

Establishment and Evaluation of a Novel Method Based on Loop-Mediated Isothermal Amplification for the Rapid Diagnosis of Thalassemia Genes

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Purpose: Currently, thalassemia is commonly detected using gap-polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) reverse dot blot, which have high requirements of space, instruments, and personnel. Therefore, it is necessary to develop a new method for thalassemia detection with high sensitivity, low cost, and simple and fast operation. In this study, we aimed to design and evaluate a new method for detecting three α -thalassemia genes including –Southeast Asian (SEA), $-\alpha 3.7$, and $-\alpha 4.2$ and five β -thalassemia genes including 654M, 41/42M, $-28M$, 17M, and 27/28M based on loop-mediated isothermal amplification (LAMP).

Methods: Primer sequences were designed using Primer Explorer V4 software. Blood samples (5 mL) were collected from all participants in EDTA. DNA was extracted using Chelex 100 and was subjected to LAMP. LAMP products were detected by fluorescence development in ultraviolet light.

Results: We found that LAMP assays for positive samples of thalassemia reached a plateau before 60 minutes, whereas the negative control samples entered the plateau after 70 minutes or showed no amplification. The concentration range of positive reactions was between 20–60 pg/ μ L and 20–60 ng/ μ L. Additionally, there were no cross-reactivities among 8 thalassemia subtypes. For clinical samples, the positive sample tube showed strong green fluorescence, whereas the negative tube showed light green fluorescence. According to these results, the LAMP method has high sensitivity for detecting thalassemia (252/254). However, 43 false-positive results were obtained in the LAMP test. The LAMP assay was also of low cost and with simple and fast operation.

Conclusion: The novel LAMP assay can be completed within 60 min using a heating block or a water bath, and the result can be read visually based on color change to detect thalassemia. The LAMP assay fulfills the requirements of field application and resource-limited areas, especially those with primary hospitals and rural areas.

Keywords: loop-mediated isothermal amplification, diagnosis, thalassemia genes, fast, low cost

Introduction

Thalassemia is one of the most common hereditary blood diseases that pose a serious threat to human health with α - and β -thalassemia being the most common.¹ The most effective way to control thalassemia is to prevent the birth of severely ill children; thus, rapid and accurate screening for thalassemia is the key to its prevention and control. At present, the deletions of α -thalassemia are detected by gap-polymerase chain reaction (PCR) and α - and β -thalassemia mutations are

detected by DNA reverse dot blot (RDB).² These methods have high requirements for space, instruments, and personnel, and it is difficult to promote their use in primary hospitals and rural areas.³ Therefore, it is necessary to develop a new method for thalassemia detection with high sensitivity, low cost, and simple and rapid operation.

Loop-mediated isothermal amplification (LAMP) is a novel method for nucleic acid amplification. It is performed by designing 4–6 specific primers for 6 regions of the target gene, and using *Bacillus stearothermophilus* (Bst) deoxyribonucleic acid (DNA) polymerase to catalyze the nucleic acid amplification reaction for 30–60 min under isothermal conditions (60–65°C).⁴ Compared to traditional PCR, LAMP does not require a special PCR instrument, and does not need detection by electrophoresis. The complete experiment only needs a constant temperature water bath or metal bath. The results can be directly observed under a UV lamp with the naked eye to judge the green fluorescence emitted by Calcein.⁵ Therefore, compared with other nucleic acid amplification methods, LAMP has the advantages of specificity and rapidity. In recent years, the LAMP assay has been widely used in food safety, pathogen and microbiological testing, clinical diagnosis, and so on.^{6–9} LAMP assays are also an effective tool for screening genetic mutations.¹⁰ A previous study detected the -SEA (Southeast Asian) deletion, and IVSII-I G>A mutation indicating thalassemia using LAMP assays.^{11,12} Thus, new LAMP methods capable of detecting thalassemia could be alternative methods to other techniques, and could be used in primary hospitals and rural areas.

