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Detection of Common Beta Globin Gene Mutations on Heterozygous Beta Thalassemia Including Haemoglobin E among High School Students

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Abstract: Beta thalassemia is one of the most common genetic disorders worldwide with a defect in haemoglobin synthesis including Myanmar. This study was conducted to determine the proportion of high school students who have heterozygous β-thalassaemia including Hb E and different types of common beta globin gene mutations by multiplex polymerase chain reaction. This community-based cross-sectional descriptive study was done in Myanmar adolescents in 2017. A total 290 apparently healthy high school students in which male 140 (48.3%) and female 150 (51.7%) studying in Basic Education High School, Anisakhan, Pyin Oo Lwin Township, Mandalay District, Myanmar were involved. Thalassaemia screening tests were done by combined NESTROFT/DCIP precipitation and confirmed test was done by Isoelectric Focusing (IEF). PCR- based genetic confirmatory by using mutant genes IVS 1-1, CD 41/42, IVS 1-5, CD 17, and nt-28. Total 131 cases (131/290, 45.2%) were positive with either of the two screening tests. These cases were considered as thalassaemia trait (carrier) and/or haemoglobin E carrier. Isoelectric focusing was done to find Hb E by which 45 cases (45/131, 34.3%) were found to have Hb E carrier. Remaining 86 cases were further run multiplex- PCR to detect beta thalassaemia carrier. Total 44 out of 86 cases were β-thalassaemia traits (25 CD 41/42 (-TCTT), 13 IVS 1-5 (G-C), 1 IVS 1-1 (G-T); 5 compound heterozygous (IVS 1-5(G-C) /CD 41/42 (-TCTT)). CD 17 (A-T) and nt 28 (A-G) mutations were not detected in this study. NESTROFT screening test has 87% sensitivity and 82% sensitivity for the detection of Hb E carrier and β-thalassaemia carrier respectively. DCIP screening test has 67.1% sensitivity and 68% sensitivity for the Hb E carrier and β-thalassaemia carriers respectively. Combined both screening tests have 100% sensitivity for the detection of Hb E and β-thalassaemia carriers. This study has highlighted that both screening tests should be used for population screening of thalassaemia and haemoglobinopathies in Myanmar.

Key Words: beta thalassaemia, beta globin gene, haemoglobin E, high school students

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Introduction

Human beta globin (HBB) gene maps in the short arm of chromosome 11 position 15.5 containing the delta globin gene, the embryonic epsilon gene, the fetal A-gamma and G-gamma genes, and a pseudogene. Mutations in HBB cause genetic disorders such as β-thalassaemia as well as sickle cell disease, Haemoglobin C and Haemoglobin E [1] . The highest carrier frequency of thalassaemia is reported in Cyprus (14 %), Sardinia (10.3 %), and Southeast Asia (3-5 %). The most common combination of β-thalassaemia with abnormal Hb is Hb E/β-thalassaemia which is most prevalent in Southeast Asia where the carrier frequency is around 50 percent [2]. According to the data from Thalassaemia International Federation (TIF) publication 2013, expected figure of β-thalassaemia syndromes births per year in Myanmar is 2398, Thailand is 6983 and Bangladesh is 64 [3]. Heterozygotes (i.e., carriers) are usually asymptomatic. Carrier testing for individuals at risk (including family members, gamete donors, and members of at-risk populations) is possible [4].

Various screening tools are used for detection of β-thalassaemia trait such as electronic blood cell counts, high performance liquid chromatography (HPLC), mean corpuscular volume (MCV), naked eye single tube red cell osmotic fragility test (NESTROFT) and dichloro indolphenol precipitation test (DCIP). It is important to evaluate the simple and effective screening strategy for detection in large population [5,6].

A combined NESTROFT/DCIP test is widely performed for mass screening as it is a simple, reliable, cost-effective and practical method and could be used in any primary health care setting where a programme of prevention and control is needed [5]. The most commonly used electrophoretic methods in thalassaemia population screening programmes are Capillary Electrophoresis, Cellulose Acetate Electrophoresis, Citrate Agar Electrophoresis and Isoelectric focusing (IEF) [7].

However few studies have been performed to find out the prevalence of β-thalassaemia in Myanmar. In 1968, a study done on 232 Myanmar volunteers reported thalassaemia trait was 4.3 percent [8] and β-thalassaemia mutations detected were CD 41/42 in 21 cases, IVS I-1 in 15 cases, IVS I-5 in 13 cases, CD 17 in 8 cases and 1 case was IVS II-654 [9]. In 2004, eleven different β-thalassaemia mutations have been identified in 61 out of 90 family members of transfusion dependent beta thalassaemia major cases and suspected cases of thalassaemia intermedia [10].

