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RAPID DETECTION OF β-THALASSEMIA MUTATIONS IN THAILAND USING MULTIPLEX ARMS

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ABSTRACT

The number of mutations underlining β-thalassemia generate a wide variety of different clinical phenotypes. An understanding of the genotype is important for medical personnel in order to provide proper counseling to patients and their families. Characterization of these mutations should aid the planning of a prenatal diagnosis program for β-thalassemia. The heterogeneity of the mutations makes it difficult and time consuming to identify the mutation in some individuals. We developed a single-tube multiplex amplification refractory mutation system (multiplex ARMS) to identify common ethnic-specific β-thalassemia mutations. Confirmation of multiplex ARMS results was carried out using direct sequencing. Three thousand three hundred twenty two people from Phitsanulok province were screened for the β-thalassemia trait by quantitation of HbA_2 with microcolumn chromatography and the genotypes of mutations were characterized using multiplex ARMS and direct sequencing. We found that the deletion at codons 41/42 (-TCTT) was the most frequent (48%), codon 17 (A→T) (30%), -28 (A→G) (6%) and IVS-I-1(G→T) (6%) were the second and third in frequency respectively. A -87 (C→A) mutation (4%), IVS II-654 (C→T) (2%), codons 71/72 (+A) (2%) and codon 35 (C→A) mutations (2%) were also found. These techniques were found to be a valuable tool for analysis of β-thalassemia mutations because they are accurate, simple, and speedy in operation. The application for the diagnosis of severe thalassemia in high-risk pregnancies is promising.

Keywords: β-thalassemia, multiplex ARMS, Thailand

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1. INTRODUCTION

β-Thalassemia is the most common genetic disorder in Thailand. The prevalence of β-thalassemia gene carriers varies from 1 to 9% [11]. There are more than 170 different β-thalassemia mutations that have been characterized world-wide [13]; they generate a wide variety of different clinical phenotypes. The identification of the genotype is important for providing proper counseling to patients and their families. Characterization of these mutations should aid the planning of a renatal diagnosis program for β-thalassemia.

In Thailand, many molecular techniques have been employed for screening and diagnosis of β-thalassemia. Most of the molecular diagnostic laboratories utilize PCR amplification with allele-specific amplification using an amplification refractory mutation system (ARMS) [5, 9, 12], allele-specific oligonucleotide probes (ASO) [10, 14, 15, 18], or reverse dot-blot hybridization [24, 32] to define the known mutations that cause β-thalassemia. However, for unknown mutations of the β-globin gene, single-strand confirmation polymorphism (SSCP) [4] and direct sequencing [18, 23] may be used. Recently, more sophisticated techniques such as real-time PCR and oligonucleotide microarray analysis have been described for the rapid analysis of thalassemia and hemoglobinopathies; however, these techniques are more laborious and expensive than current screening tests [2].

The ARMS technique is a simple PCR approach for β-thalassemia [1, 20, 26]. The ARMS protocol usually detects one mutation per reaction and can be laborious and expensive to use when looking for many common mutations individually in one sample. Our purpose was to establish a single-tube multiplex ARMS protocol to identify common β- globin gene mutations in Thailand.

More than 40 different β-globin gene mutations have been identified in the Thai population [6 - 8, 10, 18, 19, 22, 25, 29, 30] but only eight [codon 17 (A→T), IVS I-1 (G→T), codons 41/42 (-TCTT), codons 71/72 (+A), IVS I-5 (G→C), IVS II-654 (C→T), -87 (C→A), -28 (A→G)] that account for 99% of β-thalassemia in Thailand. [15, 18, 23, 24, 29]. The multiplex ARMS was developed using two sets of ARMS primers. The first battery of multiplex ARMS has been established to identify the most common β-globin gene mutations including codon 17(A→T), IVS I-1 (G→T), codons 41/42 (-TCTT), and codons 71/72 (+A). The second set of multiplex ARMS was to identify other β-thalassemia mutations: -87 (C→A), -28 (A→G), IVS I-5 (G→C), and IVS II-654 (C→T). Each ARMS primer was designed to produce different amplified fragments that can be easily differentiated in a single-tube reaction. Uncharacterized samples were further analyzed by direct sequencing. Mutations of the β-globin gene in all samples were confirmed by direct sequencing of the β-globin genes.