Previous studies have found that the mutations found in thalassemia differ between ethnic groups and regions in China because it has a vast territory with a complicated geography leading to significant variation in hereditary traits. In Wuhan China, -SEA/deletion, - α 3.7/deletion, and - α 4.2/deletion were the most common α -thalassemia mutations and IVS-2-654 mutation, CD41-42, CD17, CD26, and CD27-28 were the most common β -thalassemia mutations.¹³ In Quanzhou region of Southeast China, -SEA/deletion, - α 3.7/deletion, and - α 4.2/deletion are the most common β -thalassemia mutations whereas β IVS-II-654/ β N, β CD41-42/ β N, β CD17/ β N, β CD26/ β N, and β -28/ β N are the most common α -thalassemia mutations.² In Ganzhou, Jiangxi, -SEA/deletion, - α 3.7/deletion, and - α 4.2/deletion were the most common α -thalassemia mutations whereas 654M, 41/42M, -28M, 17M, and 27/28M were the most common β -thalassemia mutations. In this study, we aimed to design and evaluate a new method for

detecting three α -thalassemia mutations including -SEA, - α 3.7, and - α 4.2, and five β -thalassemia mutations including 654M, 41/42M, -28M, 17M, and 27/28M using LAMP.

Materials and Methods

This study was approved by the Ethics Committee of First Affiliated Hospital of Gannan Medical University (No.201317) and conducted in accordance with the Declaration of Helsinki.

Design of LAMP Primers

The LAMP primer sequences, including forward inner primer (FIP), backward inner primer (BIP), forward outer primer (F3), and backward outer primer (B3), were designed using Primer Explorer V4 software to detect the 3 deletions of α -thalassemia including -SEA, - α 3.7, and - α 4.2 and five mutants of β -thalassemia including 654M, 41/42M, -28M, 17M, and 27/28M. The design principle of LAMP primers was shown at <http://loopamp.eiken.co.jp/e/lamp/primer.html>. Besides LAMP primers, blocker was designed to reduce the probability of non-specific amplification for detecting 654M. Blocker can bind to wild-type genomic DNA before LAMP primers, thus preventing LAMP primers non-specifically binding to wild-type genomic DNA. In addition, loop primer B (LB) and loop primer F (LF) were designed to improve the amplification efficiency for detecting - α 4.2. The design principle of LB and LF was shown at <http://loopamp.eiken.co.jp/e/lamp/loop.html>. The primer sequences used for LAMP were synthesized by Sangon (Shanghai, China), and are shown in Table 1.

Clinical Specimens and Genomic DNA Extraction

For this study, 400 individuals were recruited between January 2016 and December 2018 from First Affiliated Hospital of Gannan Medical University. All participants provided written informed consent. All individuals were diagnosed by α -thalassemia Deletion Test Kit (China Medical Device Registration: 20,173,403,211) and β -thalassemia Test Kit (China Medical Device Registration: 20,173,401,093) (Zeesan Biotech, Xiamen, China), then all individuals were diagnosed by LAMP for detecting the three α -thalassemia deletions including -SEA, - α 3.7, and - α 4.2, and five β -thalassemia mutations including 654M, 41/42M, -28M, 17M, and 27/28M. The procedures for sample collection and processing were as follows: Briefly, 5 mL blood

