

HPLC IN THE DIAGNOSIS OF HAEMOGLOBINOPATHIES & THALASSAEMIAS

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Abstract

Problem Statement: It is important to identify carriers of haemoglobin disorders in order to assess the risk of a couple having a severely affected child and to provide information on the options available to avoid such an eventuality. Ideally, screening should be performed before pregnancy. Preconception screening is directed at couples planning a pregnancy, while antenatal screening focuses on pregnant women. Common modes of prenatal diagnosis are Chorion villus sampling (CVS), amniocentesis and foetal blood sampling under ultrasonic guidance. **Methods:** The diagnosis of Haemoglobinopathies & Thalassaemias was Electrophoresis, both agarose gel and cellulose acetate electrophoresis were used. But now with advancing technology, the world wide reference method of Hb typing is HPLC or HIGH PRESSURE LIQUID CHROMATOGRAPHY based upon the principles of Cation Exchange chromatography. It is a technique that offers fast and easy Thalassaemia testing along with simultaneous detection of the commonly occurring abnormal haemoglobins (Hb E, S, D & C). **Results:** Our experience and from a study done in Sri Krishna Medical College, Muzaffarpur, observed haemoglobinopathy is Hb E followed by HbS encountered in the tea garden labour community. **Conclusion:** The haemoglobin disorders are the most common clinically serious single gene disorders in the world. Most affected children are born in countries with limited resources where priority is given to tackling mortality from infections and malnutrition. Hereditary disorders receive little attention. The haemoglobin disorders are often regarded as incurable and hence “hopeless” and expensive to treat. Thus prevention is always better in such a case and the burden thalassaemia places on families are a key driving force in establishing the prevention programmes.

INTRODUCTION

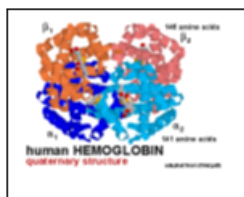


Figure 1

Haemoglobin is a conjugated protein of molecular weight 64000, consisting of two pairs of polypeptide chains. In a normal adult, Hb A₀ comprises about 97% of the total Hb. It consists of two alpha chains and two beta chains. Hb A₂ is the minor Hb in the adult, having two alpha chains and two delta chains. It is usually not detected at birth and reaches adult levels of 1.5- 3.5% at one year of age. Hb F is the major Hb during intrauterine life and has the structural formula of alpha₂, gamma₂. At birth Hb F amounts to 70- 90% of the total Hb. This rapidly

falls to 25% at 1 month and 5% at 6 months. The adult level of less than 1% is not reached in some children upto puberty.

Inherited disorders of haemoglobin synthesis are divided into two groups. One, which is characterized by structurally abnormal haemoglobin variants is referred to as HAEMOGLOBINOPATHIES. The other group in which there is a genetically determined reduction in the rate of synthesis of one or more of the normal Hb polypeptide chains, is THALASSAEMIAS. Accordingly, if there is a reduction/ absence of Alpha chains, the result is ALPHA THALASSAEMIA and in case of absence/ reduction of synthesis of beta chains- the result is BETA THALASSAEMIA.

The genetic defect in case of Alpha Thal. is usually deletion. According to the number of genes affected, the phenotypic expression of the disease varies from HYDROPS FOETALIS where there is 4 gene deletion and the condition is not compatible with life; to the least severe form or the Silent Carrier where there is just one gene deletion and the

affected individual is asymptomatic. In case of Beta thalassaemia, the common genetic defect is a mutation, either a point mutation or a frame shift mutation and the phenotype can again vary from a minor form or Beta thalassaemia Trait(BTT) to a Major form according to the severity of the genetic defect.

