



Naked Eye Detection of α^0 Thalassemia --^{SEA} Type using Hydroxynaphthol Blue Loop-mediated Isothermal Amplification

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Abstract

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BACKGROUND: Alpha-thalassemia is a major health problem for worldwide populations due to its recent large-scale global movements to many other parts of the world. The most effective method to prevent the spread of the worst form of α -thalassemia disorder, hemoglobin Bart's hydrops fetalis, is to identify the genotypes of an α^0 thalassemia carrier and then conduct subsequent genetic counseling.

AIM: This research was aimed to develop a new method for the detection of α^0 thalassemia (Southeast Asia or SEA) --^{SEA} type using hydroxynaphthol blue loop-mediated isothermal amplification (HNB-LAMP) and by visually reading results using the naked eye.

METHODS: This experimental research was conducted at Rangsit University, Pathum Thani Provinces, Thailand, from 2016 to 2018. Two sets of LAMP primers were used, one set of primer was used to detect the two existing α genes and the other set used in the detection of two α genes deletion --^{SEA} types which were designed based on the nucleotide sequence of two α genes deletion from four cases of the α^0 thalassemia carrier --^{SEA} type. HNB-LAMP was developed, and then, 77 cases from subjects were evaluated by comparing them with a conventional polymerase chain reaction (PCR).

RESULTS: The newly developed HNB-LAMP shows a high diagnostic sensitivity and diagnostic specificity, 100% in correct interpretation, from 10 cases of α^0 thalassemia carriers --^{SEA} type, 29 cases of α^+ thalassemia carrier -- $\alpha^{3.7}$ type, 8 cases of hemoglobin constant spring/hemoglobin Pakse' (HbCS/HbPS), and 30 standard cases.

CONCLUSION: This newly developed HNB-LAMP is suitable for in the field applications for detecting the presence of α^0 thalassemia --^{SEA} type due to its high diagnostic sensitivity, high diagnostic specificity, and, most notably, the ability to be detected with the naked eye.

Introduction

Alpha-thalassemia, one of the most common human genetic disorders, is a major health problem in the Mediterranean area, the Middle East, the Indian subcontinent, Africa, and Southeast Asia including Thailand. The common α^0 thalassemia deletions are --^{SEA} type in Southeast Asia and --^{MED} type in the Mediterranean, the data suggest that approximately 5% of the population worldwide is effected [1]. Although the highest frequency of α^0 thalassemia --^{SEA} type was found in tropical regions, particularly North Thailand (estimates as high as 14–15% [2], [3]), the large-scale global population movements in recent decades have led to the spreading of α -thalassemia to many other parts of the world including North Europe and North America [1], [4]. In one program, 406 cases of HbH disease and five cases of hemoglobin Bart's hydrops fetalis were reported in California, USA, after universal screening for α -thalassemia was conducted in 1998 [5]. The interaction between both parents with α^0 thalassemia carrier genes results in the most

severe form called hemoglobin Bart's hydrops fetalis which is lethal either *in utero* or soon after birth [6], [7]. The most effective method to prevent the spread of α -thalassemia disorders is to identify genotypes and perform subsequent genetic counseling.

Standard methods for genotyping of α -thalassemia using multiplex PCR have already been developed [8]. However, the limitation of PCR is that it requires advanced equipment, the high cost of reagents, well-trained technician, and tedious laboratory work. After a relatively new gene amplification technique, LAMP, was successfully devised by Notomi *et al.* [9], this versatile technique is now widely used in all fields of science and medicine with this detection method using turbidity, agarose gel electrophoresis, and colorimetric assay using the naked eye and ultraviolet (UV) light. LAMP meets all of the criteria for an ideal diagnostic test because of its sensitivity, specificity, low cost, simplicity, speed, adaptability to all kinds of climatic changes, and the availability of instruments used, as suggested by the World Health Organization [10] in a report of their findings. The previous study reported the application of LAMP for the detection of α^0 thalassemia

carrier --^{SEA} type using different intercalating dyes such as pH-sensitive dyes [11]. Based on the advantage of using HNB as a DNA intercalating dye addition before amplification and colorimetric using naked eye detection [12], we tried to develop the simplest method, HNB-LAMP, for the detection of the α^0 thalassemia carrier --^{SEA} type.

Materials and Methods

Blood sample collection

This experimental research was conducted at the Faculty of Medical Technology, Rangsit University, Pathum Thani Province, Thailand, from 2016 to 2018 and approved by the Institutional Ethics Committee of the Research Ethics Office of Rangsit University (No. 39/2558) in accordance with the Helsinki Declaration. Five milliliters of EDTA blood samples were collected from 77 volunteers after they had completed their written forms of consent. The participants of this research were medical technology students who have studied at the Faculty of Medical Technology, Rangsit University with no restrictions to sex, age, or ethnicity.

