Hemoglobin E Detection Using PCR with Confronting Two-Pair Primers

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Objective: *To develop and apply the polymerase chain reaction with confronting two-pair primers (PCR-CTPP) for detection and identification of hemoglobin E (Hb E).*
Material and Method: *Fifty unrelated northern Thais were included in the present study. DNA was extracted from peripheral blood mononuclear cells and targeted to amplify by PCR-CTPP. The amplified product was analyzed and compared with the reference hemoglobin electrophoresis and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.*
Results: *The results validated a completely concordant among these three methods consisting of 74%, 24%, and 2% identified as normal, heterozygous, and homozygous Hb E type, respectively.*
Conclusion: *Successful Hb E genotyping by PCR-CTPP was introduced. It allows for confirming and simultaneously detection with other thalassemia mutations.*

Keywords: *Hemoglobinopathy, Hemoglobin E, Beta-thalassemia, Polymerase chain reaction with confronting two-pair primers*

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Hemoglobin E (β26; Glu-Lys) is the most common hemoglobinopathy in Southeast Asia and the second prevalent worldwide[1,2]. In Thailand, the overall prevalence of Hb E is approximately 13% but rises to nearly 50% in the northeastern part of the country[3-5]. Hb E is the substitution of guanine by adenine in codon 26 of the β-globin gene. This mutation activates an adjacent cryptic splice site in codon 25 resulting in the reduction of β-globin chain production, although the existing correctly spliced mRNA can be translated to the full-length βE-globin chains. Co-inherited of Hb E and other forms of β-thalassemia gives rise to the complex thalassemia disease in which the affected individual may be symptomatic and needed to be transfused with blood components[6].

Presently, several genetic defects could be investigated by molecular typing method. According to the specific recognition site of restriction enzyme, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) allowed detection of several Hb variants. Therefore, time consuming during restriction enzyme digestion had limitations for its application. PCR with confronting two-pair primer (PCR-CTTP) was a currently genotyping method [7]. The different alleles can be distinguished on an agarose gel by designing the primers so that the mutant and wild type amplified products are of different sizes. In the present study, the authors attempted to generate the PCR with confronting two-pair primers (PCR-CTPP) for detection of a single nucleotide change of Hb E disease. This validated technique is assessed in comparison to Hb electrophoresis and the standard PCR-RFLP analysis.

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Material and Method

Blood collection

Whole blood samples were collected in a pilot tube from check-up program of fifty unrelated adult northern Thais attending the medical technology laboratory unit, Faculty of Associated Medical Sciences, Chiang Mai University from September 2002 to December 2002.

DNA extraction

Peripheral blood mononuclear cells (PBMC) were isolated using density gradient separation. Cells were lysed and genomic DNA was extracted according to the Sambrook and Russell laboratory manual[8]. DNA was dissolved in sterile distilled water and kept at -20°C until use.

PCR amplification and Hb E genotyping

Four primers designed for typing of normal and Hb E mutation were used in the present study (Table 1). Genomic DNA was subjected to amplify under a standard PCR reaction. Thermal cycling conditions were as follows: 94°C 3 min followed by 16 cycles of 94°C, 30 sec; 68°C, 30 sec; 72°C, 30 sec, and the annealing temperature was touched down 0.5°C for each cycle. The PCR profile was further continued with 18 cycles of 94°C, 30 sec; 62°C, 30 sec; 72°C, 30 sec with the final extension of 72°C, 5 min. The amplification products (10 µl) were visualized on 2% agarose gel with ethidium bromide staining. The 237-bp band specific for the normal allele was extended by FWE2 and RE1 and the 168-bp band specific for the Hb E mutant type was extended by FE1 and RME2. In addition, the 365-bp band extended by FE1 and RE1 might be amplified as a common band.

Hemoglobin electrophoresis and PCR-RFLP for Hb E detection

Hemoglobin electrophoresis used in the present study was previously described elsewhere[9,10]. For PCR-RFLP technique, Primers, designated as Hb E1 and Hb E2, were used to amplify under the standard procedure. Extended product was digested with *Mnl* I restriction enzyme and analyzed on agarose gel electrophoresis[11].