As a group Beta Thalassaemia comprises the most common inherited disease in the world, affecting 3% of the world's population. It is particularly prevalent in the Mediterranean region, Middle East, India, Pakistan & the Far East. In Assam there have been very few studies of a large number of cases. In 1975, Das and Deka found a high percentage of HbE trait and disease in Ahoms (55%) and

METHODS

THE HPLC SYSTEM



Figure 2

Till date the gold standard of diagnosis of Haemoglobinopathies & Thalassaemias was Electrophoresis, both agarose gel and cellulose acetate electrophoresis were used. But now with advancing technology, the world wide reference method of Hb typing is HPLC or HIGH PRESSURE LIQUID CHROMATOGRAPHY based upon the principles of Cation Exchange chromatography. It is a technique that offers fast and easy Thalassaemia testing along with simultaneous detection of the commonly occurring abnormal haemoglobins (Hb E, S, D & C). The BIORAD Variant haemoglobin typing system is a fully automated, reproducible system which can quantitate percentages of Hb A2 & Hb F in a single 6.5 min assay with accuracy and precision and has become the most practical means to diagnose carriers of Beta Thalassaemia gene. The different Hb variants like Hb E, S, D & C are identified by using retention time windows which are specified for each of these.

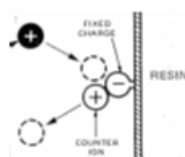


Figure 3

Kacharies (75%). Dr B C Gogoi (1990) studied 1949 cases and found the E phenotype in 50% of Mongoloid Assamese, and S in 11.2 % of S in the Tea Tribes, in the asymptomatic group, and 32% and 11.3% respectively in the asymptomatic group. R S Balgir quotes a figure of 10.4% of the prevalence of HbE phenotype in the general population. Our experience and from a study done in M.G.M. Medical College and RMRC, ICMR Dibrugarh, shows similar findings, ie the most frequently observed haemoglobinopathy is Hb E followed by HbS encountered in the tea garden labour community. According to the study conducted by RMRC, Dibrugarh the incidence of Beta Thal in N.E India is approximately 3.8%

Hemolysed specimens of blood are maintained at 12°C in the automatic sampler chamber. Two dual piston pumps and a pre-programmed gradient control the elution buffer mixture passing through the analytical cartridge. The ionic strength of the elution buffer mixture is increased by raising the percent contribution of elution buffer 2. As the ionic strength of the mixture increases, more strongly retained hemoglobins elute from the analytical cartridge. A dual wavelength filter photometer (415 and 690 nm) monitors the hemoglobin elution from the cartridge, detecting absorbance changes at 415nm. Changes in absorbance are monitored and displayed as a chromatogram of absorbance against time. To aid in the interpretation of results, windows (eg ranges) have been established for the most frequently occurring hemoglobins based on their characteristic retention times.

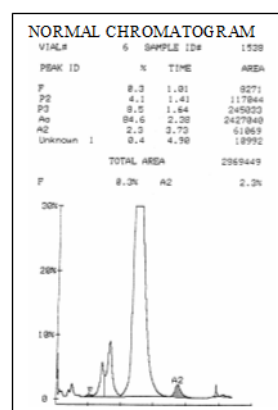


Figure 4

CHROMATOGRAMS

Normal adult chromatogram shows primarily Hb A, a small percentage of Hb A2 (less than 3.5%) and traces of foetal haemoglobin (less than 1%). P2 and P3 are normal associates of Hb A. Hb E elutes in the A2 window, differentiation between the two can be made by observing the percentage of Hb A2/E window. If A2/E is greater than 20% it is considered to be Hb E and no Hb A2 is involved. If along with Hb E of 20-35%, Hb A is present and A is greater than E; the condition is Hb E Trait. If the E peak

amounts to 85-90% and there is no Hb A, the condition is Hb E disease

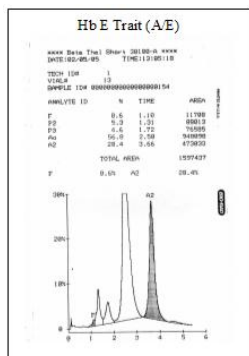


Figure 5

If the A2/E peak ranges between 10-14%, it is likely to be a case of Hb Lepore. Hb E heterozygotes are clinically normal with minimal changes in blood counts and red cell indices. RBC morphology is similar to BTT with slightly microcytic red cells and a few target cells in the PBS. Hb E levels are reduced by co-inherited alpha thalassaemia to 19-21%. Hb E homozygotes usually have normal Hb levels although some may be mildly anemic. Clinical symptoms like jaundice and hepatosplenomegaly are rare. Bone changes are not present and reticulocyte counts are consistently normal. Nucleated RBC are absent from the circulation, but 20-80% target cells are characteristically found in the PBS.

