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Diagnosing Beta Thalassemia trait in a developing country

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ABSTRACT

Background: Beta Thalassemia trait (BTT) is diagnosed by detecting hemoglobin A2 (HbA_2) >3.8% on either High Performance Liquid Chromatography (HPLC) or cellulose acetate electrophoresis (CAE). HPLC is an accurate and reproducible but costly alternative to more conventional CAE which is labor intensive but easy to interpret and inexpensive. **Objective:** To determine the sensitivity of CAE and HPLC keeping PCR as gold standard for the diagnosis of BTT. **Study Design:** Cross sectional. **Place and Duration of Study:** Armed Forces Institute of Pathology Rawalpindi. May 2014 to January 2015. **Patient and Methods:** Five ml EDTA anti-coagulated blood was collected from 100 PCR proven cases of BTT. HbA_2 levels were measured by running samples directly on HPLC. But for CAE, first a hemolysate was prepared which was then applied to cellulose acetate membrane at an alkaline pH (7.9). After elution of HbA_2 band in Tris EDTA borate buffer (pH of 8.9), HbA_2 concentration was calculated by measuring its absorbance in a photometer at a wavelength of 416 nm. **Results:** Mean age of the patients was 28.8 ± 8.1 year. The most common mutation was Fr 8–9 (35%) followed by IVS1-5 (25%) mutation. Mean HbA_2 levels by CAE and HPLC were 4.97 ± 0.42 and 5.54 ± 0.59 respectively. All the patients had $HbA_2 > 4\%$ on both CAE and HPLC. None of our patients had false negative result either on CAE or HPLC. **Conclusion:** CAE has comparable sensitivity with HPLC for detection of Beta Thalassemia Trait.

Introduction

Beta Thalassemia (BT) is one of the most prevalent inherited hemoglobin disorders in the world [1], with a carrier rate of 5% among the Pakistani population [2]. It is estimated that with a 5% carrier rate, over 5000 infants with Beta Thalassemia Major (BTM) are born every year in Pakistan [3–5]. Screening for Beta Thalassemia trait (BTT) and identification of its compound heterozygotes with variant hemoglobins is essential for diagnosis and genetic counseling that holds the key for prevention and control of BTM.

BTT is suspected on finding hypochromic and microcytic red cell indices with near normal hemoglobin levels and

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slightly raised red blood cell count on routine complete blood count [1]. Laboratory confirmation of the BTT depends primarily on detecting elevated levels of hemoglobin A_2 ($HbA_2 > 3.8\%$) in the patient's blood sample [6]. The HbA_2 levels can be quantified by many methods but the two most commonly used are Cellulose Acetate Electrophoresis (CAE) followed by elution and the automated method of High Performance Liquid Chromatography (HPLC) [7]. CAE is the conventional and commonly used technique for diagnosis of BTT. It is reproducible, inexpensive and relatively easy to interpret as shown in Fig. 1. Additionally this procedure can be performed in smaller and resource constrained laboratories. The only drawback is that it is labor intensive [8].

The cation exchange HPLC is a rapid, accurate, reproducible and less labor intensive alternative method for detection of many hemoglobinopathies including BTT [9]. HPLC offers the distinct advantage over conventional CAE as it can identify and quantify HbA_2, fetal hemoglobin (HbF) and other hemoglobin variants more accurately. It is also very useful for pediatric group of patients, as only 5 μl of blood is sufficient for analysis. Its utility is more in diagnostic centers where there is increased workload. However major drawbacks include cost considerations, heavy processing equipment and expertise to interpret results. Additionally it has been proven to overestimate HbA_2 percentage especially in the presence of sickle hemoglobin (HbS) [10]; which is a variant of Beta globin found in a significant percentage in different ethnicities of Pakistani population [11].

Polymerase chain reaction (PCR) is a highly sensitive and specific method for diagnosis of BTT which can clearly identify the type of mutation affecting Beta globin gene [12]. However, it requires expensive molecular equipment and high degree of technical skill due to which it is not carried out routinely during the workup of BT patients. It is therefore reserved for cases which present either with atypical red cell indices and/or are not detected on CAE or HPLC [13, 14].