2. MATERIALS METHODS

2.1 Subjects

We studied 3320 unrelated Thai subjects living in Phisanulok province who attended Thalassemia Research Unit at Naresuan University Hospital, Phisanulok, for thalassemia screening. After informed consent was obtained, 2 mL of peripheral blood samples were collected with EDTA as anticoagulant. All samples were screened for β-thalassemia with microcolumn chromatography on DEAE-Sephadex for quantitation of hemoglobin A2. Fifty out of 3322 samples had HbA_2 concentrations between 3.5% and 9% and were considered β-thalassemia trait. The multiplex ARMS was developed using two sets of ARMS primers, one to identify the most common β-globin gene mutations including codon 17 (A→T), IVS I-1 (G→T), codons 41/42 (-TCTT), and codons71/72 (+A), and the second to identify other β-

thalassemia mutations: -87 (C→A), -28 (A→G), IVS I-5 (G→C), and IVS II-654 (C→T). Each ARMS primer was designed to produce different amplified fragments that easily differentiated the mutations in a single tube reaction.

2.2 Genomic DNA extraction

The samples who had been diagnosed with β-thalassemia were further extracted the DNA with Chelex-100 method [27]. Briefly, 30 µl of EDTA blood was added to 1 ml of 0.5% Triton X-100, mixed and centrifuged at 12 000 *g* for 1 min. the supernatant was removed and the pellet was washed with 1 ml of sterile water. Five per cent chelex-100 suspension was added on top of the pellet, followed by 110 µl of deionized sterile water. The solution was incubated at 56°C for at least 2 hours and boiled for 10 minutes. The extracted genomic DNA was used as a template for multiplex ARMS and kept at 4°C until β-globin gene analysis.

2.3 Primer design

Sixteen primers were designed according to the ARMS protocol for β-thalassemia mutations commonly found in Thai populations [5, 12, 28]. Primers for -87 (C→A) and codons 71/72 were designed using a computer program, www.PrimerBiosoft.com. The genomic sequence of the β-globin gene was obtained from the GenBank database under the name HUMHBB, accession number U01317. The primer sequences were synthesized from Qiagen (Qiagen GmbH, Germany) and are shown in Figure 1 and Table 1.

Table 1: *Primer sequences for the multiplex ARMS set I and II* **.**

Primer	Nucleotide sequence (5'→3')	Position[a]	Product size (bp)
ARMS Set I			
1A	CAATGTATCATGCCTCTTTGCACC	63224-63247	861
1B	GAGTCAAGGCTGAGAGATGCAGGA	64061-64084	
1C	ACCTCACCCTGTGGAGCCAC	62028-62047	
Codon 17	CTCACCACCAACTTCAGCCACGTTCAGCTA	62238-62267	239
IVS I-1	TTAAACCTGTCTTGTAACCTTGATACCGAA	62279-62308	281
Codons 41/42	GAGTGACAGATCCCCAAAGGACTCAACCT	62441-62470	439
Codon 71/72[b]	AGGTTGTCCAGGTGAGCCAGGCCATCAATT	62533-62562	535
ARMS Set II			
C	AGTGCTGCAAGAAGAACAACTACC	35677-35700	326
D	CTCTGCATCATGGGCAGTGAGCTC	35981-36004	
F	CCCCTTCCTATGACATGAACTTAA	62680-62703	
G	ACCTCACCCTGTGGAGCCAC	62028-62047	
H	GAGTCAAGGCTGAGAGATGCAGGA	64061-64084	
-87[b]	CACTTAGACCTCACCCTGTGGAGCCACACA	62021-62050	683
-28	AGGGAGGGCAGGAGCCAGGGCTGGGCTTAG	62080-62109	624
IVS I-5	CTCCTTAAACCTGTCTTGTAACCTTGTTAG	62312-62283	285
IVS II-654	GAATAACAGTGATAATTTCTGGGTTAACGT	63256-63285	829

[a]Position according to the GenBank accession number U01317.[b]These sequences were designed by the software from www.PrimerBiosoft.com