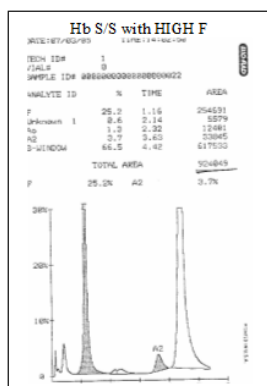


Figure 6

The Sickle cell Trait (A/S) is an asymptomatic sickling disorder with 30- 45% Hb S. The interaction of alpha thalassaemia reduces the percentage of Hb S and the red cell indices. The Hb A2 level is often slightly above normal(3.5- 4.0%) but this never signifies the presence of associated Beta Thalassaemia.

Hb S in the homozygous state or in combination with either Hb C, Hb D or Beta Thalassaemia causes sickle cell disease/ anemia. Hb S is less soluble than normal Hb in deoxygenated states and causes the classical sickle shaped deformation of the red cells leading to vascular complications. However presence of a high Hb F in these Hb S homozygotes is said to be protective against the sickling crisis.

HbS/D double heterozygous condition is a moderately severe sickle cell disease. Patients have mild to moderate anemia with plenty of target cells in the PBS and occasional painful crisis. The electrophoretic pattern of Hb S/D is confused with homozygous S/S and the two can be differentiated only at acidic Ph, while with HPLC the two can be distinguished with their relative percentages in a single assay.

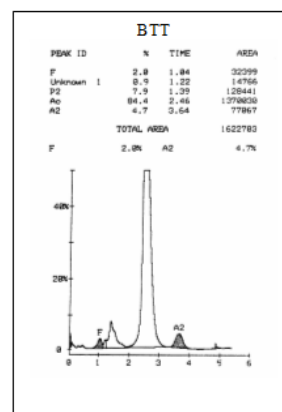


Figure 7

Clinical identification of the Beta Thal carriers is important as any offspring between individuals with BTT are at risk of being homozygous for Beta Thalassaemia(Major). Beta Thal Carriers can be diagnosed only by HPLC, where we can quantify Hb A2 and F levels. Hb A2 levels of 4- 9% irrespective of the Hb F levels are diagnostic of BTT in an asymptomatic individual with no anemia.

At birth the Beta Thal homozygotes are asymptomatic because of the high levels of Hb F, but as this declines affected children present with severe anemia during the first or second year of life. Treatment is by frequent blood transfusion to maintain a Hb level above 10 gm/dl coupled with iron chelation therapy to control iron overload. This treatment does not cure Beta Thalassaemia.

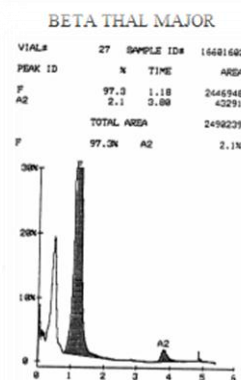


Figure 8

Only some patients have reached the age of 40 in good health. With the prospects for gene therapy remaining as distant as ever, the only cure for Beta thal is Bone marrow transplantation.

Hb E Thalassaemia, the compound heterozygous state of Hb E and Beta Thal, is a common disease in Southeast Asia. It results in a variable clinical picture similar to that of homozygous Beta Thalassaemia, ranging from a condition indistinguishable from Thal major to a mild form of Thalassaemia intermedia. In the severest form Hb F ranges from 40- 60% the remainder being Hb E.

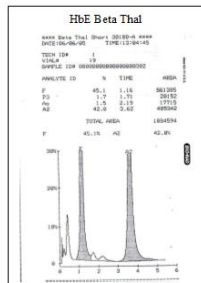


Figure 9

In most population groups, a small number of apparently healthy adult subjects with normal or near normal haematological findings have a raised Hb F level and is referred to as HEREDITARY PERSISTENCE OF FOETAL HAEMOGLOBIN (HPFH). The HPFH syndromes cause diagnostic difficulties as they resemble Beta Thal. The most important diagnostic feature is absence of anemia and normal indices.

RESULTS

EXPERIENCE AT OUR CENTRE

We have had the opportunity to see more than 1000 cases in our centre in the last year referred to us by the various departments and physicians, and the findings that we have got are an eye opener.

