Original Article

Detection of α-Thalassemia of Southeast Asian ($^{--SEA}$) Deletion by Novel Multiplex PCR

Wirawit Piyamongkol, MD, PhD[1], Supranee Upanan, PhD[1], Sirivipa Piyamongkol, PhD[2], Pimlak Charoenkwan, MD[3], Theera Tongsong, MD[1]

[1] Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

[2] Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand

[3] Department of Pediatrics, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

Background: Southeast Asian ($^{--SEA}$) deletion of α-thalassemia is the most common α-thalassemia-1 mutation in Thailand and neighborhood countries. The absence of α-globin gene production in a homozygous fetus is the most serious and leads to death in utero or soon after birth and life-threatening maternal complications. Therefore, sensitive and accurate assays for detecting α-thalassemia^{--SEA} deletion are crucial for thalassemia diagnosis.

Materials and Methods: The present study evaluated multiplex polymerase chain reaction (PCR)$^{--SEA}$, comparing with the routinely performed gap-PCR for α-thalassemia diagnosis in prenatal thalassemia prevention and control strategy commonly used in Thailand. Four primers were employed to detect the deleted region of α-globin gene family. This, thus, provides high accuracy in discriminating heterozygous and homozygous $^{--SEA}$ deletion.

Results: Multiplex PCR^{--SEA} assay showed the results with 100% sensitivity, specificity, positive predictive value, negative predictive value, and efficiency in comparison to the routinely performed gap-PCR used in prenatal thalassemia prevention and control strategy. In addition, the multiplex PCR^{--SEA} assay displayed lower detection limit for heterozygous detection.

Conclusion: The novel multiplex PCR^{--SEA} in the present study was proofed to be a good alternative for thalassemia diagnosis in thalassemia prevention and control strategy. The advantage of the assay is the capability to identify three wild types and one deleted fragment comparing with one wild type and one deleted fragment in the routinely performed gap-PCR. This is very useful, especially in the genes where polymorphism is common. Therefore, the multiplex PCR^{--SEA} provides a better protocol for α-thalassemia^{--SEA} diagnosis.

Keywords: α-thalassemia, Hemoglobin Bart's hydrop fetalis, Multiplex PCR, Polymerase chain reaction, Prenatal diagnosis, $^{--SEA}$ deletion

Received 13 Feb 2020 | Revised 1 Jun 2020 | Accepted 2 Jun 2020

J Med Assoc Thai 2020;103(8):741-7
Website: http://www.jmatonline.com

The α-Thalassemia (OMIM: 604131; 16p13.3) is one of the major public health problems in Thailand and neighborhood countries[1] and the world most prevalent inherited hemoglobin disorder.

Correspondence to:

Piyamongkol W.

Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University, 110 Intawaroros Road, Sripoom, Mueang, Chiang Mai 50200, Thailand.

Phone: +66-53-935553, **Fax**: +66-53-936112

Email: wirawit.p@cmu.ac.th

How to cite this article:

Piyamongkol W, Upanan S, Piyamongkol S, Charoenkwan P, Tongsong T. Detection of α-Thalassemia of Southeast Asian ($^{--SEA}$) Deletion by Novel Multiplex PCR. J Med Assoc Thai 2020;103:741-7.

doi.org/10.35755/jmedassocthai.2020.08.11126

The α-thalassemia-1 (α-thal-1) with both α1 and α2 globin genes deletion results in the absence of α-globin chains production[2]. Homozygous deletion (homozygous α-thal-1), also called as hemoglobin Bart's (Hb Bart's) hydrops fetalis, is the most severe form and the major target for prevention and control program[3,4]. Hb Bart's (γ4) possesses high oxygen affinity and does not release oxygen to tissues. The fetuses suffer tissue hypoxia, skin edema, ascites, heart failure, and die in utero or soon after birth. Toxemia of pregnancy is also a common dangerous maternal complication in all pregnancies with the Hb Bart's hydrops fetalis[5,6]. Southeast Asian ($^{--SEA}$) deletion is the most common mutation of α-thal-1 covering 99.7% in one major series[7].

