

Validity of DCIP Method for Rapid Screening of Haemoglobin E Trait

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ABSTRACT

Background: Hemoglobin E B thalassemia is the commonest thalassemia syndrome in Bangladesh. All tests for diagnosis of Hb E trait are expensive, time consuming and require sophisticated equipment. The need therefore, is a simple, low cost, rapid and reliable test which can be used for mass screening of E trait. The present study was designed to see the validity of DCIP test as screening test for detection of E Trait.

Methodology: A cross sectional observational study was conducted at Dhaka Shishu (Children) Hospital from December 2008 to November 2009. Total 150 subjects attending Dhaka Shishu Hospital Thalassemia centre were selected and evaluated for Hb estimation, RBC indices, Hb Electrophoresis and DCIP test. According to electrophoresis results patients were divided into 3 groups. Group I comprised of 50 subjects with Hb E trait, group II comprised 51 subjects with β thalassemia trait and Group III comprised of 49 normal subjects. In all 3 groups DCIP test was done and in positive cases, precipitated haemoglobin was visualized by naked eye at the bottom of the test tube. Negative samples with normal electrophoresis and β thalassemia trait were used to see the samples gave false positive result.

Results: The test successfully detected 48 subjects among total 50 subjects of E trait. DCIP sensitivity, specificity, positive predictive value and negative predictive values were 96%, 97%, 94.12% and 97.97% respectively.

Conclusion: DCIP has high sensitivity, specificity, positive and negative predictive value. So, it might be considered as a valid single screening test to detect E Trait in areas with limited laboratory facilities and economic resources.

Key words: Hb E Disease, Hb E Trait, β -Thalassemia trait.

Introduction

Hemoglobinopathies and thalassaemia are heterogeneous group of hereditary disorders prevalent worldwide.¹ They represent a major public health problem in many areas of the world including South East Asia.² The hereditary disorder of haemoglobin usually present as either a reduced rate of production of one or more of the globin chain (thalassaemia) nor those in which there is structural changes in a globin chain like Hb E, Hb C, Hb D etc.³ Hb E is the second most prevalent Hb variant in the world and has a worldwide carrier of 53

million.⁴ It is estimated that about 250 million people (4.5% of the population) carry a potential pathological haemoglobinopathy gene and about 3,00,000 infants are born with major haemoglobinopathies.⁶ Haemoglobin E is the second most common variant haemoglobin worldwide and it is the hallmark of South East Asia and extends from Eastern part of India, Bangladesh, Burma, Laos, Thailand and Cambodia. It is estimated that 30 million South East Asians are heterozygous of Haemoglobin E.⁷ It has

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been noted to be an important health problem in the Indian subcontinent and South east Asia. It has replaced B thalassemia as the most common thalassemia disorder in many regions. The frequency of Hb E approaches 60% in many regions of Thailand, Laos and Cambodia. The World Health Organization (WHO) estimated that in Thailand at least 1,00,000 new cases of Hb E β thalassemia are expected in the next few decades.⁸

There is no definite data regarding carriers of hereditary disorder existing in Bangladesh. A conservative World Health report has estimated that 3 percent (3.6 million) carriers of β thalassemia and 4 percent (4.8 million) are carriers of Hb E in Bangladesh. More than two thousand thalassemic children are born every year in Bangladesh.⁹ A study showed that carrier status of Hb E is 6.1 % and about 40% from tribal children of Bangladesh.¹⁰

Hb E is caused by a substitution of glutamic acid by lysine at position 26 of the β globin chain.¹¹ Patient of Hb E trait has no clinical significance. Patient may have mild microcytosis without anemia.

