

Original Research**HPLC as a classical tool for screening of β -Thalassemia and Hemoglobinopathies in Kanpur**

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Abstract

Background: Hemoglobinopathies and beta- thalassemia is one of the most common autosomal disorders worldwide different molecular mechanisms, most of which are base substitution or small deletions or insertions of one or two nucleotides in the globin genes. It has been found that hemoglobinopathies and β -thalassemia mutations are relatively populations specific; each ethnic group has its own set of common mutations. **Aim:** High Performance liquid chromatography (HPLC) forms an important tool for accurate and speedy diagnosis of various haemoglobin disorders. About 50 cases have been studied for various haemoglobin variants from Kanpur and adjoining area. **Materials and Methods:** The study was performed Agilent 1220 Infinity LC (Agilent Technologies) a High Performance Liquid Chromatography (HPLC) using beta thalassemia program. **Results:** Abnormal haemoglobin fractions on HPLC were seen in 50 cases. Of this beta-thalassemia was the predominant. There were 16 (32%) cases of beta-thalassemia major and 34 (68%) cases of beta-thalassemia carriers possessing HbA_{1c}(0.16), HbF(0.72),HbA₀(0.5), HbE(0.44), HbD(0.36), HbS(0.5), HbA₂(0.46), and HbC(0.42). Automated HPLC and beta thalassemia program is an appropriate approach for the screening and presumptive identification of patients as well as carrier of beta-thalassemia prior to DNA studies for definitive diagnosis.

Keywords: HPLC, Thalassemia, MCV, Beta Thalassemia**Introduction**

India is an ethnically diverse country with an approximate population of 1.2 billion. The inherited disorders of hemoglobin are the single-gene disorders commonly encountered in humans. They fall into three wide but overlapping groups: structural variants; thalassemias characterized by reduced rate of synthesis of one or more globin chains; and conditions in which fetal hemoglobin synthesis persists beyond the neonatal period, collectively known as hereditary persistence of fetal haemoglobin. Of these, thalassemia syndromes particularly beta thalassemia major and certain alpha thalassemia are serious and a major cause of morbidity [1].

Thalassemia is a single gene hereditary haemoglobin disorder in human. It has been reported that nowadays approximately 1 out of 14 people are carriers for thalassemia [2]. WHO figures state that about 370,000 severely affected homozygotes or compound heterozygotes of thalassemia are born every year [3]. The frequency of β -thalassemia in India ranges from 3.5 to 15% in general population [4]. There are certain mutations responsible for >90% of thalassemia cases and about 10% mutations which are unknown to us and or considered as the rare ones [2]. In developing countries like India, hemoglobinopathies increase at an alarming rate

due to lack of proper health care and knowledge. About 10% of total world thalassemia patients belong to Indian sub-continent out of which 3-4% are carriers. In India 32,400 infants are born with hemoglobinopathies [5]. The carrier rate for β -thalassemia gene varies from 1 to 3% in Southern India and 3 to 15% in Northern India [6,7,8]. The frequency of beta-thalassemia trait (β TT) has variously been reported from <1% to 17% and an average of 3.3% [9]. In India where there is high mortality from infections and malnutrition in the first year of life, many of the hemoglobinopathies go unrecognized. Thalassemia major can cause life threatening situation and chronic ill health. They pose economical and psychological burden on the affected individual and his/her family, and the society as a whole. Hence, the population needs to be screened for haemoglobin.

2a. Material and Methods

This was a prospective study carried by the Department of Paediatrics, G.S.V.M Medical College Kanpur and Central Research Lab, Rama Medical College & Research Center, Rama University Mandhana, Kanpur for 6 months period. A total of 50 cases were screened for presence of thalassemia or any structural variant. These included all cases of microcytic hypochromic anaemia (MCV < 80 fl, MCH < 27pg, and RBC count > 5million/cubmm) not responding to conventional treatment, clinically

suspected cases of hemoglobinopathy, antenatal, and other cases coming to the department for thalassemia screening. A 5 ml. intravenous blood sample was collected in EDTA anticoagulant

Hemoglobin Variants	Number(%)
HbA _{1c}	16(0.16)
HbF	72(0.72)
HbA ₀	50(0.5)
HbE	44(0.44)
HbA ₂	46(0.46)
HbD	36(0.36)
HbS	50(0.5)
HbC	42(0.42)

Haemoglobin, red blood cell counts and red cell indices were measured on an automated blood counter. Hematological parameters: Hb, PCV, MCV, MCH, MCHC, RBC count and WBC count were measured using. Peripheral smear was evaluated for features of red cell morphology [10]. Classical red cell indices for β -thalassaemia trait are indicated by a MCV <75 fl and MCH <27 pg [11]. We used the Agilent 1220 Infinity LC (Agilent Technologies) a High Performance Liquid Chromatography (HPLC) under the experimental conditions specified by the manufacturer.

2b. Principle and sample collection : Agilent 1220 Infinity LC (Agilent Technologies) a High Performance Liquid Chromatography (HPLC) uses a double beam photometer for detection in the range of 190–600 nm. This cationic exchange column chromatography enables qualitative determinations of HbA₂, Hb F and abnormal haemoglobins. Here we used β -thalassaemia kit by Gordion Diagnostik Ltd., Turkey. The kit contained Buffer A, Buffer B, Hemolysis Reagent, and one analytical column for β -thalassaemia. Only 10 μ l hemolysis mixture prepared by diluting 5 μ l of whole blood with 1 ml hemolysis reagent was injected into HPLC system. A flow rate of 1.5 ml/min, with an analytical run time of 6.5 min as recommended in kit protocol, was set. A chromatogram of a case with beta-thalassaemia trait detected on the Agilent 1220 Infinity LC is shown in figure 1.