The present effective strategy in prevention and control α-thalassemia for the sake of maternal safety is population carrier screening, prenatal

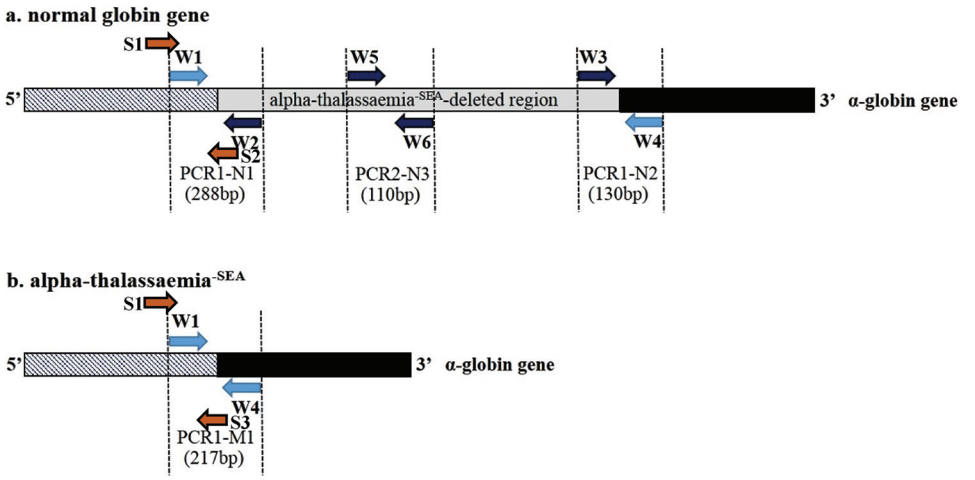

Figure 1. Diagram of normal and α-thalassemia^{--SEA} α-globin gene family with primers map for the multiplex PCR^{--SEA} assay and the routinely performed gap-PCR. Normal allele gave rise to PCR1-N1 (288 bp), PCR1-N2 (130 bp) and PCR2-N3 (110 bp) fragments, α-thalassemia^{--SEA} allele gave rise to PCR1-M1(217 bp) fragment for the multiplex PCR^{--SEA} assay. Normal allele gave rise to S-normal (287 bp) fragments, α-thalassemia^{--SEA} allele gave rise to S-deleted (194 bp) fragment for the routinely performed gap-PCR.

diagnosis for couples at risk, and termination of affected pregnancy[8]. Effective screening test for $^{--SEA}$ deletion of α-thalassemia is essential to identify α-thalassemia carrier for the prevention of severe α-thalassemia. Primers or polymerase chain reaction (PCR) protocol for α-thalassemia^{--SEA} deletion is essential not only for carrier screening but also for prenatal diagnosis of α-thalassemia. The present PCR protocol for α-thalassemia^{--SEA} deletion is based on the routinely performed gap-PCR, which has been used since 1991[9,10]. The routinely performed gap-PCR is also employed in the thalassemia prevention and control strategy, which is commonly used in Thailand and worldwide[3,4,10,11]. Multiplex gap PCR for detecting various α-globin gene deletions of α-thal-1 i.e., $^{--SEA}$, $^{-THAI}$, $^{-FIL}$, $^{-MED}$ is the combination of the same routinely performed gap-PCR with primers for other mutations[12-14]. A recently developed one-tube multiplex fluorescent PCR was used in pre-implantation genetic diagnosis (PGD) of α-thalassemia^{--SEA} protocol[15]. This PCR protocol employs novel set of primers that are different from the routinely performed gap-PCR to identify α-thalassemia^{--SEA} deletion. It demonstrated high accuracy and efficiency in situation with low copy number of DNA templates. However, the techniques need expensive fluorescent labelled primers and sophisticated automated DNA sequencer equipment.

The present study aimed to modify and test the recently developed multiplex PCR^{--SEA} protocol in detecting α-thalassemia^{--SEA} deletion in comparison to the routinely performed gap-PCR, so that it can be cheaper and less sophisticated for thalassemia prevention and control program.

Materials and Methods

DNA samples

DNA samples (n=100) with known mutation, extracted from blood using spin column techniques as the company's instruction (QIAamp DNA Blood Mini Kit, QIAGEN Thailand, Bangkok, Thailand), were obtained from Thalassemia Center, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand. The study methodology was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University, Thailand (Study Code: OBG-2560-04616/Research ID: 4616) and the patients who were couples coming through for thalassemia screening and prenatal diagnosis signed an informed consent. The DNA samples were already identified for α-thalassemia^{--SEA} mutation as homozygous normal, heterozygous, and homozygous α-thalassemia 1 using the routinely performed gap-PCR according to the thalassemia prevention and control strategy of Maharaj Nakorn Chiang Mai Hospital[4,10].