Previously no local study has done comparison between CAE and HPLC based on HbA_2 levels in PCR confirmed BTT patients. The aim of this study was to compare the sensitivity of CAE against HPLC to establish that CAE can still be used as an effective diagnostic tool not only in District Hospital laboratories (Level B) but also in Central/Regional hospital laboratories of under resourced countries like Pakistan [15]. Pakistan despite having a high prevalence of BT has inadequate resources to cater for BT screening and diagnosis at mass level. Additionally fragmented and substandard transfusion system in Pakistan is inept to cater for the transfusion needs of BTM patients in the country [16]. So inexpensive but effective diagnostic modalities like CAE are required to screen the masses for BTT in Pakistan.

Objective

To determine the sensitivity of CAE and HPLC keeping PCR as gold standard for the diagnosis of BTT.

Material and methods

It was a cross-sectional study carried out from May 2014 to January 2015 in the hematology department of Armed Forces Institute of Pathology (AFIP) Rawalpindi. A total of 100 patients were included in this study.

Sample selection

All those cases reporting for extended family screening and for prenatal diagnosis and found to be positive for one of the Beta globin gene mutations on PCR were included in this study. For control group, PCR negative cases for BT mutations were tested. While all cases having silent mutations

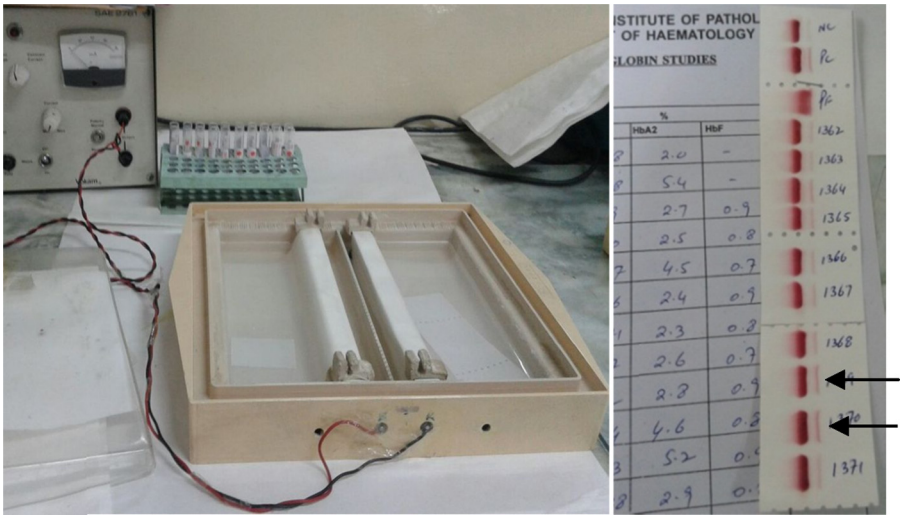

Fig. 1 – Conventional Cellulose Acetate Electrophoresis tank with buffer and electrical supply and cellulose acetate strip showing normal control (NC), positive control (PC) and Positive for HbF (PF). Arrows indicating Raised HbA_2 levels on strip. PC and PF on the strip showing BTT and BTM respectively

like CAP+1 and history of recent blood transfusion were excluded.

After the approval of the study by institute's ethical committee for medical research, all the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000. After an informed consent and recording of demographic data, 5 ml of blood was collected in EDTA anticoagulant from all patients. Complete blood counts (CBC) were performed using Sysmex KX 21 automated hematology analyzer.

Molecular characterization

PCR for BT gene mutations was performed by multiplex amplification refractory mutation system (ARMS) in 3 separate reaction mixtures for IVSI-5 (G-C), Fr 8–9 (+G), Fr 41–42 (-TTCT), IVSI-1 (G-T), Del 619 bp, Cd 5 (-CT), Fr 16(-C), IVSI-1 (G-T), Cd30 (G-C), Cd 30 (G-A), IVSII-1 (G-A), Cd 15 (G-A) and Cap +1 (A-C). These genotypes cover almost 98% of BT gene mutations/deletions in Pakistani population. DNA extraction was done by using PUREGENE genomic DNA purification kit (gentra systems, USA). DNA amplification was carried out in a 20 μl reaction mixture containing 5 pM of the common primer and 5 pM each of the two forward primers, 0.5 units of Taq polymerase (Fermentas Life Sciences, Lithuania), master mixture containing 30 mM of each dNTP, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 mg/ml gelatin and 0.1–0.3 μg of genomic DNA.