Genomic DNA preparation

Genomic DNA was extracted from the white blood cells of 77 subjects by utilizing the salting out and ethanol precipitation method [13]. The optical densities (OD) at 260 and 280 nm were measured to calculate the concentrations and the purity of DNA. The DNA (OD₂₆₀/OD₂₈₀) with purity higher than 1.8 was further subjected to genotyping of α^0 thalassemia --^{SEA} type, α^+ thalassemia - $\alpha^{3.7}$ type, and the stop codon mutation of the α -2 globin gene called HbCS/HbPS.

Alpha-thalassemia genotyping

The duplex gap-PCR for α^0 thalassemia --^{SEA} type and gap-PCR used for the detection of α^+ thalassemia - $\alpha^{3.7}$ type were modified as previously described [14], [15]. The PCR mixture was performed according to the manufacturer's instructions (Finnzymes, Finland). PCR for α^0 thalassemia --^{SEA} type was performed in a Biometra model T personal thermocycler (Technique, USA) with the following profile: Initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 10 s; annealing at 62°C for 30 s; extended at 72°C for 30 s, and a final extension at 72°C for 10 min. PCR for α^+ thalassemia - $\alpha^{3.7}$ type was conducted as mentioned above with this exception: Initial denaturation at 98°C for 3 min, followed by 25 cycles of denaturation at 98°C for 10 s; annealing and extension at 72°C for 1.30 min with an

additional extension at 72°C for 10 min. PCR-restriction fragment length polymorphism (RFLP) was used in the detection of HbCS/HbPS as described elsewhere [16]. Amplified products of α^0 thalassemia --^{SEA} type and α^+ thalassemia - $\alpha^{3.7}$ type were electrophoresed in 1.5% agarose gel, while the RFLP product of HbCS/HbPS was run through 3% NuSieve agarose gel, staining with RedSafe (iNtRON, Korea), and then visualized under UV light.

DNA sequencing and LAMP primers design

Four PCR products that tested positive for α^0 thalassemia --^{SEA} type were sent to a commercial company for DNA sequencing (Bioneer Corporation, Korea). Two sets of LAMP primers were designed by PrimerExplorer public software [17] based on the nucleotide sequence of α gene deletion.

LAMP and HNB-LAMP

LAMP reaction mixture was made according to the manufacturer's instructions (New England Biolabs, USA). Reaction tubes for the detection of two α genes were made in 25 μ L amounts containing 1x ThermoPol Buffer, various 6–10 mM MgSO₄, 1.4 mM each dNTP (Promega, USA), 0.2 μ M each F3N and B3N, 1.6 μ M each FIPN and BIPN, 8 units of the large fragment *Bst* DNA polymerase, and 100 μ g of genomic DNA. Reaction tubes of --^{SEA} type contained the same reagents as above with the exception of 0.2 μ M of F3D and B3D and 1.6 μ M each for FIPD and BIPD. LAMP mixtures were then incubated in a 65°C water bath at various times at 15, 30, 45, 60, and 90 min. The reaction was then stopped at 80°C for 20 min. LAMP product was later analyzed with a 2% agarose electrophoresis stain using RedSafe.

Naked eye detection of HNB-LAMP was modified from the previously described method [12], [18]. The LAMP mixture mentioned above was added to 120 μ M HNB (Merck, Germany), then incubated and the reaction was halted as previously mentioned. This HNB-LAMP product was observed using the naked eye to look for the color change from violet to blue indicating a positive reaction.

Results

Genotyping, sequencing of DNA of α -thalassemia and design of LAMP primer

Here, we studied 77 subjects, of which 16 cases were male and 61 cases were female. All of them were Thai people and the average age was within 21.7 \pm 1.2 years of age (range 19–26 years old).

DNA samples used in this research were genotyped as 10 cases of α^0 thalassemia --^{SEA} type, 29 cases of α^+ thalassemia - $\alpha^{3.7}$ type, 8 cases of HbCS/HbPS, and 30 standard cases (data not shown).

After analyzing the DNA sequences from 4 cases of α^0 thalassemia --^{SEA} type, 19,624 bp gene deletions were found starting from the location 135,416–155,039 of GenBank accession number DQ431198 (data not shown).

Two sets of primer were used, including one set of primer used for detecting two existing α genes and the other set used for the detection of two α genes --^{SEA} type deletions which were successfully designed based-on the nucleotide sequences from our DNA sequencing (Table 1).

Titration of LAMP

After analyzing LAMP reactions at various times, the optimal time for the incubation period in the LAMP reaction was found to be 60 min (Figure 1). From the various concentration of Mg²⁺ as 4, 6, 8, and 10 mM, the optimal concentration of Mg²⁺ of LAMP reaction was found to be 8 mM, as shown in Figure 2.

