖ HbA2 : 1.5%-3.5%
❖ HbF: < 2% (age-dependent)
❖ HbC: Absent
❖ HbS: Absent

Complete blood count (CBC) Blood cell indices were measured using Sysmex XS- 800i fully automated blood cell counter &Cellenium 19 which was calibrated with commercially available control.This kit provides the electrophoresis separation of haemoglobin on a cellulose acetate strip. After electrophoresis, different haemoglobin types forms different band on cellulose acetate strip. These bands are then read by densitometer automatically by the electrophoretic apparatus.

Figure 2 : Showing Bands of different haemoglobin on cellulose acetate strip on Alkaline gel electrophoresis. (Bands of HbA, HbS,HbF,and HbA$_2$

IV. RESULTS & DISCUSSION

A Cross sectional study with 100 patients of young age group was undertaken to study the role of electrophoresis in diagnosing the microcytic hypochromic anemia cases. The study group 54 were males and 46 were females. Use of electrophoresis showed out of 100 microcytic hypochromic anemia cases, 66% were iron deficiency anemia and 34% cases had shown abnormal haemoglobin bands on electrophoresisindicatinghaemolytic anemia.

1. **Age distribution of patients :**

Age distribution of the patients studied is shown in Table 1 and Graph 1

Age in years	Number of patients	Percentage
1 years	4	4%
1-10 years	40	40%
11-25 years	45	45%
25-60 years	11	12%
Total	100	100.0

Graph 1: Age distribution of patients
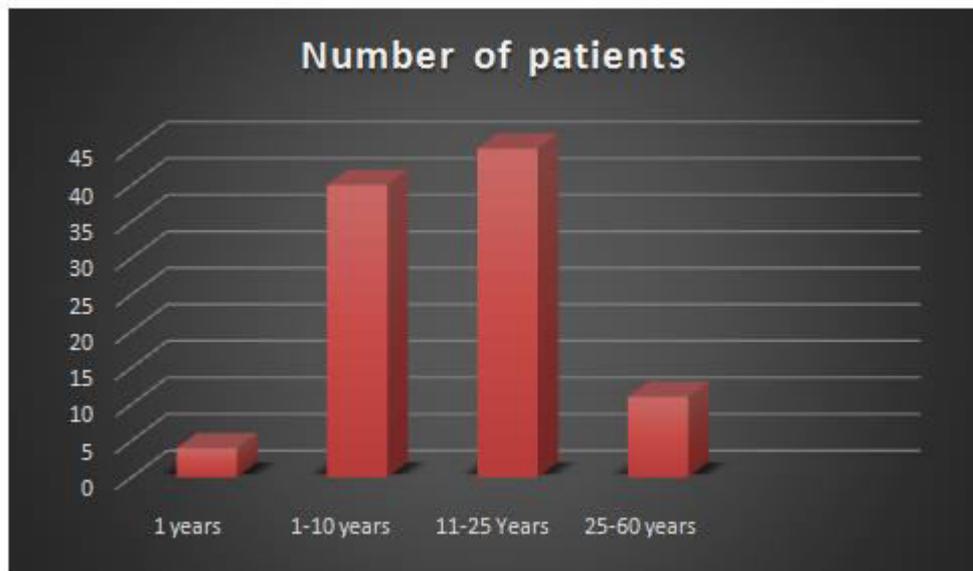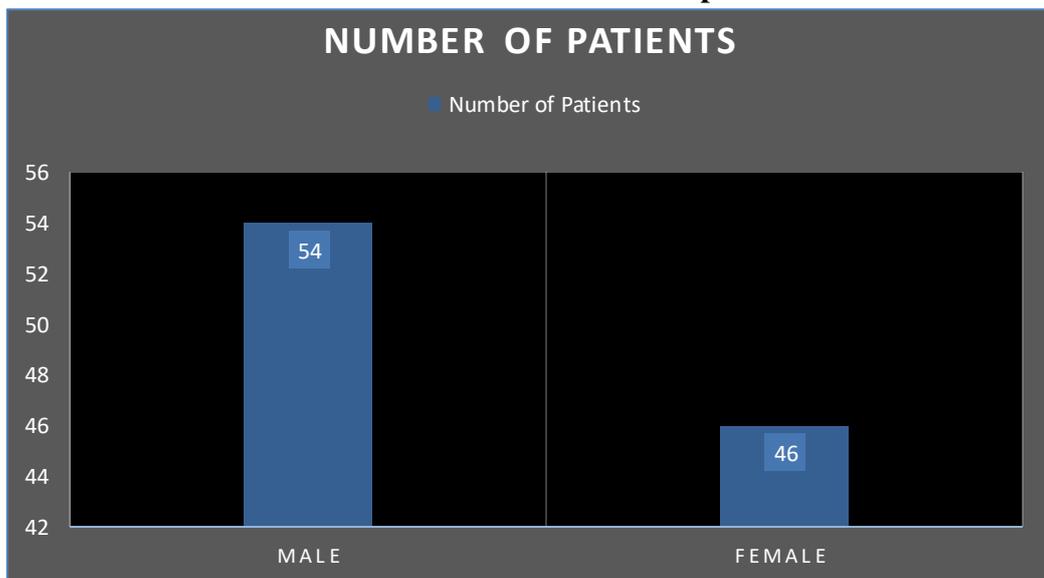