In 2010, a study done at Yangon General Hospital found out 4 common mutations of β-thalassaemia such as CD 41/42, IVS I-1 , IVS I-5 and CD 17 which is compatible to the results of the other studies which were done in South East Asia Region [11]. Among the studies done in β- thalassaemia of Myanmar, the commonly detected mutations of β-globin gene are IVS I-1, CD 41/42, IVS I-5, CD 17, CD 26 and nt-28 [10,11,12,13,14]. The previous studies have focused only on the patients and their family members by using amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) [10,11,12,13, 14]. The ARMS protocol usually detects one mutation per reaction and can be laborious and expensive to use when looking for 5 or 6 common mutations individually in one sample [15]. Multiplex-PCR is useful to detect various molecular defects in a single tube. In this study, multiplex-PCR will be used to detect ethnic-specific common mutations and only requires targeted PCR amplication and gel electrophoresis of the amplicon.

The results of this study may provide the data for further research. Moreover, this study may be useful for future early diagnosis and effective management of β-thalassaemia. If β-globin gene mutations positive rate is identical to the positive combined NESTROFT/DCIP test, this screening test can be applied as a main screening test of heterozygous β-thalassaemia in the large population of Myanmar where the human resources and laboratory capacity are limited.

Materials and Methods

This community-based cross-sectional descriptive study was done at Pathology Research Division, Department of Medical Research (Pyin Oo Lwin Branch) and Basic Education High School, Anisakhan, Pyin Oo Lwin Township**.** A total of 290 apparently healthy Grade IX students, totally unrelated whose parents/guardian had given the written informed consent were recruited by using systematic sampling procedure during January to December 2017**.** Then, three millilitres of venous blood samples were taken and 2 ml of blood was separated for haemogram and NESTROFT and DCIP screening tests were done. Isoelectric focusing (IEF) method was done for confirmation of Hb E to above either of the two positive screening samples. Then, diagnosis of Hb E was obtained. Heterozygous β-thalassaemia of common β-globin gene mutations was detected by PCR in remaining cases to detect five common beta globin gene mutations such as IVS 1-1 (G-T), CD 41/42 (-TCTT), IVS 1-5 (G-C), CD 17 (A-T), and nt-28 (A-G). If IVS 1-1 or IVS 1-5 mutation occurred, ARMS PCR was used for differentiation of these two mutations. Data entry and analysis was done by SPSS software 20.0 version. Regarding

the Ethical consideration, the approval was obtained from the Ethical Review Committee of the Department of Medical Research before the study was conducted.

Results

Background subjects' characteristics revealed total 290 Grade IX high school students aged between (14-15yrs) in which there were 140 male (48.3%) and 150 female (51.7%). Female was more preponderant than male with male to female ratio of approximately 1: 1.1. The 131 (male 52 and female 79) were possible carrier of heterozygous β-thalassaemia, female was predominant than male (52.6% vs 37.1%). Contribution of races among β-thalassaemia carrier were 92 cases (49.7%) of Bamar, 15 cases (48.4%) of Kachin, 3 cases (42.9%) of Kayin, 3 cases (23.1%) of Chin, 14 cases (32.6%) of Shan, and 4 cases (36.3%) of Indian respectively.

Table 1. Distribution of NESTROFT screening test on Heterozygous β-thalassaemia including Hb E (n =131)

NESTROFT	Heterozygous β-thalassaemia including Hb E		Total number of patients
	Positive	Negative	
Positive	76	38	114
Negative	13	4	17
Total	89	42	131

Among 290 participants, 114 participants (39.3%) were positive in NESTROFT screening test and the rest 176 (60.7%) were negative. Among the 114 positive NESTROFT screening tests, 76 cases were positive cases of heterozygous β-thalassaemia including Hb E and 38 cases were negative. Among the NESTROFT negative cases, 13 cases were positive of heterozygous β-thalassaemia including Hb E. The remaining 4 cases were negative for carriers of β-thalassaemia.

Positive DCIP screening was observed in 78 participants (26.9%) and negative in 212 participants (73.1%). Among the 78 positive DCIP screening tests, 60 cases were positive cases of heterozygous β-thalassaemia including Hb E and 18 cases were negative. Among the NESTROFT negative 53 cases, 29 cases were positive of heterozygous β-thalassaemia including Hb E. The remaining 24 cases were negative for carriers of β-thalassaemia.