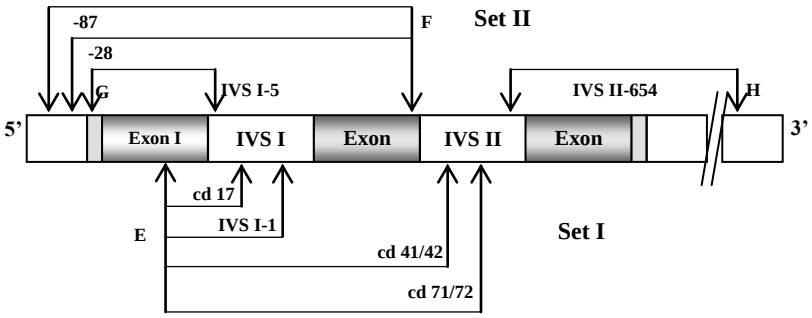

Fig. 1: *Schematic diagram showing the β-globin genes and eight PCR fragments amplified for the multiplex ARMS assay. The β -globin gene is composed of three exons (grey boxes) and two introns (white boxes). Set I was the multiplex ARMS to identify the most common β -globin gene mutations [codon 17 (A→T), IVS I-1 (G→T), codons 41/42 (-TCTT), and codons 71/72 (+A)] and set II was the multiplex ARMS to identify other β -globin gene mutations [IVS I-5 (G→C), IVS II-654 (C→T), -87 (C→A), and -28 (A→G)] found in Thai population. Arrows indicated 5'→3' directions of the primer pair to identify each β -globin gene mutation.*

These primers were coupled with one of the common primers depending on the location of the mutation in the β-globin gene and the direction of the allele-specific primer (Fig.1). A pair of control primers was also used as internal control for the PCR for all the mutations (A and B for the multiplex ARMS set I, and C and D for set II). In set I, [codon 17 (A→T), IVS I-1 (G→T), codons 41/42 (-TCTT), and codons 71/72 (+A)], the four most common mutation, were amplified in a single reaction with the common primer E. For set II, oligonucloetide primers were designed for four mutations, namely, -87 (C→A), -28 (A→G), IVS I-5 (G→C), and IVS II-654 (C→T), were amplified in a single reaction with the common primer F, G, and H. These eight mutations account for 99% of mutations in Thailand.

2.4 Multiplex amplification REFRACTORY mutation system (Multiplex ARMS)

Each sample was initially screened for the most common mutations [codon 17 (A→T), IVS I-1 (G→T), codons 41/42 (-TCTT), and codons 71/72 (+A)]. If no mutations was detected, the sample was analyzed for other mutations reported in Thais [IVS I-5 (G→C), IVS II-654 (C→T), -87 (C→A), and -28 (A→G)]. The multiplex ARMS set I was carried out in a 25-μl reaction containing 1X PCR buffer, 3.0 mM $MgCl_2$, 2.5 U HotStarTaq Polymerase (Qiagen GmbH, Germay), 200 μM dNTPs, 200 pmol of each primer set, and 250 ng template DNA. After incubation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 7 min. For the other β□-thalassemia mutations the multiplex ARMS set II was performed with the same condition, except the primers set II was used instead. The PCR products were analyzed by eletrophoresis on a 3% agarose gel containing ethidium bromide and were visualized by ultraviolet illumination. ARMS negative alleles were subjected to nucleotide sequencing of the PCR product.

2.5 Direct DNA sequencing

Two segments of the β-globin gene that covered all mutations previously characterized in Thai

population were amplified with the primers previously described [23] The primers sequences are shown in Figure and table 2.

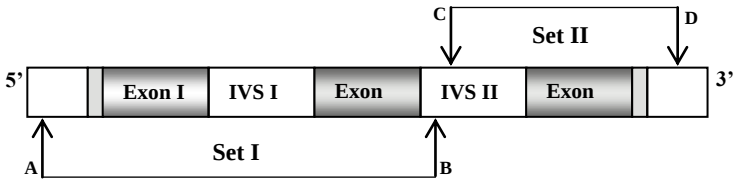

Fig. 2: *Schematic diagram showing the β -globin genes and two fragments amplified for the direct DNA sequencing. The β -globin gene is composed of three exons (grey boxes) and two introns (white boxes). Set I represents the primer pair that amplified exon I and II region produced the fragments amplified sizes 760 base pairs (bp). Set II represents the primer pair that amplified exon III region produced the fragments amplified sizes 690 bp.*