1. **Gender distribution of patients**

Gender distribution of the patients studied is shown in Table 2 and Graph 2

Gender	Number of patients	Percentage
Male	54	54.0
Female	46	46.0
Total	100	100.0

Table 2 : Gender distribution of patients
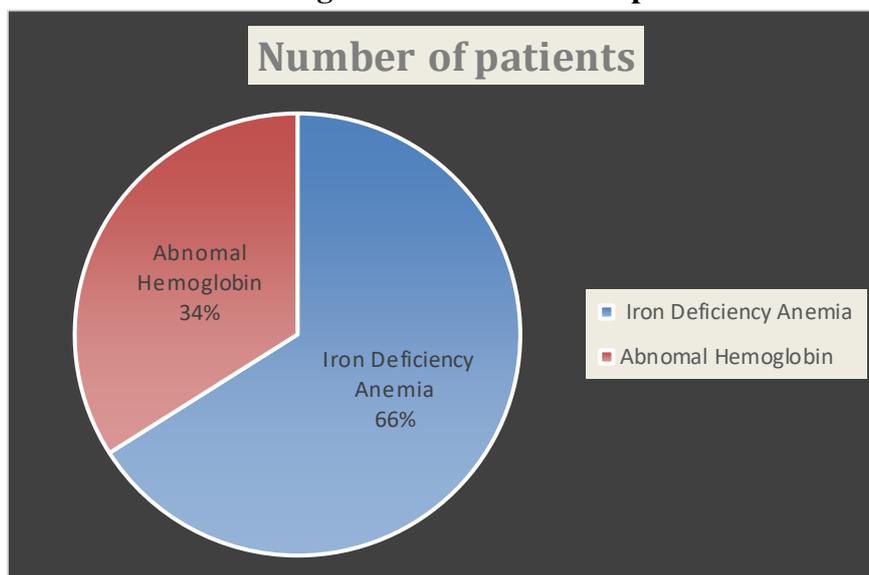

Graph 2 : Gender distribution of patients

1. Diagnosis based on electrophoresis:-

Diagnosis based on electrophoresis studied is shown in Table 3 and Graph 3

Diagnosis	Number of patients	Percentage
Iron Deficiency Anemia	66	66.0
Abnormal Haemoglobin	34	34.0
Total	100	100.0

Table 3: Diagnosis based on electrophoresis

Graph 3: Diagnosis based on electrophoresis

Of the 100 samples Microcytic hypochromic anemia tested, 66 were Iron deficiency Anemia (66%) and 34 Abnormal haemoglobin (34%) , sickle-beta thalassemia is the most common disorder 14 , Followed by beta thalassemia trait 6, and in other hemoglobinopathies were sickle cell trait 7, sickle cell disease 4 & beta thalassemia major 3 (Table 4 Graph 4)

The major abnormality observed was of high Hb A2. A cut-off of over 3.9% was taken for diagnosis of βTT. Distribution of various hemoglobinopathies is as shown in (Table 5)