Development of multiplex PCR^{--SEA} condition

Primer sets for multiplex PCR^{--SEA} are listed in Figure 1 and Table 1[15]. DNA samples were mixed with two separate reaction mixtures, i.e., PCR1 and PCR2. PCR1 was for identification of 5' and 3' α-thalassemia 1–SEA breakpoints and PCR2 mixture

Table 1. Primer sequences for the multiplex PCR^{--SEA} assay[15]

Primers	Sequences
W1 (forward)	5'-GAA GGA GGG GAG AAG CTG AG-3'
W2 (reverse)	5'-TGT GGA AAA GTT CCC TGA GC-3'
W3 (forward)	5'-TGC ACA CCT ATG TCC CAC TT-3'
W4 (reverse)	5'-TTG AGA CGA TGC TTG CTT TG-3'
W5 (forward)	5'-GCC ACT GCC TGC TGG TG-3'
W6 (reverse)	5'-AGG TCA GCA CGG TGC TCA C-3'

was for identification of an internal control fragment of α-1 exon 3 (OMIM: NG_000006). The PCR mixture (10 µl of total volume) includes 100 ng of DNA templates, 0.2 mM dNTP (Roche Diagnostics (Thailand) Ltd., Bangkok, Thailand), 1 µM primers (W1, W2, W3 and W4 primers for PCR1 generating PCR1-N1 288bp, PCR1-M1 217bp and PCR1-N2 130bp; W5 and W6 for PCR2 generating PCR2-N3 110bp, Figure 1) (Ward Medic Ltd., Part., Bangkok, Thailand), 0.5 units of Taq polymerase and 1X buffer (Vivantis, Bang Trading Co., Bangkok, Thailand). The reactions were operated on a PCR machine (Mastercycler, Eppendorf, Germany) as following conditions: Taq DNA polymerase activation at 95°C for four minutes; PCR amplification in 40 cycles of 95°C for 30 seconds and 60°C for 30 seconds and 72°C for 45 seconds; and then final extension at 72°C for 10 minutes. The PCR products were analyzed using 2% agarose gel electrophoresis, 100 volts, 50 mA, 30 minutes. The product bands were stained with DNA-Dye NonTox (Bang Trading Co., Bangkok, Thailand) and visualized under an ultraviolet trans-illuminator. Normal allele gave rise to PCR1-N1 (288 bp), PCR1-N2 (130 bp) and PCR2-N3 (110 bp) fragments. The SEA allele gave rise to PCR1-M1 (217 bp) fragment.

The routinely performed gap-PCR condition

The routinely performed gap-PCR employs three gap-PCR primers[10]. The S1 and S2 primers amplify the 5' breakpoint region of the normal allele while the S1 and S3 primers amplify the breakpoint region of the deleted allele (Figure 1). PCR condition was the same as multiplex PCR^{--SEA} condition except for the use of the gap-PCR primers i.e., S1-5'-GTG TTC TCA GTA TTG GAG GGA A-3', S2-5'-GAC ACG CTT CCA ATA CGC TTA-3', S3-5'-CTA CTG CAG CCT TGA ACT CC-3'[10]. Analysis was performed on 2% agarose gel electrophoresis, 100 volts, 50 mA, 30 minutes.