Table 2. Distribution of DCIP screening test on Heterozygous β-thalassaemia including Hb E (n=131)

DCIP	Heterozygous β-thalassaemia including Hb E		Total number of patients
	Positive	Negative	
Positive	60	18	78
Negative	29	24	53
Total	89	42	131

Among 131 either of the two positive cases, both positive (Positive/ Positive**)** 61 cases gave 47 cases (77%), (Positive/Negative) 53 cases gave 29 cases (54.7%) and (Negative/Positive) 17 cases gave 13 cases (76.5%) as identified as heterozygous β-thalassaemia including Hb E of total 89 cases.

Among 45 cases of Hb E carriers and 44 cases of β-thalassaemia carriers confirmatory cases, NESTROFT test positive (+,+) and (+,-) cases were found 39 cases in Hb E carriers and 37 cases in β-thalassaemia trait and positive DCIP test (+,+) and (-,+)was 29 cases and 31 cases in Hb E carriers and β-thalassaemia carriers respectively. Therefore, NESTROFT was positive in 87% and 84% of Hb E carriers and β-thalassaemia carriers respectively. DCIP was positive in 64% and 70% of Hb E carriers and β-thalassaemia carriers respectively.

Table 3. Either of the two positive NESTROFT and DCIP tests on Heterozygous β-thalassaemia including Hb E

Heterozygous β-thalassaemia including Hb E	NESTROFT positive	DCIP Positive
Hb E carriers (45)	(39/45) 87%	(29/45) 64%
Heterozygous β-thalassaemia (44)	(37/44) 84%	(31/44) 70%

After using combined NESTROFT and DCIP screening tests, out of 290 participants, 131 samples with positive in either one of the two screening tests or both were determined for heterozygous beta thalassemia including haemoglobin E by using IEF method; 45/131 (34.4%) showed Hb E carrier status such as 41 samples (31.3%) were Hb E trait and 4 samples (3.1%) were Hb E disease.

Out of 290 students, 86 (29.7%) were selected for multiplex PCR methods. Multiplex PCR was used to detect five common β-globin gene mutations. This study identified three out of five common mutations except CD 17 and nucleotide -28 (A _ G) mutations and positive in 44 samples (51.2%); the 4 bp deletion (-TCTT) in codons 41/42 was the most common mutation detected in 25 (29.1%) samples. The IVS 1-5 (G-C) mutation was the second most common and accounted for 13 (15.1%) samples followed by, 1(1.2%) sample with IVS 1-1 (G-T) mutation; 5 samples (5.8%) revealed both IVS 1-5 and CD 41/42 (-TCTT) mutations. Therefore, this study found out the CD 41/42 (-TCTT) mutation in 30 samples and IVS 1-5 in 18 samples, altogether these two common mutations (i.e. CD 41/42 and IVS 1-5) accounted for 43/ 44 (97.9%) among five common mutations.

Table 4. Distribution of Hb, MCV, MCH, MCHC and Screening tests in compound heterozygous β-thalassaemia (n= 5)

No	Hb	MCV	MCH	MCHC	NESTROFT	DCIP
1.	9.6	66	20.3	30.7	+	-
2.	10.5	63	19.6	31.0	+	-
3.	11.2	59	17.9	30.4	+	-
4.	9.7	66	20.7	31 6	+	-
5.	7.8	4	12.7	29.7	+	-

In this study, compound heterozygous (i.e. IVS 1-5 and CD 41/42) were found out 5 samples. The haematological indices such as Hb, MCV, MCH, MCHC and their screening results were seen as above. All of the haematological parameters were reduced in above 5 cases.

Discussion and Conclusions

Out of 44 participants with common beta globin gene mutations, 15 samples (34.1%) were male and 29 samples (65.9%) were female. In this study, the most commonly found CD 41/42 mutation was predominantly observed in 72.0% (18/25) as female and 28.0% (7/25) as male.

The overall detection rate of common beta globin gene mutations in this study was 15.2% (44/290), in which 30 (68.2%) and 14 (31.8%) were Bamar and other ethnics respectively. Codon 41/42 (-TCTT) was the most identified common mutation in these population and this frame shift mutation is also the most common mutation found in Thailand, People's Republic of China, and South East Asia. While codon 17 (A-T) was the second most common mutated allele in central, northern, and northeast Thailand [16]. This study does not cover for all states and divisions of Myanmar. It is necessary for wider population study in all states and divisions of Myanmar.

In this study, 114 out of 290 samples (39. 3%) were positive NESTROFT and that of 176 samples (60.7%) were negative. The 66.7% (76/114) of β-thalassaemia trait were detected by m-PCR above 114 NESTROFT positive cases. And the remaining 33.3% (38/114) were false positive NESTROFT results that could be α-thalassaemia trait, iron deficiency anaemia and other β-thalassaemia gene mutations but IDA had not been excluded by serum ferritin in this study.

