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Original Article

Identifying the Lower Limit of Haemoglobin A2 in Individuals with Beta Thalassemia Trait

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ABSTRACT

Objective: To determine the sensitivity, specificity and diagnostic accuracy of High Performance Liquid Chromatography in the detection of β-thalassemia trait. To determine the lower cut-off limit of HbA2 in the detection of β-thalassemia trait by using High Performance Liquid Chromatography.

Study Design: Validation study.

Place and Duration of Study: Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, from Aug 2021 to Mar 2022.

Methodology: A total of 243 patients with suspected β-thalassemia were included in this study. Inclusion criteria included patients with a mean corpuscular volume of less than 75 fl and a mean corpuscular haemoglobin of less than 25 pg. Those patients who had received blood transfusions within the past three months, had any other haemoglobinopathy, abnormal iron studies or β-thalassemia major were excluded.

Blood samples were obtained from all patients which were tested for the presence of HbA2 using High Performance Liquid Chromatography and genetic mutation of beta globin chain was confirmed by PCR methods. Data was analyzed by SPSS 26.0.

Results: Our patients had a mean age of 25.57±13.12 years with a female majority: 132(54.3%). A total of 132(54.3%) patients tested positive for β-thalassemia trait with a cut-off of 4.0% using HPLC, while 130(53.5%) tested positive for beta globin gene mutation using PCR. High Performance Liquid Chromatography was found to have a sensitivity of 96.2%, a specificity of 93.8% and a diagnostic accuracy of 95.1% with a cut-off of 4.0%. The ROC curve analysis showed that the optimum lower limit cut-off for the detection of β-thalassemia trait on High Performance Liquid Chromatography was HbA2 level of 3.95%.

Conclusion: High Performance Liquid Chromatography is an excellent modality for the detection of β-thalassemia trait.

Keywords: Beta Thalassemia Trait, Haemoglobin A2, High Performance Liquid Chromatography, Polymerase Chain Reaction.

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INTRODUCTION

β-thalassemia minor is the carrier form of beta-thalassemia in which a patient has one normal and one pathological β-globin gene each, resulting in mild anaemia which is usually asymptomatic.[1] This condition is associated with a raised total red blood cell count, and a reduced mean corpuscular volume, mean corpuscular haemoglobin and normal red cell distribution width on complete blood counts.[2] The presence of these abnormalities in red cell indices usually prompts the clinician to test the patient using a method of haemoglobin A2 (HbA2) quantification, to establish the correct diagnosis.[3]

Beta thalassemia trait is one of the most prevalent genetic abnormalities present in Pakistan, the population has a carrier rate of approximately 5% to 7%, with an estimated 9.8 million asymptomatic carriers and nearly 5,000 to 9,000 new cases of β-thalassemia major are born per annum.[4] It is usually diagnosed on the basis of the presence of a HbA2 fraction between 3.5% to 8% of the total haemoglobin.[5] Methods to assess the quantity of HbA2 present in the blood of a patient include haemoglobin electrophoresis using cellulose acetate membrane (CAM) followed by densitometry, high-performance liquid chromatography (HPLC), capillary zone electrophoresis and gel electrophoresis. Method for detection of beta globin gene mutation is polymerase chain reaction (PCR).[6,7] However, it is pertinent to note here that cost effective methods such as CAM, while they are easier to use but have demonstrated to have a variable degree of diagnostic accuracy when it comes to diagnosing thalassemia trait, while HPLC is known to have confounding within the results in patients with other aberrations of haemoglobin β-chains.[8] Methods such as PCR for beta globin gene mutations are more accurate, but can be very expensive.[9]

A large number of haemoglobin variants which contain β-chain abnormalities have been described in Pakistan, which may produce confounding within the

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diagnostic accuracy of HPLC in the detection of β-thalassemia trait.[10] The country has a high disease burden when it comes to β-thalassemia trait, and the detection of Beta thalassemia trait is imperative: pre-marital screening of cases can help to reduce the number of marriages between affected individuals which can translate into a reduction in the burden of β-thalassemia major, resulting in a major reduction in morbidity, mortality and financial costs. HPLC represents a potentially accurate, cheap and easily applicable test in this regard. This study was conducted with the purpose of determining the optimal cut-off level for HbA2 levels on HPLC in diagnosing the presence of β-thalassemia trait in suspected cases, in the environment of heavy burden of other β-chain abnormalities in a resource restraint set up.