Results and Discussion

Herein, the authors evaluated the PCR-CTTP for one-tube detection and genotyping of Hb E variant in comparison to the hemoglobin electrophoresis and the reference PCR-RFLP technique. Fifty blood samples were preliminary screened for abnormal Hb typing with hemoglobin electrophoresis prior to detection by PCR-CTPP. The normal and Hb E mutant type was verified by PCR-RFLP. The results showed that all three methods validated a complete concordant with the result of 74% (37 samples), 24% (12 samples) and 2% (1 sample) identified as normal hemoglobin type, heterozygous and homozygous Hb E type, respectively (Fig. 1). Although the overall frequency of Hb E carrier in Thailand was previously reported to be 13%[3-5], approximately one-fourth of individual samples were

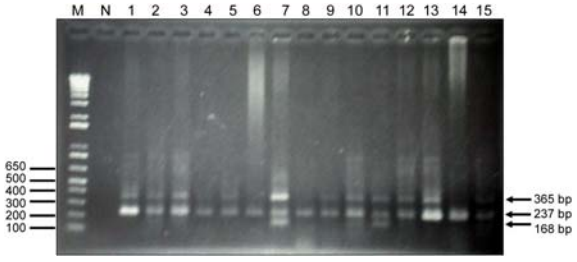

Fig. 1 2% agarose gel electrophoretic pattern of the PCR-CTTP from normal hemoglobin type (lane 1-6, 8-10 and 12-15) and the heterozygous Hb E genotype (lane 7 and 11), lane M is standard DNA marker and lane N is PCR reagent control

Table 1. Sequence primers used for PCR-CTTP and PCR-RFLP analysis

Primer name	Sequence (5'-3')	Nucleotide positions
FE1	ggc aga gcc atc tat tgc tta c*	-70 - -49
RE1	AAC AGC ATC AGG AGT GGA CAG A	278 - 299
FWE2	ACG TGG ATG AAG TTG GTG GTG	62 - 82
RME2	agc aac ctG CCC AGG GCC TT	82 - 102
Hb E1	CCT GAG GAG AAG TCT GCC GTT AC	16 - 38
Hb E2	CAT CAC TAA AGG CAC CGA GCA CT	331 - 353

* Non-coding sequences are in lower cases

assessed to be positive for Hb E genotype in the present study. Misinterpretation determined by hemoglobin electrophoresis has been previously reported when analyses with some other abnormal hemoglobins such as Hb C including the high level of Hb A_2[12]. However, PCR-RFLP confirmed that all positive samples detected by hemoglobin electrophoresis were Hb E variant. Although, PCR-RFLP was preferred to be a reference technique, in comparing with the PCR-CTPP, time-required for incubation with selected restriction enzyme is needed.

Based on the same logic of bi-directional DNA amplification as PCR-CTTP for a single nucleotide polymorphism detection, bi-directional PCR amplification of a specific allele (Bi-PASA)[13] and the tetra-primer amplification refractory mutation system-PCR (the tetra-primer ARMS-PCR)[14] have been described previously whereas the slightly difference in sequence design for the allelic primers among these methods was noticed. The recent approaches for the high throughput for single nucleotide polymorphism detection included, particularly, microarray-based[15] and mass spectrometric-based techniques[16]. Relative to PCR-CTPP, both incur much higher cost. However, the former allows distribution in many smaller centers.

Nowadays, screening for hemoglobin disorders carriers among pregnant women is an interesting aspect of antenatal counseling in Thailand. The PCR-CTTP described here allows simple, reliable and will be well suited for further detection of Hb E variant.