2c. Interpretation of Reports

Reports and chromatograms generated were studied and interpreted by observing HbA₂ and HbF concentration for beta thalassaemia and retention time and area percentage of other peaks and windows for structural variants. Each chromatogram shows peaks of Hb A₀, A₂, and Hb F along with C window, D window, S window, and

two minor peaks, P2 and P3. Several hemoglobin variants elute same window; they were provisionally diagnosed by retention time and area percentage keeping in mind the ethnicity of the patients.

Figure 1:

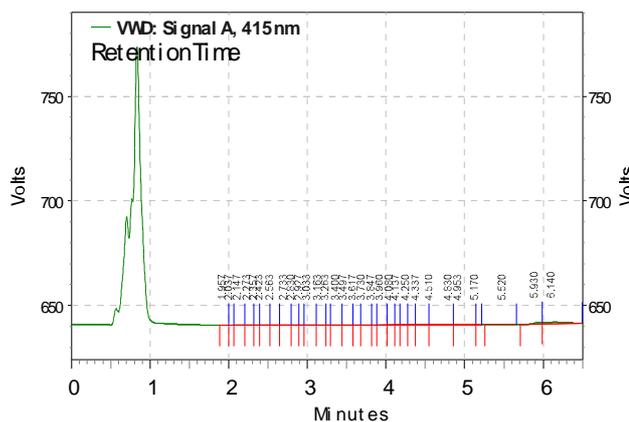


Table2: Distribution of Hemoglobin variants.

Result:

About fifty beta-thalassaemia samples were studied in which sixteen samples were thalassaemia major and thirty four samples were thalassaemic carriers in which HbA_{1c}(0.16%), HbF (0.72%), HbA₀(0.5%) HbE(0.44%), HbD(0.46%), HbS(0.5%), HbD(0.3%) HbA₂(0.46%), and HbC (0.42%) were predominant. Presumptive identification of haemoglobin variants was made primarily by retention time (RT) windows and area percent; however geographical factors, ethnicity and clinical presentation were taken into consideration. Distribution of haemoglobin variants identified is shown in as expected and prenatal study and diagnosis was done to find out carrier status.

Discussion:

India is an ethnically diverse country with marked regional variation. This diversity is reflected in the presence of different hemoglobin variants in different ethnic groups. Due to migration, there is constant mixing of peoples from different regions. Many of these abnormal variants are of little clinical significance in heterozygous state, but when combined with other variants they may give rise to severe disease. The hemoglobinopathies and thalassaemia are the most commonest inherited single gene disorder in India. Therefore there is always a need for a screening method which can detect maximum variants. HPLC has the advantage

of quantifying HbF and HbA₂ along with detecting other variants in a single screening test^[12]. Cation exchange HPLC is emerging as one of the best methods for screening and detection of various hemoglobinopathies with rapid and reproducible and precise results ^[13]. Beta thalassemia trait formed the largest sub group of abnormal hemoglobin (8.9%). The characteristic hematological findings in a typical case of beta thalassemia trait include microcytosis with raised RBC counts.

Hemoglobin rate is quite reduced than normal. The mutations common in an Indian setting include IVS1-5(G-C), 619 bp deletion, IVS 1-1(GT), CD8/9(+G), CD41/42(-CTTT), CD15 (G-A), CD30 (G-C) ^[14]. HbA₂ levels >7% are usually seen with being the major concern in this study, quantification of HbA₂ and HbF along with other variant levels by HPLC was of prime importance in our laboratory where facilities for genetic studies are available. In our study HbF frequency was higher (0.72) in the cases of beta-thalassemia. Presence of HbF variant may be useful for detection of homozygous β-thalassemia variants. HbE and HbA₂ traits were found in 44 (0.44) and 46 (0.46) cases respectively.

Detection of other variants becomes important due to complex interactions in cases with double heterozygous and homozygous states, which may lead to severe hematological abnormalities. Findings must be supplemented by hemogram findings, family/ sibling studies, hemoglobin electrophoresis, other confirmatory techniques and molecular studies based on HPLC findings and on a case-to-case basis.

Conclusion:

The hallmark of classical beta-thalassemia is the presence of an elevation of HbA₂ and HbF, where the recommended method of measurement is done by automated HPLC. HPLC forms a rapid, accurate and reproducible tool for early detection and management of hemoglobinopathies and thalassemia. This is especially important in view of high incidence of beta-thalassemia. Early detection of traits will prevent occurrence of thalassemia major in offspring. Detection of other variants become important due to complex interaction in cases with heterozygous and homozygous states which may lead to severe hematological abnormalities. Due to high prevalence of Hb disorders, premarital screening routinely done for prevention of high risk marriages. The present study conducted using HPLC reflects the magnitude of hemoglobinopathies and thalassemia

in a hospital based small population which may be in fact the tip of an iceberg, but this type of study can definitely help to increase awareness among patients suffering from these disorders.

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