TABLE 1: SUMMARY OF 1000 CASES OF HAEMOGLOBIN TYPING BY HPLC

NORMAL ADULT Hb (A/A)	360	(36.0%)
HAEMOGLOBIN E TRAIT	269	(26.9%)
HAEMOGLOBIN E DISEASE	263	(26.3%)
HAEMOGLOBIN S DISEASE	24	(2.4%)
HAEMOGLOBIN S TRAIT	21	(2.1%)
HAEMOGLOBIN S/D	01	(0.1%)
HAEMOGLOBIN D TRAIT	04	(0.4%)
HAEMOGLOBIN J	02	(0.2%)
BETA THALASSAEMIA TRAIT	33	(3.3%)
BETA THALASSAEMIA MAJOR	02	(0.2%)
Hb E BETA THALASSAEMIA	19	(1.9%)
HPFH	02	(0.2%)

These were symptomatic cases as they had been referred to us with a history of anemia or splenomegaly etc. A very high incidence of HbE disease or trait (53.2%) was observed. Sick cell anemia or trait was detected in 4.5% cases. Beta Thalassaemia Trait was detected in 3.3% cases, while there were 2 cases of Beta Thal Major. 19 cases were diagnosed to be of HbE – Beta Thalassaemia. The significant findings were detection of unusual variants like Hemoglobin S/D, D trait, J Hemoglobin, and a family of HPFH, variants which have not been detected or reported from this part of the country.

With this CME in mind, we also conducted this test in 100 consecutive antenatal cases with the help and permission of the department of Obstetrics and Gynecology, Sri Krishna Medical College, Muzaffarpur. Though the sample size was too small for a definite conclusion, a significant number (21%) of HbE disease and trait were detected and a single case of BTT was found.

DIAGNOSTIC DILEMMAS IN HPLC

At times the Hb A2 levels are below the Thal range, but above the normal level i.e 3.5- 3.9%. The red cell indices often show a mild microcytic hypochromic picture. These are borderline cases of BTT. A family study is helpful in this case.

Again there are other causes of a raised A2 besides BTT like Megaloblastic anemia, so looking into the indices and the PBS helps here. Likewise there are haematological conditions like leukemia, aplastic anemia and megaloblastic anemia where there is raised HbF, so a clinical correlation is very essential.

Clinical history like the age of the patient, the ethnic origin, clinical presentation and the history of blood transfusion are crucial. Blood transfusion within the last 3 months may distort the picture and mask the diagnosis. Serum iron profile is required to be done at times as Iron Deficiency is known to lower HbA2 levels. As all Haemoglobinopathies and Thalassaemias are genetic disorders, family studies are required to come to a conclusion. Thus we need to have a holistic approach.

Compared to previous methods of diagnosis, HPLC has definitely offered us some added advantages. It is a technique that offers fast and easy Thalassaemia testing along with simultaneous identification and quantification of the commonly occurring abnormal haemoglobins and double heterozygous conditions like S/D which are liable to be missed by conventional methods. BTT cannot be diagnosed by Gel Electrophoresis.

CONCLUSION

PREVENTIVE ASPECTS

It is important to identify carriers of haemoglobin disorders in order to assess the risk of a couple having a severely affected child and to provide information on the options available to avoid such an eventuality. Ideally, screening should be performed before pregnancy. Preconception screening is directed at couples planning a pregnancy, while antenatal screening focuses on pregnant women. Common modes of prenatal diagnosis are Chorion villus sampling (CVS), amniocentesis and foetal blood sampling under ultrasonic guidance. Currently only CVS gives reliable results during the first trimester of pregnancy and is the preferred method of sampling for DNA analysis for Haemoglobinopathies. By CVS a small sample of the developing placenta is obtained which has the same genetic make up as the foetus. The technique can be used at any stage after 11wks. But by all means prevention is good public health practice and cost effective.

The haemoglobin disorders are the most common clinically serious single gene disorders in the world.

Most affected children are born in countries with limited resources where priority is given to tackling mortality from infections and malnutrition. Hereditary disorders receive little attention. The haemoglobin disorders are often regarded as incurable and hence “hopeless” and expensive to treat. Thus prevention is always better in such a case and the burden thalassaemia places on families are a key driving force in establishing the prevention programmes.

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