One study in Pakistan 2012 stated that NESTROFT used as a marker of clinical differentiation between β-thalassemia trait and iron deficiency anaemia (IDA); NESTROFT was concomitantly positive in 13% of IDA cases while it remained negative in 88% of subjects with IDA. The diagnostic accuracy of NESTROFT was 94.6 % [17]. Many researchers in published data concluded that NESTROFT could be effectively used as screening marker for detection of β-Thalassemia trait [17, 18, 19, 20, 21].Chakrabarti and his colleagues also stated that 16 cases out of 17 cases of β-thalassemia trait were found to be NESTROFT positive [22].

NESTROFT was positive in 87% of Hb E carriers (either EE or EA) proved by IEF. NESTROFT was positive in 84% of β-thalassaemia trait proved by PCR. Bobhate, Gaikwad & Bhaledrao, 2002 showed that NESTROFT with 0.36% saline could detect 96-100% of heterozygotes β-thalassaemia [23]. A study published in India concluded that NESTROFT found to be 92.5% sensitive and 95.2% specific for screening of red cell microcytosis. Therefore, the NESTROFT screening test proves to be simple, cheap, easy to perform and adaptable for mass screening.

Among 290 participants, single DCIP precipitation test alone was positive in 78 samples (26.9%) and negative in 212 samples (73.1%) in this study. Then, IEF and m-PCR were done in 78 DCIP positive samples, 76.9% (60/78) were heterozygous β-thalassaemia including Hb E. Eighteen samples revealed false positive DCIP test and it would be the effect of techniques of DCIP which were done by manually dissolved reagents. The negative results of DCIP test were 212 cases in which positive β-thalassaemia was 22.8% which showed false negative DCIP test.

This screening result was similar with other study which was done on 2004 in Thailand described that 40 of 301 participants (13.3%) had DCIP positive [6]. Sangkitporn and co-workers studied that 188 out of 436 unrelated Thai subjects (43.1%) described DCIP positive cases for diagnosis of thalassemia and hemoglobinopathy [24].

In this study, DCIP was positive in 64% of Hb carriers and 70% of β-thalassaemia trait which were proved by IEF and PCR respectively. By doing this screening test, all cases of β-thalassaemia trait and Hb E carriers can be detected. Thus, DCIP test was more appropriate than erythrocyte indicies in screening for Hb E carriers and this screening test which can be used for a mass population.

When combined positive for screening tests were confirmed with both IEF and m-PCR, total 61 cases had positive of 47 cases (77.0%). For NESTROFT test positive alone, 66.7% of carrier for β-thalassaemia could be detected. For DCIP test positive alone, 76.5% were carrier for β-thalassaemia. The combined positive for NESTROFT and DCIP results showed 77.0% of β-thalassaemia carrier. So, NESTROFT test screening was not sensitive but it was specific and DCIP screening test was more sensitive and the combined NESTROFT and DCIP test was more suitable for screening of β-thalassaemia. Therefore, it can be used as preliminary screening test for identifying the carriers of heterozygous β-thalassaemia including Hb E in population screening. By doing these screening tests together, the diagnosis for β-thalassaemia was more accurate for asymptomatic cases.

These findings were agreement with the retrospective study in Thailand, conducted from 1999 to 2008 and analysed 13,745 cases to describe the magnitude of false positive screening of combined NESTROFT/ DCIP precipitation tests for thalassemia in a primary care setting. Cases with α-thalassemia trait, β-thalassemia trait, and haemoglobin E carrier status were confirmed with HPLC and PCR analysis and the remaining were considered as false

positives. The false positive rate was in the range of 20.1-36.1% [25].

Hb E occurs at a high frequency in parts of North East India and throughout South East Asia. In Thailand and other South East Asian countries, thalassaemia is very common with 20-30% of the population having the α-thalassaemia trait, 3-9% having β-thalassaemia trait whereas 20-30% having the Hb E trait [26]. According to a previous study done in Sri Lanka, HbE/β-thalassaemia (CD26 G-A) mutation was observed at a frequency of 26.3% [27]. This study was agreement with other study which was done on 152 participants with normal Hb level and normal MCV of healthy individuals, there was 24 Hb E traits (15.8%), no α-thalassaemia trait and 1 case of β-thalassaemia trait [28].