Table 1 The Sequences of Primers Used for LAMP Method

Name of Primers	Sequence of Primes
To Detect -SEA F3 B3 FIP BIP	CGATCTGGGCTCTGTGTTTC TGGAGTGCAGTGTGTAGTC GGACGACCGAGTTCTCGCATTTGAGGGAAGGAGGGGAGAAG CCTTGGGGAGGTTCACTTGGAGAGCCTTGAACCTCTGGACTT
To Detect -α3.7 F3 B3 FIP BIP LF LB	CGCGCCCCAAGCATAA GCCTGCGCTACACCTCC GAGACAGCACCATGGTGGGTTC-CTGGCGCGCTCGatGG GCGATCTGGGTGAGGGGGC-CGCAACCCGCGTGATCC TCTCTGAGTCTGTGGGGACC CTTCTCGCAGGGCAGA
To Detect -α4.2 F3 B3 FIP BIP LF LB	GTGTGTGCCTGGGTTCTC ACCCTCCCCAAGCTCAA CATGCAGCTGCAGAGAGGTCCGTCGCCGAATGTGCCAACAG CGGTGCTGAAGTCTGGAATCCGCAGGGACTGGGTTGGAA GGTCTGAGACAGGTAACACCTCCA GCTGGGAAGTTGCACAGCCC
To Detect 654M F3 B3 FIP-LNA BIP BLOCKER	TGCCTCTTTCACCATTTC TTTGCCAAAGTGATGGGC TGTAGCTGCTATTAGCAATATGAAACCAGTGATAATTTCTGGGTTAAGG+T CCAGCTACCATTCTGCTTTTATTTTGAAGATAAGAGGTATGAACATG TCTGGGTTAAG+G+C+AATAGCAATATCTC
To Detect 41/42M FIP BIP F3 B3	CCTTACCTTAGGGTTGCCCATCCTTGGACCCAGAGGTaG+A TCATGGCAAGAAAGTGCTCGGTAGGTGCCCTTGAGGTTGT AGGCTGCTGGTGGTCTAC GCAGCTCACTCAGTGTGG
To Detect 28M F3 B3 FIP-LNA BIP	ATCTACTCCCAGGAGCAG TGTAACCTTGATACCAACCT CAGTTGTGTGAGAAGCAAATGTAAGCCAGGGCTGGGCaA+G GACACCATGGTGCATCTGACCCAACCTTCATCCACGTTCA
To Detect 17M F3 B3 FIP BIP	CAATCTACTCCCAGGAGCAG GTCTTGTAACCTTGATACCAACC TGCTAGTGAACACAGTTGTGTCAGCAGGGCTGGGCATAAAAGTC CATCTGACTCCTGAGGAGAAGTCTCCAACCTTCATCCACGTTCCaA
To Detect 27/28M F3 B3 BIP FIP-LNA	GGCAAGGTGAACGTGGATG GGACAGATCCCCAAGGACT TGGGTTTCTGATAGGCACTGACTGGGTAGACCACCAGCAGC CCACATGCCAGTTTCTATTGGTTGGTGGTGAGGCCgC+T

(Continued)

Table 1 (Continued).

Name of Primers	Sequence of Primes
To Detect Internal Control	
FIP	ATGCAGAGATATTGCTATTGGCACCATTCTAAAGAATAACAGT
BIP	AGGTTTCATATTGCTAATAGCAGCTCTTATCCCAACCATAAAAATAAAGC
F3	GATACAATGTATCATGCCTCTT
B3	TGGACTCAGAATAATCCAGC

Note: Base which preceded by a "+" sign indicating locked nucleic acid (LNA) modification.

Abbreviations: LAMP, Loop-mediated isothermal amplification; SEA, Southeast Asian; BLOCKER, BLOCKER is an oligonucleotide that hybridizes perfectly to the wildtype template, and thereby suppressing unintended hybridization of the LAMP primer to the wildtype; FIP, forward inner primer; BIP, backward inner primer; F3, forward outer primer; B3, backward outer primer; LF, forward loop primers; LB, backward loop primers.

samples in EDTA were collected from all participants and subjected to DNA extraction using Chelex 100. Briefly, 300 μL of whole blood was added to 200 μL NaOH/SDS and then centrifuged at $13,000 \times g$ for 3 min. The precipitate was collected, 5% Chelex-100 was added, and incubated at 56°C for 30 min. The samples were vortexed at high speed for 5–10 s and then centrifuged at $13,000 \times g$ for 2 min; the supernatant was collected and stored at -20°C until use.

LAMP Reaction and Analysis of LAMP Products

To carry out LAMP reaction, 25 μL LAMP reaction system was prepared as shown in Table 2. The tube to

detect internal control HBB gene was named as control tube, and the tubes to respectively detect 3 deletions of α thalassemia ($-\text{SEA}$, $-\alpha 3.7$, and $-\alpha 4.2$) or five mutants of β -thalassemia (654M, 41/42M, -28M , 17M, and 27/28M) were named as detection tube. The DNA template added in control tube and detection tube is same DNA from same patient. The LAMP reaction system mixture was incubated at 56°C for 45 min in a water bath (Kuansons, Shanghai, China). LAMP products in the control tube and the detection tube were detected by fluorescence color development under ultraviolet light. The definition of the positive and negative samples is shown below: The definition of the positive and