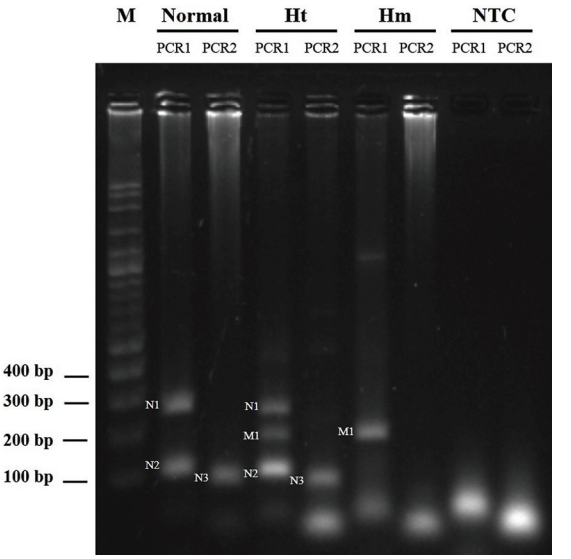

Figure 2. PCR product sizes of the multiplex PCR^{--SEA} assay for α-thalassemia diagnosis: 100 bp Plus DNA Ladder (M) (Vivartis, Bang Trading Co., Bangkok), fragments for normal (N1:288 bp, N2:130 bp, N3:110 bp), fragments for hetero-zygous (Ht) (N1:288 bp, M1:217 bp, N2:130 bp, N3:110 bp), fragment for homozygous (Hm) affected (M1:217 bp) and non-template control (NTC).

Evaluation of multiplex PCR^{--SEA} assay

The validity of the multiplex PCR^{--SEA} assay was determined by diagnostic indices using a two-by-two table compared with the routinely performed gap-PCR[10] for α-thalassemia diagnosis in Chiang Mai Strategy to evaluate sensitivity, specificity, positive predictive value, negative predictive value, and efficiency. McNemar's test was used for correlation analysis.

The detection limit of the multiplex PCR^{--SEA} assay was also estimated to compare with the routinely performed gap-PCR[10] using 2-fold dilution of pooled heterozygous DNA templates (24 ng, 12 ng, 6 ng, 3 ng, 1.5 ng, 750 pg, 375 pg, and 187.5 pg).

Results

Agarose gel electrophoresis of multiplex PCR^{--SEA} assay

Agarose gel electrophoresis revealed the size of PCR products as shown in Figure 2 and summarized in Table 2. Samples with homozygous normal showed N1:288 bp and N2:130 bp bands in PCR1 and N3:110 bp band in PCR2; heterozygous (Ht) samples showed N1:288 bp, M1:217 bp and N2:130 bp bands in PCR1 and N3:110 bp band in PCR2; homozygous (Hm) affected samples showed only M1:217 bp band in PCR1 (Figure 2).

Table 2. Multiplex PCR^{--SEA} results interpretation

Genotypes	PCR products			
	PCR1-N1 (288 bp)	PCR1-M1 (217 bp)	PCR1-N2 (130 bp)	PCR2-N3 (110 bp)
Normal	Yes	No	Yes	Yes
Heterozygous	Yes	Yes	Yes	Yes
Homozygous	No	Yes	No	No

PCR=polymerase chain reaction

Table 3. Two-by-two table for diagnostic indices of the multiplex PCR^{--SEA} assay for α-thalassemia^{--SEA} diagnosis

Multiplex PCR^{--SEA} assay	α-thalassemia^{--SEA} diagnosis in Chiang Mai Strategy (routinely performed gap-PCR)		
	Positive	Negative	Total
Positive	44	0	44
Negative	0	56	56
Total	44	56	100

PCR=polymerase chain reaction

Validation of multiplex PCR^{--SEA} assay

The recently developed one-tube multiplex fluorescent PCR is used in PGD of α-thalassemia $^{--SEA}$[15]. The protocol was modified by separating the reaction into two separate tubes so that the DNA bands can be identified easily on the agarose gel electrophoresis. Multiplex PCR (PCR1) was performed for mutation analysis of the 5' and 3' breakpoints and a control PCR reaction (PCR2) was performed for identifying the presence of the internal control fragment of α-globin gene family. Therefore, four sets of primers were employed in the present protocol to amplify the deleted region of α-globin gene (Figure 1), while the routinely performed gap-PCR in thalassemia prevention and control strategy employs only one set of primers to detect the deleted region[9]. Theoretically, this alternative protocol should provide an improved accuracy and sensitivity, consequently, a reduced the false negative results. Therefore, this would be a better alternative assay for screening α-thalassemia^{--SEA} in thalassemia diagnosis for thalassemia screening and control strategy.