The impact of problem is that haemoglobin E carriers and homozygous Hb E people can produce diseased offspring if they get married with β thalassemia carriers. The Hb E/ β thalassemia compound heterozygote is the most common abundant form of thalassemia disease in Bangladesh.¹⁰ The compound heterozygote state of Hb E β thalassemia results in a variable phenotype ranging from a complete lack of symptoms to transfusion dependency.^{13,14} Approximately one half of the patients are phenotypically similar to patients with thalassemia major who require regular transfusion therapy and the other half resembles thalassemia Intermedia.¹⁵ Transfusion dependent severe E β thalassemia patients need regular blood transfusion, iron chelation therapy and treatment of various complications which are very expensive. As a poor country, it is not possible for us to bear expense of treatment of thalassemia patient. The only available curative treatment of thalassemia is bone marrow transplantation which is far beyond our reach. Majority of the patient die due to lack of treatment.¹⁶ The present management gives a probable life expectancy beyond third or fourth decade. The quality

of life of patients and burden of the families due to treatment represents for public health service clearly underline the fundamental aspects of prevention rather than treatment.^{17,18} Prevention which includes population education, mass screening, genetic counseling and prenatal diagnosis is the only effective way to cope with such disease.^{16,18} For effective genetic counseling the population at risk needs to be identified. The aim of the carrier screening is to identify carriers of haemoglobin disorders in order to assess the risk of couple having a severely affected child and to provide information on the options available to avoid such an eventuality.¹⁹ NESTROFT (Naked Eye Single Tube Red cell Osmotic Fragility Test) is found to be very useful for detecting beta thalassemia trait.²⁰ β thalassemia trait can also be screened by using RBC indices where MCV and MCH values are low with high RBC count.²¹ Hb E heterozygote are clinically well. Screening of Hb E carrier is equally necessary as they combine with B thalassemia trait to cause Hb E β thalassemia which is the most common thalassemia syndrome in Bangladesh. There are doubtful role of blood count and RBC indices in detecting Hb E trait. The false negative result with MCV and MCH for screening is unacceptable especially in a population where there is significant high prevalence of Hb E.²² The only screening as well as confirmatory test for detection of Hb E trait is Hb E estimation by Hb Electrophoresis which needs skilled personnel and require sophisticated equipment. For countries with limited resources, mass screening can be conducted using cheaper and a less complex methods.

A simple cheaper alternative screening methods has been advocated for screening purpose using a combination of modified one tube osmotic fragility test and a modified Dichlorophenolindophenol (DCIP) precipitation test.²³ This method can be used to detect carrier at a low cost especially in underdeveloped countries. Different studies have been done in Thailand using these methods revealed excellent result with DCIP test for Hb E trait. The present study was carried out to see the sensitivity and specificity of simple, inexpensive method of DCIP as a screening test for Hb E trait.

Methodology:

This hospital based cross sectional study was carried out at Dhaka Shishu (Children) Hospital from December 2008 to November 2009. A total number of 150 subjects were selected who were older than 1 year, not suffering from acute infections and without β thalassemia major or Hb E β thalassemia attending Dhaka Shishu Hospital Thalassemia Center were tested for haemoglobin, red cell count, red cell indices, peripheral blood film and haemoglobin electrophoresis.

All subjects were divided into three groups. Group I comprised 50 subjects with Hb E traits, detected by Hb electrophoresis when Hb E level was found between 20% to 35%. Group II comprised 51 subjects with β thalassemia trait detected by Hb Electrophoresis when Hb A2 level was found $>3.5\%$. Group III comprised 49 subjects who detected normal by electrophoresis report. DCIP precipitation test was done in all subjects of above three groups.

DCIP precipitation test was done with 30 microlitre of blood within 4 hours of collection of blood. Principle: The Hb E mutation occurs from the mutation on the beta globin gene at codon 26, GAG-AAG, resulting in the amino acid change from Glu-Lys. This results in a mildly unstable haemoglobin and exposure of SH group, which can be oxidized by certain chemical agents including the dye DCIP (Dichorophenolindophenol) at the neutral pH (7.5).Hb E and other unstable haemoglobin molecules such as Hb H will be precipitated when exposed to this dye as 37 C.

DCIP reagent contains Tris base 4.36g, EDTA Na 2.2 H2O 2.68 g, DCIP 0.0276g, Saponin 0.05 g. The reagents will be dissolved in distilled water and Ph adjusted to 7.5 and volume made to 500 ml. 30 microlitre of whole blood will be delivered into 5 ml of DCIP solution and then gently mixed and incubated at 37 degree centigrade for one hour. In positive cases precipitated haemoglobin can be visualized by the naked eye at the bottom of the test tube. Negative sample with normal electrophoresis and beta thalassemia trait will be used to see whether these samples give false positive result. Data were analyzed by SPSS programme. Statistical unpaired t test was done.