Table 2 LAMP Reaction System

Component	To Detect $-\text{SEA}$, $-\alpha 3.7$, 41/42M, 28M, 17M, 27/28M, and Internal Control	To Detect $-\alpha 4.2$,	To Detect 654M
	Volume (μL)	Volume (μL)	Volume (μL)
10 \times Bst DNA polymerase reaction buffer (NEB, Ipswich, MA, USA)	2.5	2.5	2.5
dNTP Mixture (10mM) (Sangon, Shanghai, China)	3.5	3.5	3.5
MgSO ₄ Solution (100mM) (NEB)	1.5	1.5	1.5
Betaine (5M) (Solarbio, Beijing, China)	4.0	4.0	4.0
Bst DNA polymerase (NEB)	1	1	1
FIP (40 μM)	1	1	1
BIP (40 μM)	1	1	1
F3 (10 μM)	0.5	0.5	0.5
B3(10 μM)	0.5	0.5	0.5
Blocker (10 μM)	–	–	1
LF (10 μM)	–	1	–
LB (10 μM)	–	1	–
Calcein (100 μM) (Aladdin, Shanghai, China)	0.5	0.5	0.5
DNA template	2	2	2
ddH ₂ O	Up to 25	Up to 25	Up to 25

Abbreviations: LAMP, loop-mediated isothermal amplification; SEA, Southeast Asian; dNTP, deoxy-ribonucleoside triphosphate; Bst, *Bacillus stearothermophilus*; FIP, forward inner primer; BIP, backward inner primer; F3, forward outer primer; B3, backward outer primer; LF, forward loop primers; LB, backward loop primers; ddH₂O, double distilled H₂O.

negative samples is shown below: when only LAMP products of control sample show green fluorescence, this tested sample was determined to be Thalassemia negative sample; when LAMP products of both tested and control sample showed green fluorescence, the tested sample was determined to be Thalassemia positive sample, and the type of thalassemia can be estimated according which detection tube to detect 3 deletions of α thalassemia (-SEA, $-\alpha 3.7$, and $-\alpha 4.2$) or five mutants of β -thalassemia (654M, 41/42M, -28M, 17M, and 27/28M) showed green fluorescence. When LAMP products of control sample did not show green fluorescence, the reaction was considered to be failed and the sample needed to be retested.

PCR and Analysis of PCR Products

The PCR system was the same as the LAMP reaction system. The mixture was incubated for 90 cycles at 65°C for 1 min/cycle (ABI 7500). The Ct values of wild-type and mutant types were then analyzed to calculate the Δ CT values (Ct values of mutant type - Ct value of wild type).

Statistical Analyses

Using the Sanger sequencing results as the control group, the Stata 12.0 software was used to calculate the sensitivity and specificity of the assay method, and the 95% confidence interval. The consistency of the method's test results with those of Sanger sequencing was evaluated using the Kappa test method with the SPSS software.

Results

Specificity of LAMP Primers

PCR was performed using the wild-type and mutant/deficiency plasmids at concentrations of 1000/10,000/100,000 copies/ μ L as templates. Under these same real-time conditions as the LAMP reaction, successful amplification of the mutant/deficiency sequence was observed, indicating that the primers designed for the LAMP assay could be used for PCR amplification. Additionally, genes -28 and -17M of wild type had not been amplified. The Δ CT values are shown in Table 3. The results showed that the higher template concentrations ($\geq 100,000$ copies/ μ L) had lower Δ CT values whereas the lower template concentrations ($\leq 100,000$ copies/ μ L) had higher Δ CT values. These

Table 3 The Δ CT Values

Gene	Concentration (Copies/ μ L)	Δ CT
41/42	1000	12
	10,000	25
	100,000	20
27/28	1000	24
	10,000	16
	100,000	7
-28	1000	**
	10,000	**
	100,000	**
-17M	1000	**
	10,000	**
	100,000	**
654M	1000	40
	10,000	13
	100,000	4
SEA	1000	25
	10,000	15
	100,000	7
-3.7	1000	20
	10,000	13
	100,000	5
-4.2	1000	18
	10,000	11
	100,000	4

Note: Δ CT values=Ct values of mutant types - Ct value of wild type; the larger the Δ CT value, the better the discrimination between the wild type and the mutant plasmid. **Indicates that the wild type template has not been amplified.

Abbreviations: SEA, Southeast Asian; CT, cycle threshold.

results showed that LAMP primers had high specificity to distinguish between wild-type and mutant/deficiency gene.