Multiplex PCR^{--SEA} assay could identify all 100 cases of DNA from the subjects, including normal (n=56), heterozygous (n=32), and homozygous (n=12). The multiplex PCR^{--SEA} assay revealed 28% (56/200) positive and 72% (144/200) negative for α-thalassemia^{--SEA} alleles (Table 3). The validity of multiplex PCR^{--SEA} assay was determined in comparison to the gap-PCR for α-thalassemia diagnosis in Chiang Mai strategy. The diagnostic indices are presented in Table 3. The multiplex PCR $^{--SEA}$ assay displayed 100% sensitivity, specificity, positive predictive value, negative predictive value, and efficiency. This demonstrated that the multiplex PCR^{--SEA} assay could identify all carriers and homozygous samples without false-negative or false-positive results, in other words, the multiplex PCR $^{--SEA}$ assay is as efficient as the routinely performed gap-PCR in detecting normal, heterozygous, and homozygous α-thalassemia^{--SEA} deletion.

Efficiency of multiplex PCR^{--SEA} assay

For the detection limit study, the multiplex PCR $^{--SEA}$ assay could differentiate heterozygotes from normal at the amount of DNA templates of 1.5 ng or higher, while the gap-PCR assay could do at the amount of the templates of 3 ng or higher (Figure 3). These results demonstrated that the multiplex PCR $^{--SEA}$ assay was twice as sensitive as the routinely performed gap-PCR assay at low amount of DNA templates. At 1.5 ng of DNA template, the routinely performed gap-PCR gave the normal 287 bp fragment but failed to give the $^{--SEA}$ deleted 194 bp fragment (Figure 3). The results would be interpreted as normal, leading to a false negative result.

Discussion

According to Thailand's public health policy, population screening for heterozygotes or carriers is the fundamental act of prevention and control of the homozygous α-thalassemia 1 or Hb Bart's disease[1-4]. Hb Bart's hydrops fetalis is the most severe α-thalassemia, having no α-globin chain production, leading to fetus death in utero or soon after birth and toxemia of pregnancy. Unavoidably, Hb Bart's fetuses were terminated by therapeutic abortion to save maternal lives[5,6]. Thus, accurate test for α-thalassemia diagnosis is crucial. In the Chiang Mai strategy, osmotic fragility test (OFT) and gap-PCR were opted for α-thalassemia screening and diagnosis, respectively. OFT is a sensitive screening test; however, following positive OFT, gap-PCR[3,4,9-11] is needed for diagnosing α-thalassemia 1 carriers. The routinely performed gap-PCR has been used for population screening and prenatal diagnosis since 1991[9,10].

Notably, in the multiplex PCR^{--SEA} assay, six primers were employed for identifying three normal fragments and one mutant fragment while most of the gap-PCR tests employed only three primers for detecting one normal and one mutant fragment