Table 2: *The sequences of primers used for the direct DNA sequencing.*

Primer	Nucleotide sequence (5'→3')	Position*	Product size (bp)
A	AGAAGAGCCAAGGACAGGTACG	61991-61990	760
B	TGCAATCATTCGTCTGTTTCCC	62730-62751	
C	TCCCTAATCTCTTTCTTTCAGG	63190-63211	660
D	TTTTCCCAAGGTTTGAACTAGC	63829-63849	

**position according to the GenBank accession number U01317.*

The reaction was carried out on a 25 µl reaction mixture containing 1X PCR buffer, 2.5 mM $MgCl_2$, 1 U HotStarTaq Polymerase (Qiagen, Germany), 200 µM dNTPs, 400 pmol primers, and 250 ng template DNA. The thermal cycling of set I consists of heating the reaction at 95^{o}C for 15 min and 40 cycles of denaturation at 94^{o}C for 45 sec, primer annealing at 60^{o}C for 1 min and DNA extension reaction at 72^{o}C for 2 min. The set II was performed in the same condition except the concentration of 1.5 mM $MgCl_2$, and 500 pmol primers. After heating the set II reaction at 95^{o}C for 15 min, 35 cycles were performed with denaturation for 45 sec at 94^{o}C, annealing for 1 min at 55^{o}C, and elongation for 1.30 min at 72^{o}C.

Following gel electrophoresis confirming successful amplification of the correct size product, the remaining PCR product was purified using a QIAquick PCR Purification Kit (Qiagen, Germany). Purified PCR products were sequenced using an ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit protocol (Applied BioSystems, USA). Briefly, to a 0.2 ml PCR tube 5-20 ng of DNA, 3.2 pmol of sequencing primer, 8 µl Terminator Ready Reaction Mix was added and made up to a final volume of 10 µl with deionized distilled water. The sequencing products were cycled in a thermal cycler for 25 cycles of 96^{o}C for 10 sec, 50^{o}C for 5 sec and 60^{o}C for 4 min.

The extension products were precipitated using ethanol and sodium acetate. To the sequencing reaction was added 2 µl of 3M sodium acetate, pH 4.6, 50 µl of 95% ethanol and left at room temperature for $\geq$ 15 min but not overnight. Samples were centrifuged at 12 000 *g* for 20 min and the supernatant removed and discarded. To the pellet, 250 µl of 70% ethanol was added and briefly vortexed. After centrifugation for 5 min at 12 000 *g*, the supernatant was dried for 1 min at 90^{o}C with a heat block

Samples were analyzed on an automated sequencer, ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) as per the manufacturer's instructions.

3. RESULTS

We have developed the multiplex ARMS to identify the eight most common β-thalassemia mutations in Thai population and standardized on DNA samples with known mutation, the pattern was shown in Figure 3. The method was subsequently tested on 50 unrelated samples with unknown β-thalassemia mutations (Fig. 4). An internal control bands were also amplified in all the reactions, indicating successful PCR. No band other than the internal control was amplified in the negative control reaction, which was carried out with a blood normal for β-thalassemia (Fig. 4). The codons 41/42 (-TCTT) mutation is the most common β-thalassemia defect in Thai population. The following mutations are presented in decreasing frequency: codon17 (A→T), -28 (A→G), IVS I-1 (G→T), -87 (C→A), codons 71/72 (+A), IVS II-654 (C→T), and codon 35 (C→A) (Table 3).

Table 3: *Distribution of β-thalassemia mutations (n=50) detected by multiplex ARMS and sequencing techniques.*

Mutation	ARMS	Sequencing
Codons 41/42 (-TCTT)	24 (48)	24 (48)
Codon 17 (C→T)	15 (30)	15 (30)
-28 (A→G)	3 (6)	3 (6)
IVS1-1 (G→T)	3 (6)	3 (6)
-87 (C→A)	2 (4)	2 (4)
Codons 71/72 (+A)	1 (2)	1 (2)
IVS II-654 (C→T)	1 (2)	1 (2)
Codon 35 (C→A)	0	1 (2)
Uncharacterized	1	0
Chromosomes	50	50

Only mutations that we detected are described. Numbers indicating gene frequencies and percentage of each mutation are given in parentheses.