The haemoglobin values and RBC indices in different haemoglobinopathies is as shown in (Table 6)

Beta-thalassemia major patients presented with anemia with average hemoglobin concentration being just 3.9 g/dL.The patients with sickle-beta thalassemia had an average hemoglobin concentration of 6.7 g/dL.While sickle cell disease   had an average of 7.2 g/dL.Beta- thalassemia trait patients had an average 7.3 g/dL. And sickle cell trait had an average hemoglobin concentration of 5.2g/dL. (Graph 7)

	Male	Female	Total
Microcytic Hypochromic Anemia	29	37	66
Sickle-beta thalassemia	9	5	14
Beta thalassemia trait	4	2	6
Sickle cell trait	5	2	7
Sickle cell disease	1	3	4
Beta thalassemia major	2	1	3

**Table 4: Results of haemoglobin electrophoresis**
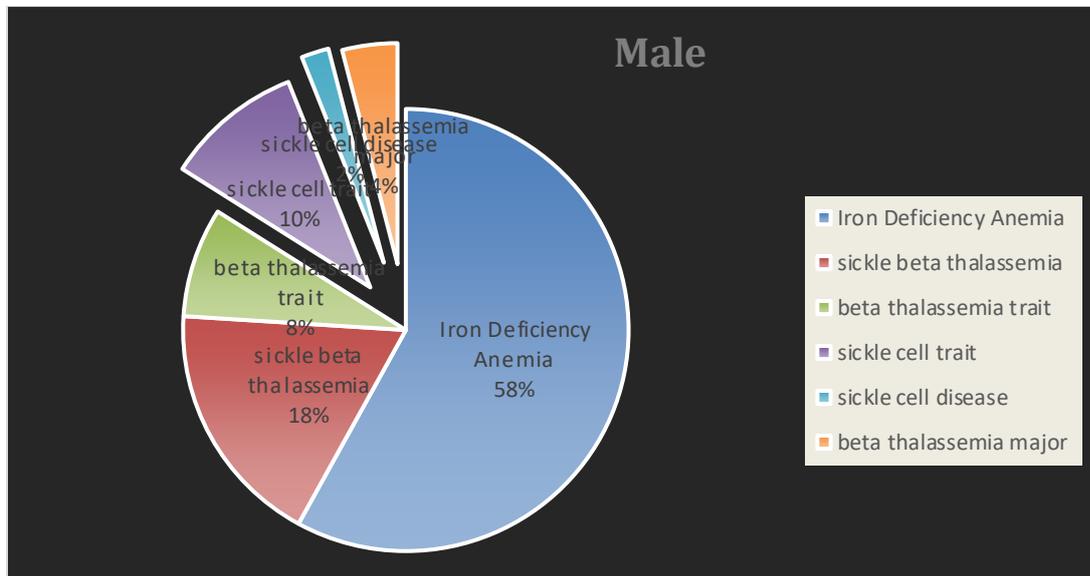
Graph 4:Results of haemoglobin electrophoresis

Presumptive Hb Electrophoresis Diagnosis	HbA (%) ±SD	Hb F (%) ±SD	HbA2 (%) ±SD	Variant Hb (%)±SD
Sickle-beta thalassemia	4.6±1.8	18.3±8.4	3.3±0.3	36.3±4.8
Beta thalassemia trait	82.3±2.3	1.0±1.1	5.6±0.6	NA
Sickle cell trait	52.7±3.8	1.0±0.6	3.5±0.6	37.4±1.4
Sickle cell disease	3.9±0.7	7.9±1.0	4.0±0.6	85.1±2.3
Beta thalassemia major	6.8±1.5	91.9±5.2	3.9±0.9	NA