In this study, according to combined positive screening results (+/+) (NESTROFT/DCIP) 23 samples (23/61) (37.7%) were β-thalassaemia carriers confirmed by m-PCR, and also 25 samples (25/61) (40.98%) were Hb E carriers confirmed by IEF. Therefore, the combined NESTROFT/DCIP test can be used as preliminary screening test for identifying the carriers of heterozygous β-thalassaemia and Hb E.

The present study showed the three common mutations such as CD 41/4 (-TCTT), IVS 1-5 (G-C) and IVS 1-1 (G-T) mutations in only 51.2% (44/86) of heterozygous β-thalassaemia. It is not agreement with the studies done by Ne Win (2002), Yi Yi Tin (2004) and Sein Win (2010) in which prevalence was higher (i.e. more than 80%) than the prevalence stated in this study. That may be due to the selection criteria for the participants used in the present study [10,11,12,13,14] .

In this study, compound heterozygous (i.e. IVS 1-5 and CD 41/42 mutations) were found out 5 samples (5/86, 5.8%). Overall β-globin gene mutations were identified in 58/86 (67.4%) in Bamar and 28/86 (32.6%) were not Bamar. Detailed correlation of genotype and ethnicity demonstrated a marked heterogeneity: 2 students of Kachin, 2 students of Kayin and one student of Bamar in compound heterozygous (IVS 1-5/ CD 41/42). This finding was agreement with Ambekar et al., 2001 reported that 3 cases of compound heterozygous of IVS 1-5 and CD 41/42 mutations was seen in total 114 β-thalassaemia carrier patients and they concluded that the frequency of blood transfusion was very less in their lifespan [29].

The nucleotide -28 (A-G) and CD 17 mutation were not identified in this study. Why these mutations were not observed in this study is that the nucleotide -28 (A-G) mutation cause the mild β-thalassemia phenotype; thus, this mutation has been observed only in thalassaemia intermedia patients. This present study was carried out in apparently healthy High School adolescents. Moreover, A-G substitution at position -28; 5' of the β-globin gene was common at Chinese origin [30] and participants from Chinese origins were not enrolled in this study.

Therefore, the strategy, first screening by combined NESTROFT and DCIP precipitation and followed by specific genetic testing like m-PCR, gave the best result on early diagnosis for β-thalassaemia carriers. Following these ways, proper screening for β-thalassaemia can be done in developing countries such as Myanmar. Therefore, it was recommended that m-PCR reaction could be able to detect common β-globin gene mutations in β-thalassaemia carrier as initial evaluation to obtain the definite diagnosis.

Limitations of the Study

All blood samples are not carried out HPLC that was not selected as a method of choice for haemoglobin analysis. Since Hb A_2 and Hb E were not separated by IEF method, the more the concentration and the thicker the IEF band were considered as Hb E in this study and using HPLC is the limitations for this study. Differentiating between types of anaemia was beyond scope of study.

According to NESTROFT screening test, 0.36% normal saline was only used in this study. Only 66.7% (76 out of 114 NESTROFT positive test) was detected β-thalassaemia trait and this study was not excluded IDA. Serum ferritin level could not be measured in this study. So, other causes of hypochromic microcytic anaemia may be included in this study. According to DCIP screening test, suspicious samples with very little turbidity were considered to be negative result in this study and manually dissolved DCIP reagents were only used.

Concomitant inheritance of Hb E with β-thalassaemia is also a common scenario amongst the Myanmar population. But it was not considered in these populations because blood samples were taken from healthy populations and they have also no history of transfusion dependent thalassaemia.

The remaining unidentified β-thalassaemia mutations rather than previously identifiable five common β-thalassaemia gene mutations are required for National data; further study is warranted to improve the molecular diagnosis of common and rare ones and so further require DNA sequencing.

Recommendations

1. To get early detection, effective screening and easily available screening tests like combined NESTROFT/DCIP and blood for complete blood count are needed.

2. In order to have better management of thalassemia cases and to educate adolescents with thalassemia minor for the purpose of preventing the increasing numbers of thalassemia major among the next generation in Myanmar, effective prevention programme are needed.

3. Mutational pattern study may help in successfully establishing a program of genetic counselling and may help in prenatal diagnosis of β- thalassaemia in order to reduce the burden of this disease in the society.

4. In the future, this type of study should be carried out in these communities of different ethnic groups in this region to get an exact rate of prevalence of these genetic diseases and in determining the common β-thalassaemia mutations of this region.

5. There are limited reports available on thalassemia mutations among healthy people in the community. Multiplex PCR method was found to be simple, rapid, accurate and cost-effective for the identification of beta thalassaemia mutations compared to ARMS not only in thalassaemia major patients but also screening for thalassaemia trait. This strategy is suitable for extensive population study of large sample size.

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