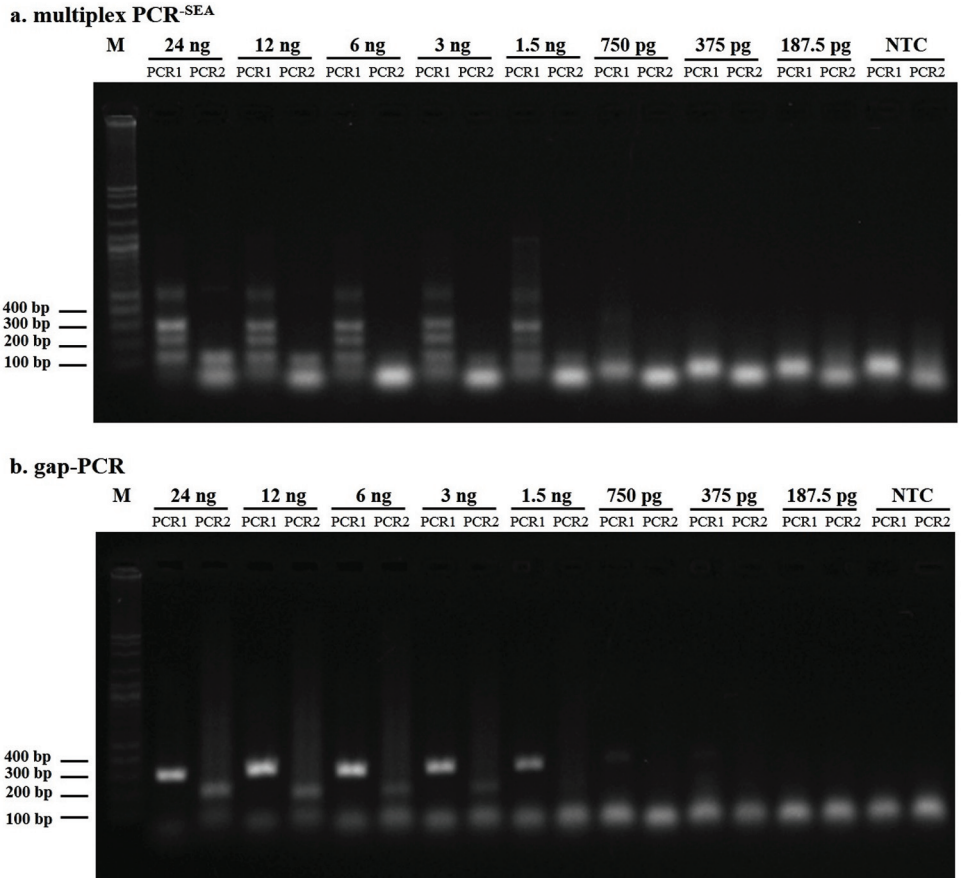

Figure 3. Comparison of the detection limit between the multiplex PCR^{--SEA} (a) and the routinely performed gap-PCR (b) to detect a heterozygous α-thalassemia^{--SEA} sample using 2-fold diluted DNA templates (24 ng-187.5 pg): 100 bp Plus DNA Ladder (M) and non-template control (NTC). The routinely performed gap-PCR result was interpreted with 287 bp in PCR1 for detecting the normal allele and 194 bp in PCR2 for detecting the $^{--SEA}$ deleted allele[9,10].

covering the breakpoints[9,10]. To detect the genotype, the multiplex PCR^{--SEA} assay employs three pairs of primers amplifying four fragments, one across the 5' wild type breakpoint (N1), one across the 3' wild type breakpoint (N2), one within the breakpoints (N3) as the backup result, and one across the deleted breakpoint (M1) (Figure 1), while the routinely performed gap-PCR uses only three primers, one pair for 5' wild type breakpoint and another for the deleted breakpoint. The α-globin gene family contains sequences with common polymorphism. In case of polymorphism, false negative of the normal allele may occur and lead to misdiagnosis as homozygous affected (Hb Bart's) conclusion for a heterozygous sample. This will result in termination of pregnancy decision of a healthy baby. The multiplex PCR^{--SEA} assay provides the analysis of three loci of the normal allele (Figure 1). The chance of false negative of the three normal allele fragments is very low and should be lower than that of the routinely performed gap-PCR. In addition, the multiplex PCR^{--SEA} exhibits the lower detection limit for heterozygous identification (Figure 3).

This advantage has also been applied for multiplex fluorescent PCR for PGD using low amount of DNA from single cells[15], however, this is using more expensive fluorescent labelled primers, analyzed on a more sophisticated and expensive automated DNA sequencer. From the single cell fluorescent PCR study, the routinely performed gap-PCR gives the efficiency of 81.6% and 37.5% allele dropout rate of the normal allele[16], while the multiplex PCR^{--SEA} assay gives 100% efficiency and 78.2%, 96.4%, and 96.4% ADO rates of the PCR1-N1, PCR1-N2 and PCR2-N3, respectively, with 100% overall diagnostic efficiency for the normal allele[15]. Therefore, the later

designed primers for multiplex PCR^{--SEA} demonstrate better performance than the routinely performed gap-PCR in single cell fluorescent PCR for PGD.

Conclusion

The novel multiplex PCR^{--SEA} was validated and proved to be a good alternative for α-thalassemia^{--SEA} diagnosis in thalassemia prevention and control strategy. The advantage of the present assay is the capability to identify three wild type and one deleted fragment comparing with one wild type and one deleted fragment in the routinely performed gap-PCR. This is very useful especially in the genes where polymorphism is common. Therefore, the multiplex PCR^{--SEA} provides a better protocol for α-thalassemia $^{--SEA}$ diagnosis.

What is already known on this topic?

There has been only one gap-PCR protocol for α-thalassemia^{--SEA} mutation since 1991 for α-thalassemia 1 screening and diagnosis. The α-globin gene family region contains various polymorphisms and GC rich sequences where the routinely performed gap-PCR protocol is sometime ineffective, therefore, some carriers escape the screening leading to the missing of prenatal counselling and diagnosis.