METHODOLOGY

This research was conducted from Dec 2021 to May 2022, as a validation study, in the Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi on 243 consenting participants (consent obtained from guardians where participant was a minor) who reported for screening for β-thalassemia trait. All patients were included via convenient non-probability sampling. The WHO sample size calculator was used to calculate the sample size keeping an expected sensitivity of 100%, expected specificity of 70.0%, expected prevalence of 35.4%, a desired precision of 2.1 and a confidence level of 95%.[11]

Inclusion Criteria: Patients of all ages (except <01 year) and genders, with a mean corpuscular volume (MCV) of less than 75 fL and a mean corpuscular haemoglobin of less than 25 pg were included in the study.

Exclusion Criteria: Participants who had received blood transfusions within the past three months, those who were known patients of other haemo-globinopathies or had abnormal iron studies or β-thalassemia major were excluded from the study.

Patients were asked for a detailed family history on enrollment, and underwent 6ml venous blood sampling which was collected in blood collection tubes containing Ethylene Diamine Tetra Acetic acid (EDTA). Patients underwent a complete blood count (CBC) and a clotted sample was used to assess the serum iron profile (including serum iron, ferritin and total iron binding capacity. This was followed by haemoglobin quantification using HPLC with a calibrated and control-tested BIORAD-D10 HPLC instrument. A cut-off of 4.0% was used as indicative of the presence of β-thalassemia trait. Subsequently, all patients underwent PCR on thermal cycler ProFlex in which deoxyribonucleic acid (DNA) was isolated by chelex method and amplified in thermal cycler and applied to an oligonucleotide probe-containing polyacrylamide gel electrophoresis strip.

Data was analyzed using the Statistical Package for the Social Sciences (SPSS) version 26.0. Mean±SD was calculated for quantitative variables which included age, haemoglobin level, MCV, MCH, and the level of haemoglobin A2 on HPLC. Qualitative variables like gender, diagnosis of β-thalassemia on the basis of HPLC and its presence on the basis of PCR were recorded in terms of frequency and percentage. A 2x2 table was constructed to calculate the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of HPLC in detecting the presence of β-thalassemia trait. A ROC curve was calculated.

RESULTS

We studied a total of 243 patients with a mean age of 25.57±13.12 years, of whom 132(54.3%) were female. The total mean haemoglobin level of the sample at the time of enrollment in the study was 9.23±0.72 g/dL. The mean MCV and mean MCH of the study population was 60.95±4.24 fL and 26.63±2.91 pg, respectively, while mean MCHC was 31.28±1.68 g/dL. The pre-test characteristics of the patients are displayed in Table-I.

Table-I: Pre-Testing Patient Characteristics According to Gender

Variable	Male	Female	*p*-value
Gender	111(45.7%)	132(54.3%)	-
Age	14.58±6.78	34.82±9.52	<0.001
Haemoglobin Levels (g/dL)	9.33±0.75	9.15±0.68	0.047
Mean Corpuscular Volume (fl)	61.50±4.42	60.49±4.04	0.066
Mean Corpuscular Haemoglobin (pg)	26.44±2.61	26.78±3.14	0.360
Mean Corpuscular Haemoglobin Concentration (g/dL)	31.17±1.66	31.36±1.69	0.386

The mean value of HbA2 on HPLC was 4.51±1.62%. A total of 132(54.3%) patients tested positive for β-thalassemia trait with a cut-off of 4.0%, while 130(53.5%) tested positive using PCR for beta

globin gene mutation. The results are shown in Table-II.