Reaction Time of LAMP Assays

DNA was extracted from clinical specimens and used for LAMP assays to determine the reaction time of LAMP assays. The results showed that LAMP assays for positive samples of thalassemia reached a plateau before 60 minutes, whereas the negative control samples entered the plateau phase after 70 minutes or showed no amplification (Figure 1). The LAMP reaction tube with a reaction time of 60 minutes was exposed to ultraviolet light for observing the development of color. The positive sample tube showed strong green fluorescence, whereas the negative tube showed light green fluorescence (Figure 2). The negative and positive samples could be distinguished visually by fluorescence intensity.

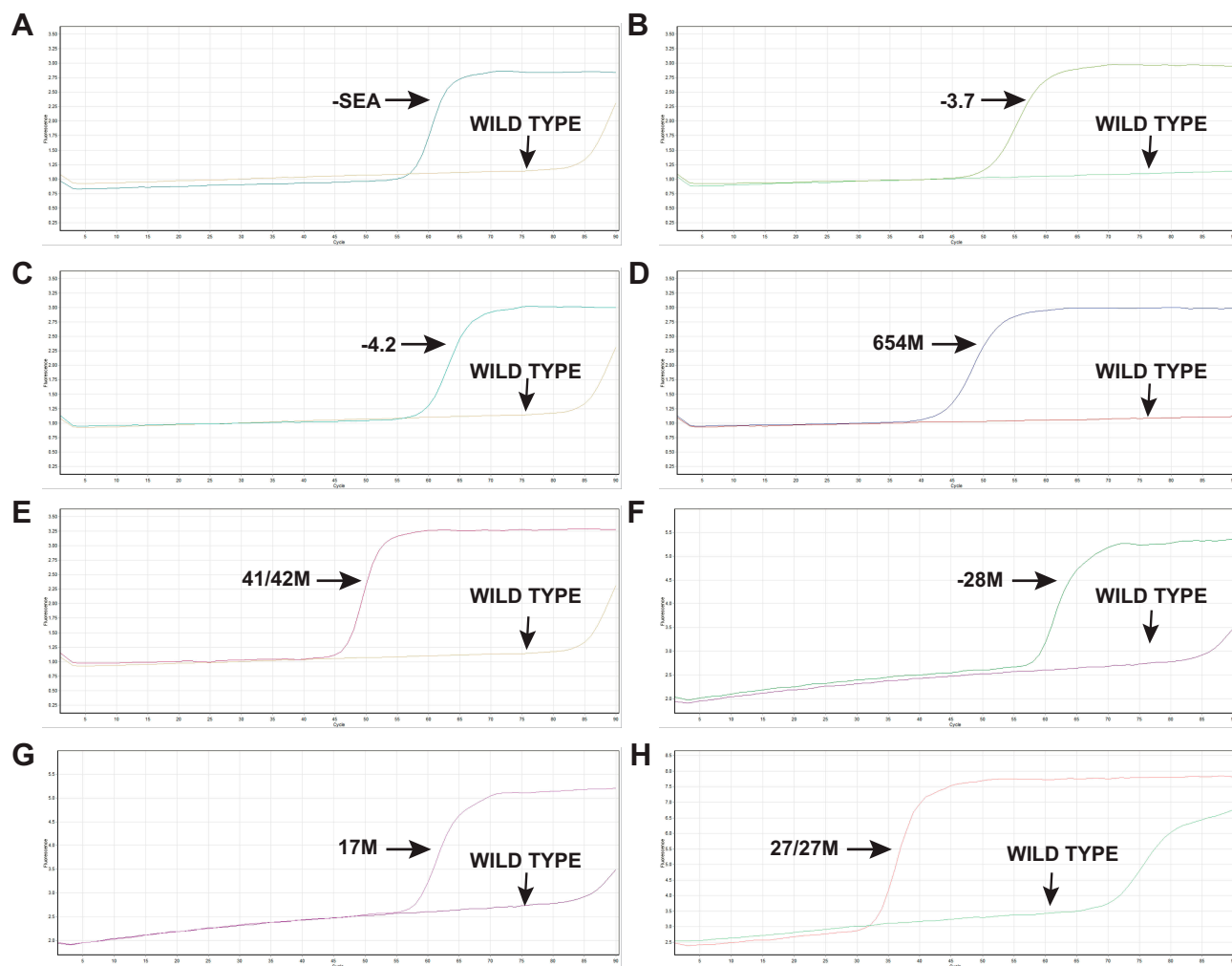


Figure 1 LAMP assays for the positive and negative samples of thalassemia. (A–H) PCR reactions were performed using positive samples and negative samples of thalassemia as templates under these same real-time LAMP reaction conditions.