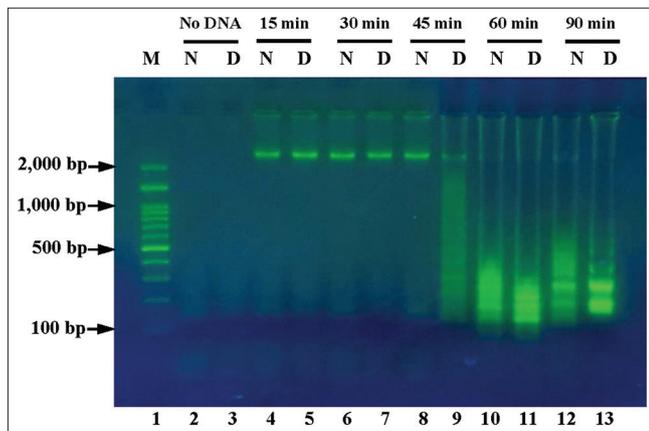


Figure 1: Time titration of LAMP

HNB-LAMP

A technique for using naked eye detection with HNB-LAMP was successfully developed. The positive reaction tubes (blue color) were found in both normal ($\alpha\alpha/\alpha\alpha$) in lane 4 (Figure 3) and α^0 thalassemia --^{SEA}

type (--^{SEA} / $\alpha\alpha$) in lane 6 (Figure 3), using normal primer sets (F3N, FIPN, B3N, and BIPN). This positive reaction represented the existence of two α genes in both normal and α^0 thalassemia --^{SEA} types. When using deletion primer sets (F3D, FIPD, B3D, and BIPD), the positive reaction

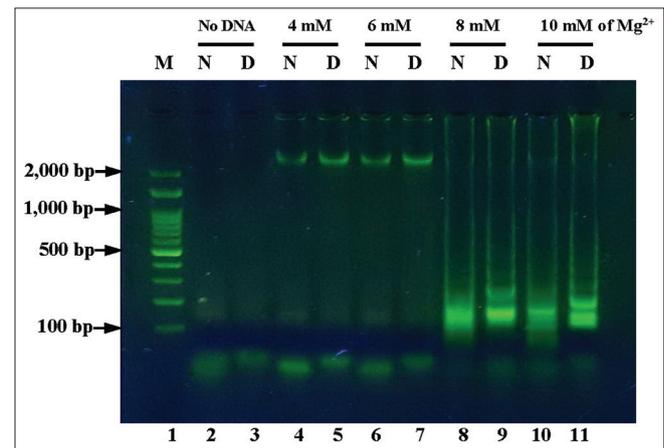


Figure 2: Magnesium concentration titration of LAMP

tube was only found in α^0 thalassemia --^{SEA} type (lane 7 of Figure 3) representing the deletion of two α gene --^{SEA} types. These results indicated that the pre-addition of 120 μ M of HNB did not interfere with the DNA amplification of the large fragment *Bst* DNA polymerase.

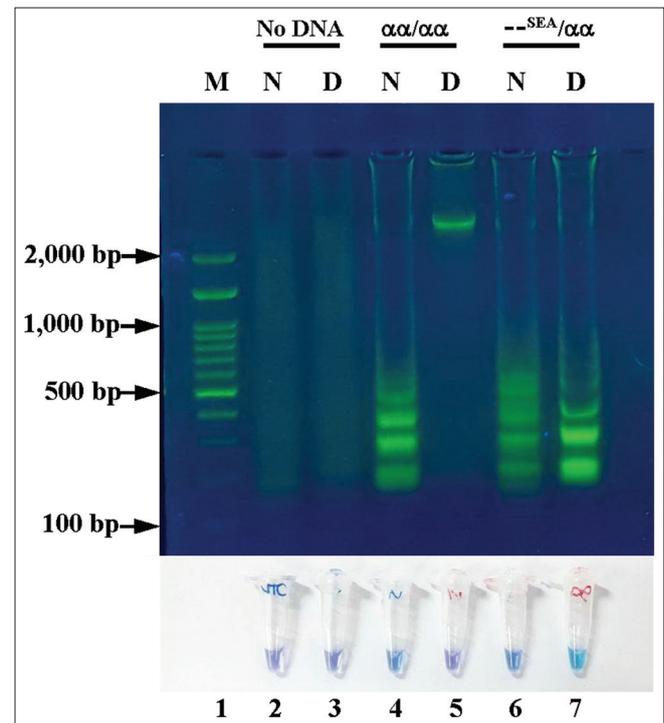


Figure 3: Detection of 2 α genes deletion SEA type by HNB-LAMP

Table 1: Sequences of primers for LAMP genotyping of α^0 thalassemia --^{SEA} type