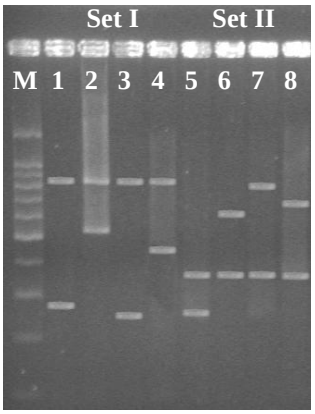

Fig. 3: *Multiplex ARMS patterns of eight common β-globin gene mutations in a 3% agarose gel. Lane 1-4, 861-bp an internal control for multiplex ARMS set I; lane 1, 281-bp of IVS I-I (G→T); lane 2, 535-bp of codons 71/72 (+A); lane 3, 241-bp of codon 17 (A→T); lane 4, 454-bp of codons 41/42 (-TCTT). Lane 5-8, 326-bp an internal control for multiplex ARMS set II; lane 5, 258-bp of IVS I-5 (G→C); lane 6, 624-bp of -28 (A→G); lane 7, 829-bp of IVS II-654 (C→T); lane 8, 683-bp of -87 (C→A); M (marker), 100 base pairs DNA ladder (Promega, USA).*

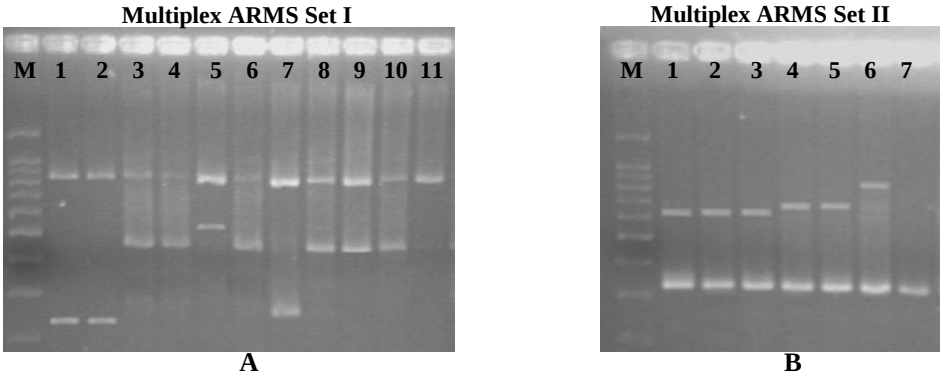

Fig. 4: *Ethidium bromide stained photograph of a 3% agarose gel showing the multiplex ARMS for the most common β-thalassemia mutations found in Thai population. (**A**) The multiplex ARMS set I ; M (marker), 100 base pairs DNA ladder (Promega, USA); the 861-bp band is the β-globin fragment used as an internal control. Lane 1 and 2 were the codon 17 (A→T) (241-bp); lane 3, 4, 6, 8, 9, and 10 were the codons 41/42 (-TCTT) (454-bp); lane 5 was the codons 71/72 (+A) (535-bp); lane 7 was the IVS I-I (G→T) (281-bp); lane 11 was the negative control. (**B**) The multiplex ARMS set II; M (marker), 100 base pairs DNA ladder; the 326-bp band is the ^{G}β-globin fragment used as an internal control. Lane 1, 2 and 3 were -28 (A→G) (624-bp); lane 4 and 5 were -87 (C→A) (683-bp); lane 6 was IVS II-654 (C→T) (829-bp); lane 7 is the negative control.*

Figure 5 shows the sequencing of the β-thalassemia mutations found in Phitsanulok. We were unable to determine the mutation in one sample with multiplex ARMS. Genomic sequencing revealed this mutation was codon 35 (C→A) (Figure 5F). The primer for this mutation was not used in our multiplex ARMS condition because this mutation was less common in the north.

Thus by using the multiplex ARMS along with sequencing, it is possible to detect all of the mutations. The strategy presented here for characterization of unknown mutations is quite simple and cost effective.