Abbreviations: SEA, Southeast Asian; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction.

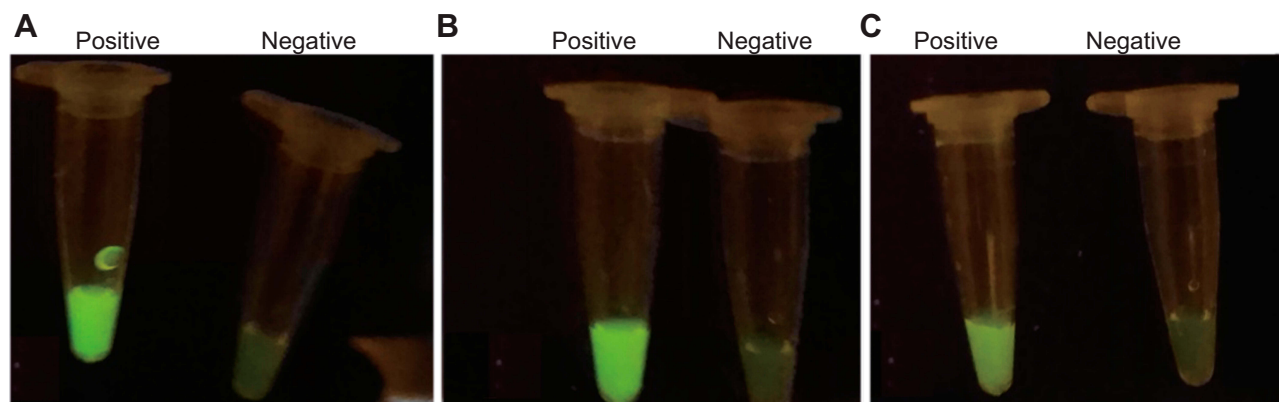


Figure 2 The LAMP reaction tube with a reaction time of 60 minutes was exposed to ultraviolet light for observing color. The positive sample tube showed strong green fluorescence while the negative tube showed light green fluorescence. (A) –SEA positive sample and negative sample; (B) 654M positive sample and negative sample; (C) 27/28 positive sample and negative sample.

Abbreviations: SEA, Southeast Asian; LAMP, loop-mediated isothermal amplification.

Sensitivity of LAMP Assays

DNA extracted from positive samples at concentrations of 20–60 ng/μL, was serially diluted 10-fold as 100% 10%, 1%, 0.1%, 0.01%, and wild-type plasmid was used as the negative control. The results showed that positive clinical samples at concentrations of 100% 10%, 1%, and 0.1% emitted bright green fluorescence, whereas positive samples at concentrations of 0.01% and the wild-type plasmid group emitted light yellow fluorescence. The concentration range of positive reactions was between 20–60 pg/μL and 20–60 ng/μL. According to these results, the detection limit of the PCR was 20–60 pg/μL.

Specificity of LAMP Assays

Eight positive and negative clinical samples were added to 8 groups of the LAMP reaction system, respectively. For example: 8 positive and negative clinical samples were added to the HBB–SEA LAMP reaction system, respectively. The results showed that only HBB–SEA positive clinical samples emitted bright green fluorescence whereas other positive and negative clinical samples emitted light yellow fluorescence. The results showed that the LAMP method which detected the HBB–SEA subtype had high specificity. Similar to these results, the LAMP method could also detect the other 7 subtypes with high specificity.

Sensitivity and Specificity of LAMP Assays in Clinical Assay

In total, 400 clinical samples were collected and tested by LAMP assay (Table 4). Among these, there were 254 positive mutation specimens (including heterozygous) and LAMP detected 252 positive mutant specimens; this indicates that the LAMP method has high sensitivity for detecting thalassemia (252/254). However, 43 false-

positive results were obtained with the LAMP test; the –3.7 and –4.2 subtypes showed especially more false-positive results, indicating that the LAMP detection method was less specific. These results showed that the sensitivity and specificity were 99.2% (252/254) and 29.5% (43/146), respectively.