HbA_2 levels measurement

Samples were run on both HPLC and CAE to measure HbA_2 within 24–48 h of the collection. Whole blood samples were run directly on HPLC which automatically quantifies the HbA_2 levels based on chromatographic separation of hemoglobin proteins following its passage through a column of polar aluminum compounds under high pressure. However to measure HbA_2 levels by CAE, first a hemolysate was prepared which was then applied on cellulose acetate membrane in an electrophoretic tank having an alkaline pH of 7.9. An electrical current was applied with a voltage of 200 V for 30 min. Afterwards HbA_2 band was eluted in Tris EDTA borate buffer at pH 8.9 for 30 min. Finally percentage of HbA_2 was calculated in this elute by measuring its absorbance in a photometer at a wavelength of 416 nm. HbA_2 levels >3.8% by CAE and/or HPLC were considered as diagnostic of BTT [17].

Data was collected and analyzed on SPSS 17. Mean and standard deviation were calculated for quantitative variables like age, Total Red Blood Cells (TRBC), Hemoglobin (Hb), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Red Cell Distribution Width (RDW) and HbA_2. Frequencies and percentages were calculated for qualitative variables like gender. Sensitivity of CAE and HPLC was calculated by using the formula "Sensitivity = TP/(TP + FN) × 100". All PCR positive patients having $HbA_2 > 3.8\%$ on CAE and/or HPLC were considered true positive, and those detected positive on PCR but negative ($HbA_2 < 3.8\%$) on CAE and/or HPLC were taken as false negative.

Results

A total of 100 patients were included in this study. Majority of the patients were between 12 and 44 years of age. Mean age of the patients in this study was 28.8 ± 8.1 years. Distribution of patients by gender showed 62 patients (62%) were male and 38 patients (38%) were female. Out of these 100 patients, 35(35%) patients had Fr 8–9 mutation which was the commonest mutation in these patients while 25 (25%) patients had IVS1-5 mutation. Rests of the mutations with their frequency are shown in Table I.

All of our patients had $HbA_2 > 4\%$ on both CAE and HPLC. And none of our patients had false negative result. Mean HbA_2 levels by CAE were 4.97 ± 0.42 while Mean HbA_2 levels as measured on HPLC were 5.54 ± 0.59. All of the patients in this study had MCH < 27 pg. Mean MCH value on CBC was 18.9 ± 1.4 pg. Mean values of different variables are shown in Table II.

As all these 100 BTT cases were detected by both HPLC and CAE thus there was not even a single false negative case on either HPLC or CAE. The sensitivity of both the methods for diagnosis of BTT was calculated by using the following formula:

Sensitivity of hemoglobin electrophoresis/HPLC

$$= TP/(TP + FN) \times 100 = 100/(100 + 0) \times 100 = 100\%$$

Discussion

Thalassemia is the commonest inherited disorder worldwide and in our country BT is the most common single gene disorder [3]. Prevention of BTM births is of vital importance as the average income in Pakistan is $1115 per year [18] whereas the average annual cost for management of a BTM patient is > 10 times the income. It is therefore important to diagnose BT accurately and timely. The laboratory diagnosis of BT can be done by a step-wise approach starting with a detailed clinical and family history; CBC including red cell indices, Reticulocyte count and Red blood cell (RBC) morphology; protein based analytic methods like alkaline and acid hemoglobin electrophoresis, HPLC and reserving PCR for difficult cases and genetic counseling [13]. An increase in the HbA_2 levels in the range of 4–6% is specific for BTT after the third month of life. And high HbA_2 concentrations are a result of BT in almost all instances [19].

Table I – Beta globin gene mutation analysis

Type of mutation	Total no. of cases	Frequency (percentage)
Fr 8–9 mutation	100	35 (35%)
IVS1-5 mutation	100	25 (25%)
Fr 41–42 (12%)	100	12 (12%)
Del 619 (10%)	100	10 (10%)
Cd 15 (6%)	100	6 (6%)
IVS1-1 (4%)	100	4 (4%)
Cd 5 (3%)	100	3 (3%)
Fr 16 (3%)	100	3 (3%)
Cd 30 (2%)	100	2 (2%)

Table II – Mean values of different variables

Variable	Number	Minimum	Maximum	Mean ± SD	P value
Age (years)	100	12	44	28.8 ± 8.1	–
Hemoglobin (g/dl)	100	8.1	14.5	11.4 ± 1.6	–
TRBC ($\times 10^9$/l)	100	4.87	8.16	6.06 ± 0.87	–
MCV (fl)	100	55.9	80.1	64.6 ± 4.0	–
MCH (pg)	100	13.7	23.6	18.9 ± 1.4	–
MCHC	100	24.5	33.3	29.3 ± 1.4	–
RDW	100	33.1	45.9	37.8 ± 2.4	–
HbA_2 on HPLC (%)	100	4.4	6.68	5.54 ± 0.59	<0.0001
HbA_2 on hemoglobin electrophoresis (%)	100	4.0	5.6	4.97 ± 0.42	<0.0001

Keeping in mind economic constraints of the country and high prevalence of the disease, cost effective, reliable and appropriate diagnostic tools hold key to eradicate or tackle this disease.