4. DISCUSSION

β-Thalassemia is the most common genetic disorder in Thailand. A previous report investigated the distribution of β-thalassemia mutations in this area based on an analysis of a fairly small number of subjects [5]. In the present study we describe a wider spectrum of β-thalassemia mutations in Phisanulok by analyzing 50 β-globin chromosomes. Eight different β-thalassemia mutations were identified in our study accounting for more than 99% of all the β-thalassemia chromosomes. These included four of the common Thai mutations: codons 41/42, codon 17, IVS I-1, and codons 71/72. In the current study, 24 chromosomes were found carrying the codons 41/42 mutation, representing 48% of the total Thai β-thalassemic chromosomes. When compared to other areas of Thailand, the distribution and types of β-thalassemia mutations in Phitsanulok were found to be generally similar; a small number of mutations predominated and the most common ones were geographically the most widespread and theoretically the oldest.

The most common mutation was the codons 41/42 and this was the same as previous findings observed in other parts of Thailand [10, 12, 14, 15, 18, 23, 24, 28, 31, 32]. The second most common mutation was codon 17, similar to the central region [31, 32], the northern region [23] and the northeastern region [10, 12], whereas IVS I-5 was the second most common mutation observed in the southern region [15, 18]. The frequency of IVS I-1 was similar to the northern

region [23], and -28 was similar to the central region [31], whereas codon 19 was the third most common mutation in the soutern region [15, 18, 31]. Furthermore, the mutations -87, codons 71/72, and IVS II-654 were similar to the north region [23], while codon 35 was similar to the central region [31, 32].