Discussion

In this study, we report a novel LAMP method to detect the three α -thalassemia deletions include –SEA, – α 3.7, and – α 4.2, and five β -thalassemia mutations including 654M, 41/42M, –28M, 17M, and 27/28M. Four primers were designed specifically to bind to six distinct regions of the target gene. In this study, for primers with low specificity, locked nucleic acid (LNA) technology was used for primer modification. LNA is a class of nucleic acid analogs that contain a methylene bridge connecting the 2' oxygen and 4' carbon in the ribose moiety, which increases the thermal stability of hybrid strands.¹⁴ The use of LNA technology in molecular diagnostic methods improves the specificity and sensitivity of assays.¹⁵ Using the primers designed for the present study, the LAMP method showed high sensitivity for detecting thalassemia (252/254). However, there were 43 false-positive results in the LAMP test; in particular, the –3.7 and –4.2 subtypes showed more false-positive results, indicating that the LAMP detection method was less specific for detecting the –3.7 and –4.2 subtypes.

LAMP assay has been widely used for screening genetic mutations and deletions. In previous studies, LAMP assays were used to detect the F167Y mutation in *Fusarium graminearum* and KRAS mutation patients with cancer.^{16,17} Additionally, LAMP assays were used to detect the haptoglobin gene deletion in alkaline-denatured blood.¹⁸ In this study, we found that the target sequence was successfully amplified and that the wild type and target could be distinguished. LAMP assays for positive samples of thalassemia reached a plateau before 60 minutes, whereas the negative control samples entered the plateau after 70 minutes or showed no amplification. The concentration range of positive reactions was between 20–60 pg/μL and 20–60 ng/μL. These results suggest that the designed LAMP assays had high sensitivity and low reaction time. Additionally, there were no cross-reactivities between 8 thalassemia subtypes, indicating that LAMP assays had high specificity. For clinical samples, the positive sample tube showed strong green fluorescence, whereas the negative tube showed light green

Table 4 Clinical Performance of LAMP Assays

Gene	Clinical +/LAMP+	Clinical +/LAMP–	Clinical –/LAMP+	Clinical –/LAMP–
41/42	34	1	2	363
27/28	3	0	0	397
–28	3	0	0	397
–17M	12	0	1	387
654M	46	1	1	352
SEA	117	0	2	281
–3.7	25	0	20	355
–4.2	12	0	17	371

Abbreviations: LAMP, loop-mediated isothermal amplification; SEA, Southeast Asian.

fluorescence; the negative and positive samples could thus be distinguished visually, based on fluorescence intensity.

At present, deletions of α -thalassemia are detected by Gap-PCR and the α - and β -thalassemia mutations are detected by DNA reverse dot blot (RDB).^{2,19,20} Compared to these methods, the LAMP assay has many advantages such as high sensitivity, and simple and fast operation, and it does not need a special instrument. Furthermore, the cost of detecting thalassemia (include $-SEA$, $-\alpha3.7$, and $-\alpha4.2$ α - thalassemia, and 654M, 41/42M, $-28M$, 17M, 27/28M β -thalassemia) using commonly clinical test kits is about 300 CNY in our hospital, while the cost of detecting thalassemia using the LAMP method in the manuscript is about 30 CNY. Compared to commonly clinical test kits, the LAMP assay has the advantage of low cost. Although the present study successfully used LAMP to detect three α - thalassemia deletions include $-SEA$, $-\alpha3.7$, and $-\alpha4.2$, and five β -thalassemia mutations including 654M, 41/42M, $-28M$, 17M, 27/28M, the -3.7 and -4.2 subtypes showed more false-positive results, possibly because the -3.7 and -4.2 deletion genes were closely related to the wild type gene sequence before mutation, which is prone to false amplification. Another important reason is that the positions and lengths of the missing fragments of -3.7 and -4.2 are not fixed, so there may occur erroneous amplification. Hence, further studies with redesigned primer sets are needed to detect these mutations with high specificity. In addition, we recommend that patients with 3.7 and 4.2 deletion mutations need to further screen in the superior hospital.

In conclusion, the novel LAMP assay can be completed within 60 min using a heating block or a water bath and the result can be read visually based on color changes to detect thalassemia. The LAMP assay had high sensitivity, low cost, and simple and fast operation, fulfilling the requirements of field application in resource-limited areas, especially those with primary hospitals and in rural areas.

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Disclosure

Fang-li Song and Shao Huang were employed by company Jiangxi Shiningmed Medical Technology Ltd. All other authors declare no competing interests in this work.

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