HPLC has emerged as an alternative method over conventional CAE for diagnosis of BTT in most of the health care setups and large diagnostic centers worldwide as well as in this country. It can also accurately identify and quantitate abnormal hemoglobins in mass screening programs. However it is expensive and costs Pakistani Rupees 1000 ($10) per test compared to conventional CAE which costs only Rupees 100–150 ($1–1.5) per test.

We conducted this study to evaluate and compare sensitivity of CAE against HPLC in PCR positive patients to ascertain its utility as a diagnostic tool in our setup. Out of our 100 PCR positive BTT cases, all were diagnosed by CAE. As all of our cases were carriers and silent mutations were excluded, none of our cases showed a borderline increase in HbA_2 (3.5–3.8%). Five hundred carriers were tested on HPLC in a validation study in India. Out of these 500 cases, only 7 cases had borderline increase in HbA_2 which on molecular testing showed presence of silent mutations [20]. Although 2003 survey of College of American Pathologist has shown superiority of HPLC over conventional gel electrophoresis but our results show that electrophoresis is comparable to HPLC [21].

Many studies have shown the precision and accuracy of CAE in diagnosis of BTT. And traditionally it has been considered as method of choice for detection and quantitation of different hemoglobin variants [6, 12]. Tyagi et al. in India showed similar results like ours in which all BTT cases were detected both on electrophoresis and HPLC [22]. Khosa et al. did a comparative analysis of CAE and HPLC for quantitative determination of HbA_2 levels in 40 BTT cases and showed 100% sensitivity for both the methods. And in this study, CAE was recommended to be more suitable for diagnosis of BTT in poor countries like Pakistan [23]. Despite being slow and labor intensive method, most (296 of 387) of the laboratories participating in the College of American Pathologists Hemoglobinopathies Survey program reported results for HbF, HbA_2 and Hemoglobin identification using conventional CAE methods. While some laboratories use a combination of CAE and HPLC to identify and quantitate hemoglobin variants.

Our study shows a CV of 8.39% for HbA_2 at mean concentration of 4.97% for electrophoresis and for HPLC it was 10.72% at a mean HbA_2 concentration of 5.54%. BTT diagnosis requires $HbA_2 > 3.8\%$ and at this concentration, CV is better for electrophoresis than HPLC. However it was 5.02 per cent ± 0.72 for HPLC and 7.01 ± 1.56% for electrophoresis in a study by Tangvarasittichai et al. [7]. The mean and SD values of HbA_2 in our study were 4.97 ± 0.42% and 5.54 ± 0.59% for electrophoresis and HPLC respectively. In our study we noted that all of our BTT cases had MCH < 27 pg which is usually recommended as an initial screening test for BTT [24–27]. So MCH can be used as an initial screening test followed by HbA_2 quantitation for diagnosis of BTT. Based on the results of our study and resource constraints of our country, we recommend that MCH < 27 pg and $HbA_2 > 3.8\%$ by CAE are the reliable and appropriate method for screening and diagnosis of BTT.

The equipment and technology has improved over the years for the diagnosis of hemoglobin disorders. HPLC has advantages in mass screening programs and high workload. The conventional CAE is time honored cheap methodology and has sensitivity comparable to HPLC as found in this study. Considering the economic restraints in developing countries like Pakistan, it is recommended that conventional CAE can be done at all levels of hospital care with good accuracy for diagnosis of BTT.

Authors' contributions/Wkład autorów

SeR – study design, data collection and interpretation, statistical analysis, manuscript preparation, literature search. SAK – data collection, statistical analysis. GUS – data interpretation. FM – manuscript preparation. NA – literature search. AS – Study concept/design.

Conflict of interest/Konflikt interesu

None declared.

Financial support/Finansowanie

None declared.

Ethics/Etyka

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving

humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

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