Table 5:Hb fractions on Hb Electrophoresis in various hemoglobinopathies

Presumptive Hb Electrophoresis Diagnosis	MCV (fl)±SD	MCH (pg)±SD	MCHC (%)±SD	RBC count (×106/µl)±SD	RDW±SD
Sickle-beta Thalassemia	70.2±5.0	21.5±2.3	30.5±2.1	3.67±0.6	17.1±3.8
Beta Thalassemia trait	68.5±6.2	21.3±2.6	28.3±1.8	5.06±0.9	15.4±6.1
Sickle cell trait	84.9±3.4	27.3±2.1	31.7±2.3	4.45±0.54	16.2±4.4
Sickle cell disease	91.2±0.9	28.9±1.1	32.1±1.7	3.4±1.5	20.4±2.1
Beta thalassemia major	54.9±6.5	16.8±3.6	26.3±2.9	2.5±0.8	31.5±5.3

Table 6: Red Blood Cells indices in various Hemoglobinopathies
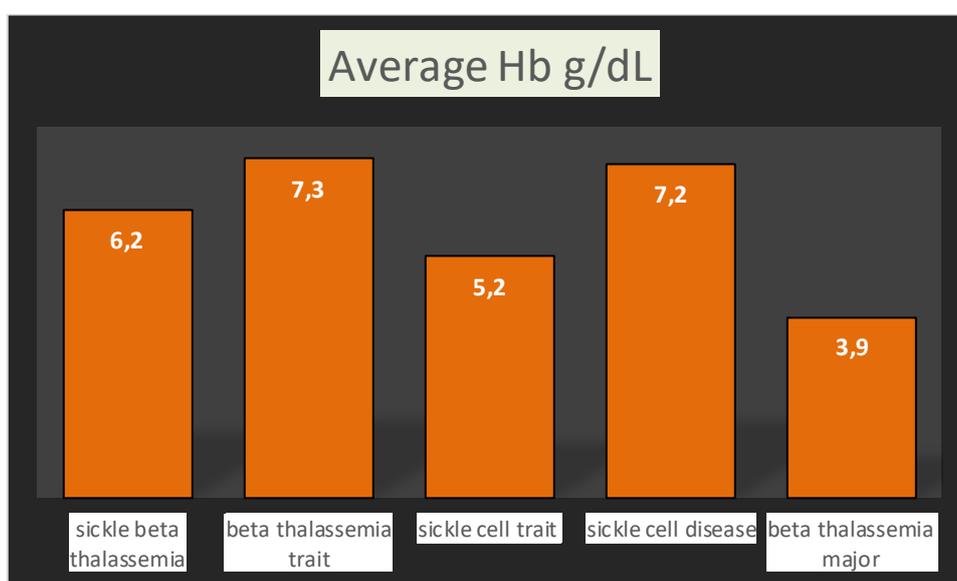
Graph 7: Average hemoglobin concentration of abnormal haemoblobin in our patients

In this study the red blood cells showed hypochromic & microcytic picture. Genetic counselling for couple at risk, for offspring with homozygous β - thalassaemia should be done in these patients.

V.DISCUSSION

Iron deficiency anemia is the most common nutritional deficiency in our country.Haemoglobinopathiesare inherited disorder of globin chain synthesis. It either reduced rate of synthesis or structurally abnormal globin chain leading to abnormal haemoglobin molecule synthesis. The diagnosis of haemoglobinopathy including thalassaemia can result from either clinical suspicion or from follow up of an abnormality detected during screening.8 In our study screening of all anaemia cases was done initially by clinical history including family history, cast & ethnicity of the patients. All cases were subjected to physical examination, a blood count & peripheral blood examination. In addition Sickle cell phenomenon & Alkali denaturation for foetalhaemoglobin was also carried out. The more confirmatory test by Cellulose Acetate Membrane electrophoresis at alkaline pH (CAM) was followed in all cases. Various Indian studies have reported that many variants of haemoglobin