For validation the number of true positive (TP), true negative (TN), false positive (FP) and false negative

(FN) were determined. The sensitivity, specificity and positive predictive value and negative predictive value were calculated.

Results:

Table I: Age distribution among the study population (n = 150)

Age (year)	Mean +- SD	Range
E Trait (n= 50)	29.53 +- 10.70	2-47
B Trait (n=51)	30.31+-9.85	1-55
Normal (n=49)	21.81+-14.26	1-52

In group I the mean age was 29.53 years and the age range was 2-47 years. In group II the mean age was 30.31 years and the range was 1-55 years. In group III the mean age was 21.81 years and the range was 1-52 years (Table-I).

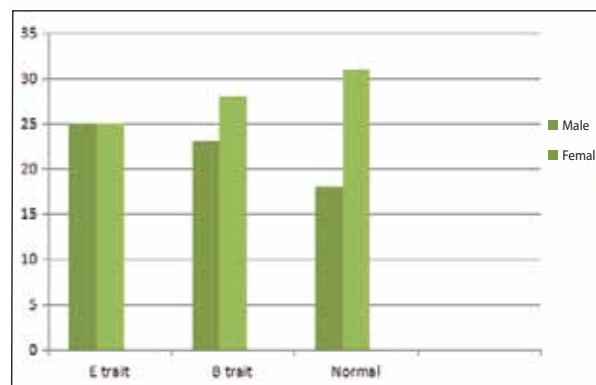


Figure 1: Sex distribution of study population (n=150)

In group I, 25 male and 25 female (n=50), in group II, 23 male and 28 female(n=51), and in group III, 18 male and 31 female (n=49) subjects were included (Figure-1).

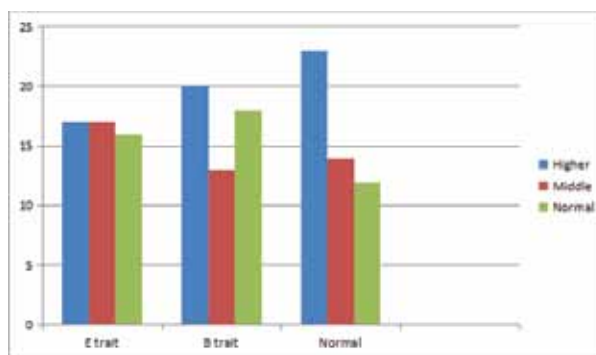


Figure -2:Socio economic status of study population (n= 150)

In group I there is no significant variation of socio economic status but in group II and in group III the majority are from higher socio economic group.(fig 2)

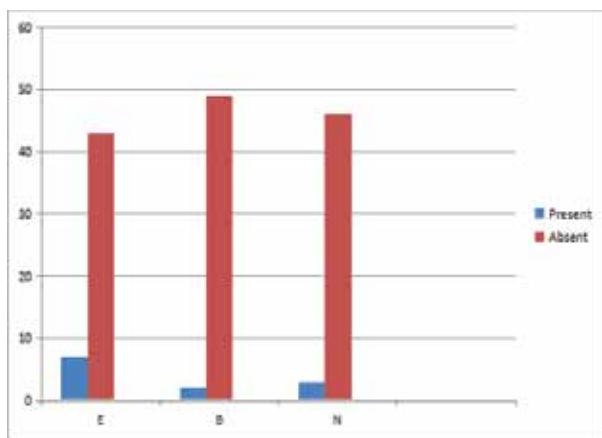


Figure 3 : Presence of consanguinity among the study population (n=150)

In all the 3 groups majority of the study population were from non consanguineous parents but in Group I ,seven of the subjects among the total 50 were from consanguineous parents (Figure-3).