Table-II: β-Thalassemia trait testing According to Gender

Variable	Male	Female	*p*-value
Mean HbA2 Levels (%)	4.58±1.78	4.45±1.48	0.533
HPLC Positive Patients	56(50.4%)	76(57.6%)	0.267
PCR Positive Patients	52(46.8%)	78(59.1%)	0.057

Comparison of results of PCR and HPLC is given below in Table-III.

Table-III: Comparison of Results of HPLC and PCR

2 x 2 Table		Presence of β-Thalassemia trait according to PCR	
		Yes	No
Presence of β-Thalassemia trait according to HPLC	Yes	True Positive: 125(51.4%)	False Positive: 7(2.9%)
	No	False Negative: 5(2.1%	True Negative: 106(43.6%)

Table-IV: Test Characteristics

Test	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Diagnostic Accuracy
HPLC	96.2%	93.8%	94.7%	95.5%	95.1%

HPLC was found to have a sensitivity of 96.2%, a specificity of 93.8% and a diagnostic accuracy of 95.1% with a cut-off of 4.0%. The specific test characteristics and likelihood ratios are displayed in Table-4 and Table-5 respectively. The ROC curve shown in Figure-1 shows that the optimum lower limit cut-off for the detection of β-thalassemia trait on HPLC was an HbA2 level of 3.95%.

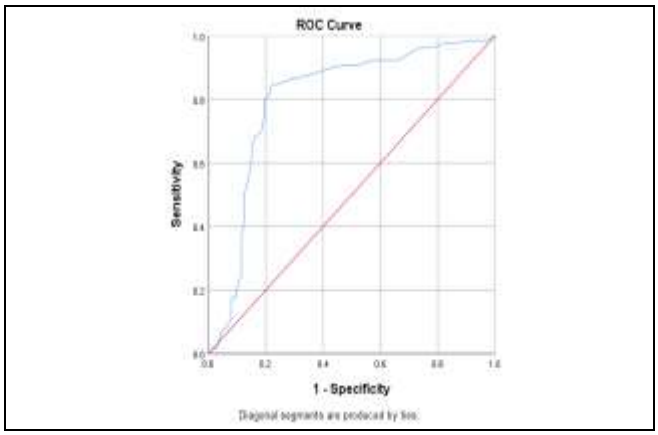

Figure-1: ROC Curve

DISCUSSION

The prevalence of β-thalassemia is on a rising trend in Pakistan, largely due to a lack of awareness, prevalent consanguineous marriages and the absence of any screening program.[12] Screening for the presence of β-thalassemia trait is an important foundation on which any national β-thalassemia control program can be based, however, the testing modality has to be cost-effective, easily implementable and accurate.[13] HPLC represents a potential test modality which can be employed in a screening role for β-thalassemia trait, but needs further evaluation before it can be committed to such a role.[14]

The mean age of our sample was 25.57±13.12 years, with females presenting for testing at a significantly higher mean age of 34.82±9.52 years versus 14.58±6.78 years for males, (<0.001). This late presentation is likely attributed to the higher incidence of iron deficiency in females secondary to menstruation, which may mask the presence of a β-thalassemia trait as demonstrated in study by Arshad *et al*.[15] However, these ages are significantly higher than Nosheen *et al*., who reported a mean age of 11.65±6.25 years for their sample.[16] This difference can be attributed to the way our study was carried out: we screened patients who had reported with microcytic anaemia who were not iron deficient, while the aforementioned study conducted a screening program. Our study had 111(45.7%) males and 132(54.3%) females, which is in keeping with the fact that females have a higher incidence of microcytic anaemia than males, and this was also seen in study by Nah *et al*.[17]