are prevalent and very common in rural Indian population. Haemoglobinopathies are one of the major public health problems in our country. On the basis of analysis of reports published in last 20 years it is observed that several tribes in various parts of India have been identified as high risk groups of Haemoglobinopathies. In India about 4635 ethnic communities have shown 05 common & 12 rare mutation In India, females are more affected with anemia than males 5,8,15 and the reasons range from high cost of healthcare facilities, poor food quality and the low status of women.This is suggests that causes other than nutritional may be more important in male anemic patients and the results of this study also suggest the same of male patients had hemoglobin disorders.This study was conducted because of the observation that most of the patients who had history of being diagnosed with severe anemia recently or in the past were given blood transfusions and/or iron supplements while ignoring the need for finding out the actual cause for such degree of anemia.In India, females are more affected with anemia than males5,8,15 and the reasons range from high cost of healthcare facilities, poor food quality and the low status of women.5 This condition becomes worse in rural areas as demonstrated by Kaur and Kochar6 in their study and also attributed poor nutritional status to lower hemoglobin levels in females. A study from Central India also concluded that females have a higher prevalence of anemia than males especially those falling under moderate to severe category.7 Various studies5,9,10 have indicated higher prevalence of malnutrition among females than males especially in poor socialeconomic status populations. This suggests that causes other than nutritional may be more important in male anemic patients and the results of this study also suggest the same as male patients had hemoglobin disorders. Central India and some other tribal areas of India are known to have a considerable degree of prevalence of hemoglobinopathies.11,12,13 Sickle cell disorders as well as beta-thalassemiaare especially prevalent in such areas. With proper and early diagnosis and providing specific treatment, the severity of anemia in these disorders can be reduced as well as frequency of complications can be brought down.2,3 This is of significance in a developing nation like India where health issues can have major social and economic impact. And since almost half of the population of India was found to be anemic, there is a need to address the approach towards anemia related disorders.Beta-thalassemia and sickle cell anemia are both genetic/ hereditary disorders and are present in general population in varying prevalence. Some populations/communities have higher prevalence of these disorders due to preference to consanguineous marriages.15 Such trend has a possibility of breeding between populations having mutations for both the diseases and resulting in a population having higher prevalence of sickle-beta thalassemia as compared to general population. This is important particularly in this study as sickle-beta thalassemia was the most common hemoglobin defect observed in this study.Beta thalassemia syndromes are a group of hereditary disorders resulting from genetic deficiency in the synthesis of beta-globin chains.16 In the homozygous state (i.e., thalassemia major), it causes severe, transfusiondependent anemia, whereas the heterozygous state (trait or thalassemia minor), causes mild to moderate microcytic anemia. Those presenting with clinical severity lying between that of thalassemia major and minor are said to have thalassemia intermedia. Patients with thalassemia minor generally don't require specific therapy whereas those with thalassemia major are transfusion dependent and need iron chelation therapy. Splenectomy, allogenic stem cell transplantation and supportive measures are also required.Sickle cell disease/anemia (SCD) and its variants are hereditary/genetic disorders resulting from thepresence of a mutated form

of hemoglobin i.e., hemoglobin S (HbS).16 Sickle cell disorders can cause significant morbidity and mortality. Morbidity, frequency of crisis, degree of anemia, and the organ systems involved vary considerably from individual to individual. SCD is suggested by the typical clinical picture of chronic hemolytic anemia and episodes of vaso-occlusive crisis. Electrophoresis is used to confirm the diagnosis by showing presence of homozygous HbS and can also document other hemoglobinopathies like HbSC, HbS-beta+ thalassemia. Management involves preventing/ treating infections, management of vaso-occlusive crises, chronic pain syndromes, maintaining hydration, prevention of stroke and renal damage, managing chronic anemia etc. and stem cell transplantationApart from various pharmacological and nonpharmacological measures for hemoglobin disorders, patient education about the illness is also of paramount importance especially counselling about various risks associated with the disease and measures to prevent marriages between atrisk populations as it has been shown that supervision of atrisk population and conducting premarital genetic screening/counselling results in reduction of burden of disease.2 This can only be made possible if proper investigations are carried out to determine cause of severe anemia and needed measures are undertaken to eventually reduce the burden of the disease.

VI.CONCLUSION

Microcytic Hypochromic Anemia is a very common problem in clinical practice, early detection of which helps in the correct treatment. Most common cause of Microcytic Hypochromic Anemia in this study was iron deficiency anemia and abnormal haemoglobin bands on electrophoresis indicating haemolytic anemia.