Table II: Comparison of hematological parameter between Hb E trait and normal subjects

Hematological Parameters (Mean)		Mean +- SD	P value
Hb% (gm/dl)	Normal (n=49)	10.77+- 1.4	0.17
	E trait (n=50)	11.76+-1.91	
MCV (fl)	Normal (n =49)	77.35 +- 10.27	0.68
	E Trait (n =50)	72.89 +-12.24	
MCH (pg)	Normal (n=49)	28.28+-2.716	0.33
	E trait (n=50)	24.84+- 3.71	
MCHC(gm/dl)	Normal (n=49)	32.97+- 2.38	0.11
	E trait (n=50)	32.82+-2.27	
RBC Count (million/cmm)	Normal (n=49)	4.61 +- .65	0.87
	E trait (n=50)	5.01+- .71	
RDW (%)	Normal (n=49)	14.83 +-1.28	0.11
	E Trait (n =50)	14.32+-2.34	

The present study revealed the mean \pm SD haemoglobin level (g/dl) in group I (E trait) and group III (normal subjects) were 11.76 ± 1.91 and 10.77 ± 1.4 respectively. Statistically there was no significant difference was found between these 2 groups ($P > .05$). The mean corpuscular volume (MCV) in mean \pm SD in group I and group III were 72.89 ± 12.24 fl and 77.35 ± 10.27 fl respectively. No statistical significance was found as $P > 0.05$. The mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin

concentration (MCHC) in group I were 24.84 ± 2.71 pg & 32.97 ± 2.27 g/dl and in group III were 28.28 ± 2.71 pg & 32.97 ± 2.38 g/dl respectively. There were no significant difference between group I and III regarding MCH and MCHC (Table-II).

The mean RBC count in E trait and normal subject group were 5.01 ± 0.71 million/cmm and 4.61 ± 0.65 million/cmm respectively. There was no significant difference was found. $P > 0.05$. Mean red cell distribution width (RDW) in group I and III were 14.32 ± 2.34 and 14.83 ± 1.28 without any statistical difference ($P > 0.05$) (Table-II)

Table III: Significance of DCIP test to detect Hb E trait.

DCIP test	E trait	β trait	Normal	Total
DCIP test positive	48	3	0	51
DCIP test negative	2	48	49	99

Among 50 patients with E trait DCIP test was positive in 48 cases and negative in 2 cases. Among 51 β thalassemia trait, DCIP test showed positive result in 3 cases and negative result in 48 cases. But DCIP test was negative among all the normal subjects. Table - III.

DCIP test was positive in total 51 cases, of them 48 were E trait (true positive) and 3 other than E trait (false positive). This test was negative in total 99 cases, among these negative cases 2 were E trait (false negative) and 97 were either β thalassemia trait or normal subjects. (true negative) (Table- IV)

Sensitivity of DCIP test was 96% and specificity was 97%. predictive value of positive test was 94.12% and predictive value of negative test was 97.97% (Table-IV).

Table IV: Adequacy of DCIP test to detect Hb E trait.

DCIP test	Hb Electrophoresis positive	Hb Electrophoresis negative
DCIP test positive	48	3
DCIP test negative	2	97
Total	50	100

Sensitivity =96%

Specificity=97%

Positive predictive value= 94.12%

Negative predictive value= 97.97%

Accuracy =94.67%

Discussion:

A number of studies have conducted by Fuchroen et al²³, Wiwanikit et al²⁴, Kannadit et al²⁵ and Siripakorn et al²⁶ and their sample size were 301,213,808 and 436 respectively to make an screening protocol for thalassemia patients in Thailand. In the present study, in Group I the mean age was 29.53 years and the age range was 2- 47 years. In Group II the mean age was 21. 81 years and the range was 1-52 years. The other study done by Fuchroen et al for simplified screening for thalassemia and Hb E in rural communities the age range was 8 - 30 years.²³

In the present study in all 3 groups majority of the study population were from non consanguineous parents. Regarding socio economic status in group I there is no significant variation of socio economic condition but in group II and in group III the majority of the subjects are from higher socio-economic group. It indicates that Dhaka Shishu (Children) Hospital Thalassemia Centre is creating awareness among all groups of people having thalassemia syndrome from different economic background. The other studies which have already mentioned earlier did not show anything about consanguinity and socio-economic status.