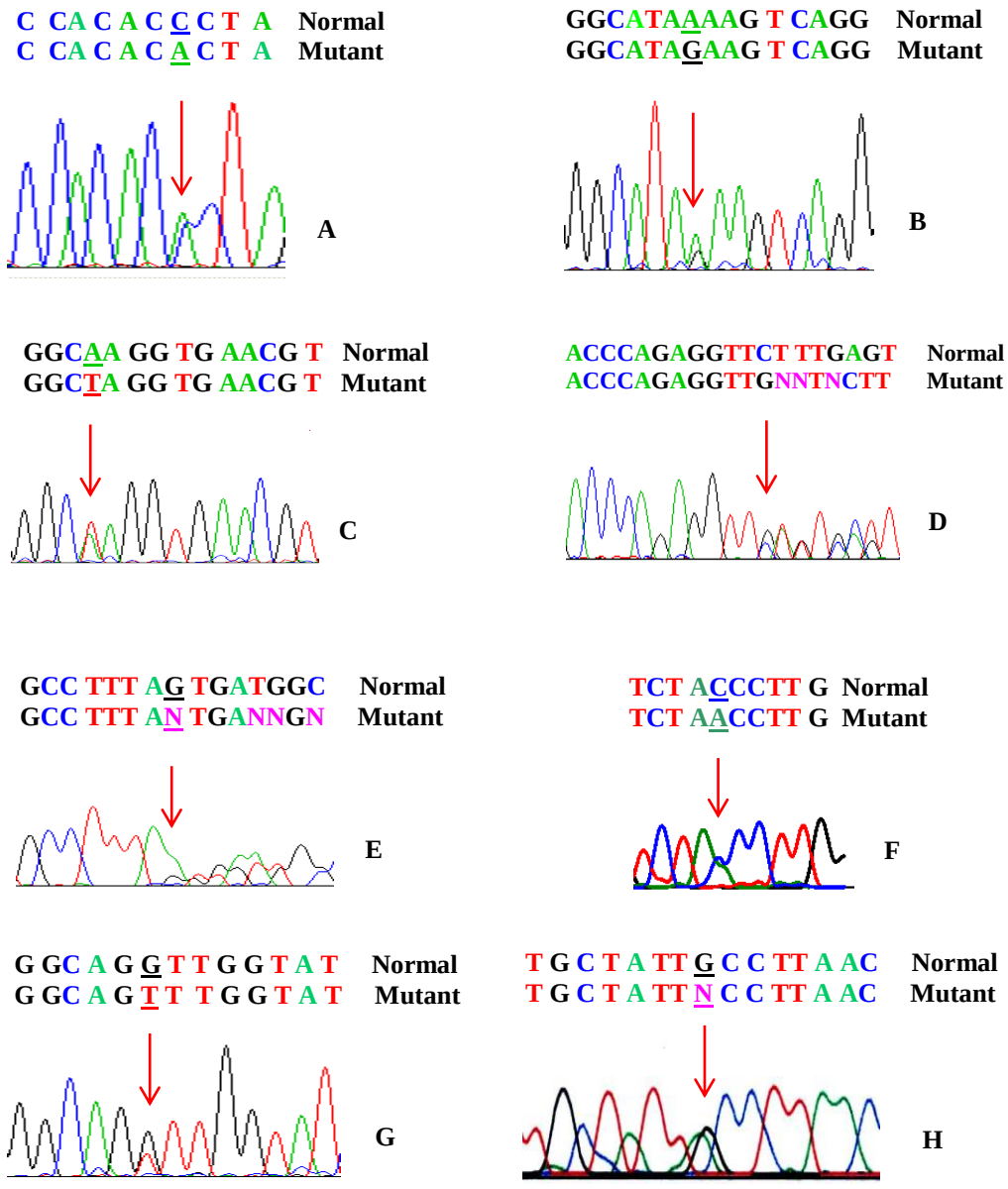

Fig. 5: *Nucleotide sequence chromatograms of the β-globin genes. (A) The forward sequence of a substitution in -87 (C→A); (B) The forward sequence of a substitution in -28 (A→G); (**C**) The forward sequence of a substitution in codon 17 (A→T); (**D**) The forward sequence of a 4 bp deletion of codons 41/42 (-TCTT); (**E**) The forward sequence of a addition in codons 71/72 (+A); (**F**) The forward sequence of a substitution in codon 35 (C→A); (**G**) The forward sequence of a substitution in IVS I-1 (G→T); (**H**) The reverse complementary sequence of a substitution in IVS II-654 (C→T).*

When the percentage of the eight most common β-thalassemia mutations in the population of Phitsanulok is compared with the overall frequency of mutations in the country, some interesting differences are revealed. Increased percentage of the mutations -28, -87, and decreased percentage of codons 71/72 were observed when compared to the northern region [23]. The IVS I-5 mutation which is frequent in the central and southern regions was absent in the samples from Phitsanulok.

Our aim was to establish multiplex ARMS to detect eight common β-thalassemia mutations in Thailand since this technique reduces the overall cost and time. Multiplex ARMS relies on the principle that oligonucleotides with mismatched terminal 3'-nucleotides will not function as primers in a PCR under appropriate conditions. As a result, DNA amplification does not occur.

We found that the most important factor for reliable and accurate results using multiplex ARMS was the primer concentration. Polymerase chain reactions were carried out in 25 □l volumes with primer concentrations of not more than 20 pmol. An increase in the primer concentrations led to non-specific DNA amplifications of the same molecular weight, although these non-specific DNA bands were less distinct compared with the specific amplified bands.

The PCR conditions for denaturation (95^{o}C), annealing (65^{o}C), and synthesis (72^{o}C) were the same for all the mutations studied. Detection of amplified DNA in our multiplex ARMS protocol was clearly observed on 3 per cent agarose gel and there was no need to use the more expensive NuSieve agarose gels. In addition, we were able to lower the concentration of enzyme Taq polymerase to 0.5 U per PCR. The cost per analysis using multiplex ARMS compared with sequencing is also greatly reduced.

The ARMS technique used in this study allowed precise identification of the mutations in all samples making detection easy, straightforward, and less time consuming with 100% accuracy in samples. The use of the multiplex ARMS technique saved both time and cost with 100% accuracy. From an economical point of view, reduced labor time and using simpler equipment decrease the cost of test per sample by multiplex ARMS.

In Thailand, with its vast spectrum of β-thalassemia mutations, a procedure such as multiplex ARMS is much more efficient and practical than single ARMS because many β-thalassemia mutations can be simultaneously screened on a single reaction in a reasonably short time. This number can be increased by designing a second set to be run in parallel, i.e. 10 mutations on 2 reactions.

Multiplex ARMS and sequencing would provide rapid screening for β-thalassemia mutations in Thailand. The multiplex ARMS protocol described in this study has provided an accurate, rapid, inexpensive, and direct diagnosis technique for β-thalassemia.

5. CONCLUSION

The identification of the spectrum of β-thalassemia mutations in our region is necessary for population carrier screening and for accurate prenatal diagnosis. Furthermore, the detailed distribution tables of β-thalassemia mutations are essential for counseling and extraction of genetic diversity estimates for similar studies in other inherited disorders.

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