Primers	Nucleotide sequences	Location on chromosome 16
Normal forward outer (F3N)	5'-CGATCTGGGCTCTGTGTTCT-3'	135,276-135,295
Normal forward inner (FIPN)	5'-ACAGTGGGGACGACCGAGTAGGGGAGAAGCTGAGTGATG-3'	135,353-135,371/135,313-135,332
Normal backward outer (B3N)	5'-AAGGACCCAGGAGACGC-3'	135,477-135,494
Normal backward inner (BIPN)	5'-GGTTCACTTGGGGACGCCGACTGCTCCGCTCCA-3'	135,385-135,403/135,447-135,462
Deletion forward outer (F3D)	5'-CGATCTGGGCTCTGTGTTCT-3'	135,276-135,294
Deletion forward inner (FIPD)	5'-GGACGACCGAGTTCTGCGAGAGGGAAGGAGGGAGAAG-3'	135,345-135,364/135,304-135,322
Deletion backward outer (B3D)	5'-TGGAGTGCAGTGTGTAGTC-3'	155,101-155,120
Deletion backward inner (BIPD)	5'-GACGCCCTGGGGAGGTTACAGCCTTGAACCTCCTGGACTT-3'	135,398-135,415, 155,040-155,041/155,069-155,088

Accession number of chromosome 16 is DQ431198.

Detection of α^0 thalassemia --^{SEA} type by HNB-LAMP

All of the 10 cases of α^0 thalassemia --^{SEA} type were correctly detected by HNB-LAMP when compared with duplex gap-PCR. There were zero misinterpretations of the existing two α genes when analyzing all 67 cases including the 29 cases of α^+ thalassemia - $\alpha^{3.7}$ type, all 8 cases of HbCS/HbPS, and the 30 standard cases (Table 2). The results indicated 100% accuracy in diagnostic sensitivity and specificity of HNB-LAMP for the detection of two α genes deletion --^{SEA} types.

Table 2: Diagnostic sensitivity and diagnostic specificity of HNB-LAMP comparison with conventional PCR

HNB-LAMP for -- ^{SEA} type	Duplex gap-PCR for α^0 thalassemia -- ^{SEA} type		
	Positive	Negative	Total
Positive	10	0	10
Negative	0	67	67
Total	10	67	77
Sensitivity	10/10	=100%	
Specificity	67/67	=100%	

Discussion

A crucial step for the development of the naked eye detection method, HNB-LAMP, was the primer designed using a DNA template based on the DNA sequence of α^0 thalassemia --^{SEA} type subjects. This study was the first to report the exact SEA gene deletion as 19,624 bp.

We successfully designed two sets of LAMP primers, whereas the previous study only reported one set of primer used in the detection of two α genes deletion --^{SEA} type [11]. The similarities in the sequence of current primers (F3D and B3D) with the previously reported primer [11] may be due to its design using the same web-based free software, however, different DNA templates were used.

The titration steps for LAMP reaction as well as the optimal incubation time and Mg^{2+} concentrations from this study were comparative with the previous report [12].

The previous studies reported the use of DNA intercalating dyes including SYBR green [19], [20], PicoGreen [21], [22], and propidium iodide [20] added to the solution after amplification. Because of the increased risk of contamination from other subsequent LAMP reaction solutions when opening the reaction tubes, the advantage of HNB-LAMP over other methods is the reduction of amplicon aerosol contamination when detecting with the naked eye in normal room light. The other DNA intercalating dyes in addition to the pre-reaction including calcein [23], malachite green [24], [25], neutral red, and phenol red [26], [27] may be optimized for visual detection. However, calcein requires the inclusion of manganese that may inhibit

the polymerase reaction. Furthermore, malachite green also appeared to inhibit the amplification of the genome exponential amplification reaction (GEAR) assay [24]. Moreover, neutral red and phenol red, which are pH sensitive indicators, may be limited because they require optimal pH for the best results.

The sample size of this research was relatively small when compared to the requirements for implementation in a clinical setting. Therefore, this novel approach in using HNB-LAMP should be further investigated using a higher sample base.

Conclusion

The identification of α^0 thalassemia --^{SEA} type in populations is necessary for the prevention of Hb Bart's hydrops fetalis. The newly developed HNB-LAMP demonstrated in this study was shown to have a significant advantage in the detection of α^0 thalassemia --^{SEA} type. Some of the many advantages of this method include naked eye detection, high diagnostic sensitivity, high diagnostic specificity, and not requiring the use of a PCR thermal cyclers. Therefore, it was suitable for use in field applications and rural low-income areas.

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