The present study revealed that the mean \pm SD haemoglobin level g/dl in Group I(E trait) and Group III (normal subjects) were 11.76 \pm 1.91 and 10.77 \pm 1.4 respectively. Statistically there was no significant difference was found between there 2 groups. Similar results were obtained by Fuchroen et al, who found normal haemoglobin level in both groups.²³

The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) in mean (\pm SD) for Group I was 72.89 \pm 12.24 , 24.84 \pm 3.71 pg and 32.82 \pm 2.27 g/dl respectively and for Group III were 77.35 \pm 10.27 fl, 28.28 \pm 2.71 pg & 32.97 \pm 2.38 g/dl respectively. No statistical significance was found regarding all parameters ($p > 0. 05$). MCV, MCH and MCHC in E trait group were closer to normal subjects. Similar results were obtained by Fuchroen et al.²³ Other study done by Sanchaisuriya et al ²² showed MCV and MCH were normal in E trait and they may give false negative result for screening of E trait if RBC indices are considered as screening method.

The present study showed that RBC count in E trait and normal subject group were 5.01 \pm 0.71 million/mm³ and 4.61 \pm 0.65 million/mm³ respectively. But there was no significant difference was found $P > 0.05$. Mean red cell distribution width (RDW) in group I and III were 14.32 \pm 2.34 and 14.83 \pm 1.28. That was also not statistically significant. $P > 0.05$. The similar result was obtained by Fuchroen et al²³ that showed no significant difference of RDW in different subjects who had different type of haemoglobin disorder. The present study found the DCIP test is highly sensitive and specific but not as sensitive or as specific as some of the other studies. Sensitivity of the test 96% is little lower than other studies (Fuchroen et al²³ , Chappel et al²⁷ , Winichagoon et al²⁸, Wiwanikit et al²⁴ ,Kannadit et al²⁵, Siripakorn et al²⁶. Specificity of the test (97%) correlates with most of the other studies.(Fuchroen et al , Chappel et al , Winichagoon et al, Wiwanikit et al ,Kannadit et al.) . Only Siripakorn et al showed low specificity in comparison to other studies.Fuchroen et al, Chappel et al , Winichagoon et al , Wiwanikit et al showed 100% sensitivity and 98.7%,92%,100%and 97.2% percent specificity respectively. Kannadit et al and Siripakorn et al also showed higher sensitivity as 97.16% and 99.5%. The negative predictive value of the test in this study was 97.98%.the result is comparable with other studies (Fuchroen et al, Chappel et al, Wiwanikit et al, Kannadit et al) who reported values 100%,100%, 100% and 95.19%. The important point to be noticed that the presence of negative test almost rules out the possibility of E trait in general population. The positive predictive value of the test of this study is 94.11% which is quite close to the result of other studies done by Fuchroen et al, Chappel et al , Wiwanikit et al , kannadit et al , who reported values 98.6%,85.7%,94.4% and 99.42%. A lower positive predictive value suggested false positive results probably due to associated other globin chain disorders. Hb H could generate false positive result. The cost of performing a single DCIP test is less than Tk 30. Its easy to perform, as much technical expertise is not required, no well equipped laboratory is needed. The stock solution once made keeps well in a stopper bottle, thus can be used in field surveys. A single individual can perform 10-20 tests in an hour.

The confirmatory tests needed for diagnosis of E trait are costly, laborious, and time consuming. By excluding control subjects and thus restricting further investigation for the precise diagnosis to the small proportion of positive subjects DCIP test reduce the time, cost and labour. The present study found DCIP test to be both sensitive and specific and of high negative predictive value. However, multicentre study with large sample size is needed to recommend DCIP as a single screening test for detection of E trait.

Conclusion:

The frequency of Hb E disease is increasing day by day, though there is no effective prevention or screening program for the disease in. Mass screening methods should be identified to reduce the mortality and morbidity due to Thalassemia. Our study has some limitations. This study was confined to a single hospital and limited to small sample size. For detection of carrier status of haemoglobin disorder ideal procedure should be DNA analysis which is unavailable in our setting. DCIP is highly sensitive and specific and have both positive and negative predictive values. Since it is less expensive and suitable for field survey, it might be considered as the single screening test to detect Hb E trait in areas with limited laboratory facilities and economic resources. Multi-centered study is required in Bangladesh among the people of different areas and ethnic origin to see effectiveness of DCIP method. As Hb E and β trait are prevalent here, DCIP method can be used align with NESTROFT for mass screening even in upazila level to prevent birth of child with E β thalassemia.

Conflict of interest: None.

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