What this study adds?

This study presents an alternative multiplex PCR^{--SEA} protocol for α-thalassemia 1 screening and diagnosis. The novel multiplex PCR^{--SEA} protocol showed the results with 100% sensitivity, specificity, positive predictive value, negative predictive value, and efficiency in comparison to the routinely performed gap-PCR. The newly introduced protocol provides the advantage of identifying three wild type and one deleted fragment comparing with one wild type and one deleted fragment detection in the routinely performed gap-PCR.

Acknowledgement

This research study was supported by the Faculty of Medicine Research Fund, Chiang Mai University, Thailand (004/2561).

Conflicts of interest

All authors declare no conflicts of interest.

References

1. Fucharoen S, Winichagoon P. Hemoglobinopathies in Southeast Asia. Hemoglobin 1987;11:65-88.
2. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ 2001;79:704-12.
3. Kor-anantakul O, Suwanrath CT, Leetanaporn R, Suntharasaj T, Liabsuetrakul T, Rattanaprueksachart R. Prenatal diagnosis of thalassemia in Songklanagarind Hospital in southern Thailand. Southeast Asian J Trop Med Public Health 1998;29:795-800.
4. Tongsong T, Wanapirak C, Sirivatanapa P, Sanguansermsri T, Sirichotiyakul S, Piyamongkol W, et al. Prenatal control of severe thalassaemia: Chiang Mai strategy. Prenat Diagn 2000;20:229-34.
5. Fucharoen S, Winichagoon P. Thalassemia in SouthEast Asia: problems and strategy for prevention and control. Southeast Asian J Trop Med Public Health 1992;23:647-55.
6. Vaeusorn O, Fucharoen S, Wasi P. A study of thalassemia associated with pregnancy. Birth Defects Orig Artic Ser 1988;23:295-9.
7. Mankhemthong K, Phusua A, Suanta S, Srisittipoj P, Charoenkwan P, Sanguansermsri T. Molecular characteristics of thalassemia and hemoglobin variants in prenatal diagnosis program in northern Thailand. Int J Hematol 2019;110:474-81.
8. Tongsong T, Charoenkwan P, Sirivatanapa P, Wanapirak C, Piyamongkol W, Sirichotiyakul S, et al. Effectiveness of the model for prenatal control of severe thalassemia. Prenat Diagn 2013;33:477-83.
9. Chang JG, Lee LS, Lin CP, Chen PH, Chen CP. Rapid diagnosis of alpha-thalassemia-1 of southeast Asia type and hydrops fetalis by polymerase chain reaction. Blood 1991;78:853-4.
10. Ko TM, Tseng LH, Hsieh FJ, Hsu PM, Lee TY. Carrier detection and prenatal diagnosis of alpha-thalassemia of Southeast Asian deletion by polymerase chain reaction. Hum Genet 1992;88:245-8.
11. Fucharoen G, Sanchaisuriya K, Sae-ung N, Dangwibul S, Fucharoen S. A simplified screening strategy for thalassaemia and haemoglobin E in rural communities in south-east Asia. Bull World Health Organ 2004;82:364-72.
12. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of alpha-thalassaemia deletions and alpha-globin gene triplication by multiplex polymerase chain reactions. Br J Haematol 2000;108:295-9.
13. Sae-ung N, Fucharoen G, Sanchaisuriya K, Fucharoen S. Alpha(0)-thalassemia and related disorders in northeast Thailand: a molecular and hematological characterization. Acta Haematol 2007;117:78-82.
14. Siriratmanawong N, Pinmuang-Ngam C, Fucharoen G, Fucharoen S. Prenatal diagnosis of Hb Bart's hydrops fetalis caused by a genetic compound heterozygosity for two different alpha-thalassemia determinants. Fetal Diagn Ther 2007;22:264-8.
15. Piyamongkol W, Vutyavanich T, Sanguansermsri T. Preimplantation genetic diagnosis of alpha-thalassemia-SEA using novel multiplex fluorescent PCR. J Assist Reprod Genet 2012;29:95-102.

16. Piyamongkol W, Harper JC, Delhanty JD, Wells D. Preimplantation genetic diagnostic protocols for alpha- and beta-thalassaemias using multiplex fluorescent PCR. Prenat Diagn 2001;21:753-9.