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References

1. Vichinsky EP. Changing patterns of thalassemia worldwide. Ann N Y Acad Sci 2005; 1054: 18-24.
2. Rees DC, Styles L, Vichinsky EP, Clegg JB, Weatherall DJ. The hemoglobin E syndromes. Ann N Y Acad Sci 1998; 850: 334-43.
3. Fucharoen S, Winichagoon P. Hemoglobinopathies in Southeast Asia. Hemoglobin 1987; 11: 65-88.
4. 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.
5. Fucharoen S, Singsanan S, Sanchaisuriya K, Fucharoen G. Molecular and haematological characterization of compound Hb E/Hb Pyrgos and Hb E/Hb J-Bangkok in Thai patients. Clin Lab Haematol 2005; 27: 184-9.
6. Weatherall DJ, Clegg JB. The thalassemia syndromes. 4th ed. Oxford: Blackwell Science; 2001.
7. Hamajima N, Saito T, Matsuo K, Kozaki K, Takahashi T, Tajima K. Polymerase chain reaction with confronting two-pair primers for polymorphism genotyping. Jpn J Cancer Res 2000; 91: 865-8.
8. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. NewYork: Cold Spring Habor; 2001.
9. Schneider RG. Methods for detection of hemoglobin variants and hemoglobinopathies in the routine clinical laboratory. CRC Crit Rev Clin Lab Sci 1978; 9: 243-71.
10. Bain B. Hemoglobinopathy diagnosis. Oxford: Blackwell Science; 2001.
11. Fucharoen G, Tungwiwat W, Ratanasiri T, Sanchaisuriya K, Fucharoen S. Prenatal detection of fetal hemoglobin E gene from maternal plasma. Prenat Diagn 2003; 23: 393-6.
12. Sanchaisuriya K, Fucharoen G, Sae-ung N, Siriratmanawong N, Surapot S, Fucharoen S. Molecular characterization of hemoglobin C in Thailand. Am J Hematol 2001; 67: 189-93.
13. Liu Q, Thorland EC, Heit JA, Sommer SS. Overlapping PCR for bidirectional PCR amplification of specific alleles: a rapid one-tube method for simultaneously differentiating homozygotes and heterozygotes. Genome Res 1997; 7: 389-98.
14. Chiapparino E, Lee D, Donini P. Genotyping single nucleotide polymorphisms in barley by tetra-primer ARMS-PCR. Genome 2004; 47: 414-20.
15. Cremonesi L, Ferrari M, Giordano PC, Harteveld CL, Kleanthous M, Papasavva T, et al. An overview of current microarray-based human globin gene mutation detection methods. Hemoglobin 2007; 31: 289-311.
16. Tsang JC, Charoenkwan P, Chow KC, Jin Y, Wanapirak C, Sanguansermsri T, et al. Mass spectrometry-based detection of hemoglobin E mutation by allele-specific base extension reaction. Clin Chem 2007; 53: 2205-9.

การตรวจหาฮีโมโกลบิน อี โดยใช้เทคนิค PCR with confronting two-pair primers

สรศักดิ์ อินทรสูต, รัชนู ทองผึ่ง, ขจรศักดิ์ ตระกูลพัว, มงคล โชตยาภรณ์

***วัตถุประสงค์**: เพื่อพัฒนาและประยุกต์ใช้เทคนิค polymerase chain reaction with confronting two-pair primer (PCR-CTPP) ในการตรวจและแยกชนิดของฮีโมโกลบิน อี*

***วัสดุและวิธีการ**: ทำการศึกษาในคนไทยในเขตภาคเหนือจำนวน 50 ราย โดยทำการแยกสกัด ดีเอ็นเอ จากเม็ดเลือดขาว แล้วนำไปเพิ่มขยายปริมาณ ดีเอ็นเอ ด้วยเทคนิค PCR-CTPP จากนั้นนำผลไปวิเคราะห์เปรียบเทียบกับวิธี hemoglobin electrophoresis และ polymerase chain reaction-restriction fragment length polymorphism*

***ผลการศึกษา**: ทั้งสามวิธีการให้ผลการตรวจที่ตรงกัน และผลการตรวจประกอบด้วย ผู้ที่ไม่เป็นพาหะของฮีโมโกลบิน อี เป็นพาหะของอีโมโกลบิน อี และฮีโมโกลบิน อี ชนิดโฮโมซัยกัส คิดเป็นร้อยละ 74, 24 และ 2 ตามลำดับ*

***สรุป**: การตรวจและแยกชนิดของฮีโมโกลบิน อี โดยเทคนิค PCR-CTPP นั้นได้ประสบความสำเร็จ สามารถนำไปใช้เพื่อตรวจยืนยันผลและใช้ร่วมกับการตรวจหามิวเตชันชนิดอื่นในโรคธาลัสซีเมีย*