Diagnosis of the Microcytic Hypochromic Anemia was achieved easily with the help of complete hemogram and peripheral blood picture. Differential diagnosis based on complete hemogram and peripheral smear is possible but special tests like serum iron profile and hemoglobin electrophoresis are a must for confirmation of diagnosis. Iron deficiency anemia shows decreased serum iron and transferrin saturation with increased TIBC. Hemoglobin electrophoresis is a must in the diagnosis of hemolytic anemia .Early diagnosis of which helps to start transfusion therapy and yield better prognosis. Iron overload being a major complication in thalassemia showed increased serum iron levels.

REFERENCE

1.Thakur S, Sharma R, Raw SN. Incidence of Thalassemia and Sickle Cell Disease in Chhattisgarh, Central India: Using Hardy Weinberg Equations. J Mol Genet Med 2015;9: 155.

2.Memish ZA, Saeedi MY. Six-year outcome of the national premarital screening and genetic counselling program for sickle cell disease and β-thalassemia in Saudi Arabia.Annals of Saudi Medicine. 2011;31:229- 235.

3.Ghani R, Manji MA, Ahmad N. Hemoglobinopathies among five major ethnic groups in Karachi Pakistan. Southeast Asian J Trop Med Public Health 2002:33(4);855-61

4.Borland EW, Dalenius K, Grummer- Strawn L and Mackintosh H et al. Pediatic Nutrition Surveillance: 2007 Report Atlanta, Ga: Centres for Disease Control and prevention; 2009

5. DeMaeyer E, Adiels-Tegman M. The prevalence of anemia in the world.World Health Stat Q 1985; 38:302-16.

6. Deborah RundetaI. Mean corpuscular Volume of Heterozygotes for β thalassemia correlates

with the severity of mutations. Blood 1992 Jan; 79(1): 238 – 243

7.   SwaroopMitra.   The   Clinical   and   Hematological   profile   of   thalassemia   and Hemoglobinopathies in India. Indian J Paed,1983: 20;25-30

8. Grow K., Abrol P., Vashist M., Yadav R., Sharma S., 2013. Associated Complications In Beta Ph ton 241 Thalassemia Patients. IOSR Journal of Pharmacy, 3(1), 22-25

9. Madan N., Sharma S., Sood S. K., Colah R., Bhatia L.H., 2010. Frequency of β-thalassemia trait and other hemoglobinopathies in northern and western India.Indian Journal of Human Genetics, 16(1), 16- 25. PMID: 20838487.

10. Menon A., Salim K.A., 1993. Sickle cell disease in South India.Journal of the Association of Physicians of India, 41(9), 617. PMID: 8307934.

11.Eastman J.W., Wong R., Liao C.L., Morales D.R., 1996. Automated 10.HPLC screening of newborns for sickle cell anemia and other hemoglobinopathies.Clinical Chemistry, 42(5), 704- 10. PMID: 8653895.

12. Wharton BA. Iron deficiency in children: detection and prevention. British Journal of Haematology.1999; 106: 270-80.

13. Verma M, Chhatwal J, Kaur G. Prevalence of anemia among urban school children of Punjab. Indian Pediatr. 1998 Dec; 35 (12): 1181-6. [18]

14. Sinha AK, Majumdar B, Yadav SK. Prevalence and significance of iron deficiency of anemia among people of Mornag District of Nepal. Journal of Nobel Medical College. 2011; 1 (1): 40-4.

15. Madan N, Sharma S, Sood SK, Colah R, Bhatia HM. Frequency of β-thalassemia trait and other haemoglobinopathies in northern and western India. Indian J Hum Genet 2010;16:16-25.

16.Dolai TK, Dutta S, Bhattacharyya M, Ghosh MK. Prevalence of haemoglobinopathies in rural Bengal, India. Hemoglobin 2012;36:57-63.

17.Meena V.K., Kumar K., Meena L.P., Bharti A., Kumar A., 2012. Screening for Hemoglobinopathies in blood donors from eastern Uttar Pradesh. National Journal of Medical Research, 2(3), 366-368

18.Saha A.K., Mohanta M.K., Khalequzzaman M., Biswas P., 2012. Detection of Thalassaemia Trait at Rajshahi Region, Bangladesh. Journal of Medical & Allied Health Sciences, 1(2), 32- 37.