Our study showed that the MCV, MCH and MCHC were all low in our study population i.e 60.95±4.24 fL, 26.63±2.91 pg and 31.28±1.68 g/dL, respectively. This is in keeping with other literature on the subject such as Abdel-Messih *et al*., who showed that patients with β-thalassemia trait have derangements in these red blood indices greater which is comparable to patients suffering from iron deficiency anaemia.[11] Moafi *et al*., noted that patients with β-thalassemia trait tended to have higher derangements in MCV than MCH which could be used as a basis for the decision to proceed for further testing such as HPLC or molecular investigations.[18] It is pertinent to mention here that it is of paramount importance to differentiate β-thalassemia trait from iron deficiency anaemia, to avoid misdiagnoses, and unnecessary iron supplementation as well as a lack of

genetic counselling and red blood cell indices such as the aforementioned ones go a long way in this regard.[19] Our study showed a mean HbA2 level of 4.51±1.62%. Abdel-Messih et al noted a slightly higher level of 5.4±0.7%,[11] while Hussain *et al*., reported a value that was closer to ours i.e 5.05±1.30.20

Our study showed that a cut-off level of HbA2 on HPLC of 4.0% was associated with a sensitivity, specificity and diagnostic accuracy of 96.2%, 93.8% and 95.1%, respectively. A lower cut-off of 3.95% was associated with adequate balance between sensitivity and specificity. Previous studies by Thong *et al*., have suggested that a minimum HbA2 level of between 3.5% and 4.0% is sufficient to diagnose the presence of β-thalassemia trait,[21] while Rauf et al determined that HPLC was 100% sensitive in the detection of β-thalassemia trait at this level.[22] However, Abdel-Messih *et al*., proposed that a cut-off lower level for HbA2 of 3.5% was better with a sensitivity of 100.0%, a specificity of 70.0% and a diagnostic accuracy of 70.0%.[11] At this level, our study showed a sensitivity of 90.0% and a specificity of 56.6% and while a compelling argument for using a lower threshold can be made while noting the fact that a significant number of trait carriers have so-called "silent" mutations whose HbA2 levels land between 3.5% and 4.0% on HPLC,[23] we believe a higher number of false positives cannot justify this low threshold. However, a compromise can be advocated: patients with HbA2 levels in the "borderline" region, i.e., between 3.5% and 3.9% may be confirmed as β-thalassemia trait by using molecular methods of testing such as PCR, which will help to establish the diagnosis definitively, without compromising the patients' well-being.[11]

LIMITATIONS OF STUDY

β-thalassemia trait is a relatively common condition that is essential to screen for and, sometimes, difficult to diagnose. Our study used HPLC as a screening test to determine whether a patient with microcytic anaemia was a carrier, however, there were certain limitations. Firstly, we used a cut-off level of 4.0% to diagnose β-thalassemia trait, however beta thalassemia trait mutations which are associated with lower levels of HbA2, or even normal ones would be missed at this level. Second, we sampled a specific population i.e., armed forces personnel and their families, which may not be representative of the general population. Thirdly, we studied patients who had been pre-screened for iron deficiency anaemia, which may not be possible in busy out-patient departments, offering the test upfront as screening may result in the decrease in the diagnostic accuracy of the test, with a resultant decrease in utility. And lastly, minimal data is available in literature on the subject, as western countries, which provide the bulk of medical research, have a very low prevalence of the disorder, due to effective screening programs, making a basis for comparison difficult.

CONCLUSION

HPLC is a useful modality that can be applied as an effective screening test in the diagnosis of β-thalassemia trait. Where the values are borderline, further testing with molecular methods such as PCR may be warranted. Further research on the subject should focus on launching pilot programs to do just that, in an effort to reduce the burden of β-thalassemia major in our population and our study has shown that HPLC is a very effective tool which can be utilized to achieve this goal.

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Authors' Contribution

The Following authors have made substantial contributions to the manuscript as under:

MWA & FA: Study design, drafting the manuscript, data interpretation, critical review, approval of the final version to be published.

MB & EM: Data acquisition, data analysis, approval of the final version to be published.

SZ & SS: Critical review, concept, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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