High Resolution Melting Real-Time PCR for Rapid Discrimination between *Brugia malayi* and *Brugia Pahangi*

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Objective: *To identify two closely related Brugia malayi and B. pahangi in cat reservoirs by using high resolution melting real-time PCR (HRM real-time PCR)*
Material and Method: *HRM analysis on the Corbett Rotor-Gene 6000 instrument was used to test 5 Brugia specimens by using five sets of specific primers for HhaI repetitive region (HR), small heat shock protein (SHP), small subunit ribosomal DNA (18S rDNA), internal transcribed spacer region (ITS), and trans-spliced leading Exon I gene (SLX1).*
Results: *HRM analysis of ITS and SLX clearly generated 2 profiles of B. malayi and B. pahangi while those of HR, 18S rDNA, and SHP could classify B. pahangi.*
Conclusion: *HRM is a simple and rapid method for identification of two closely related B. malayi and B. pahangi in which it can detect both parasites within 30 min after real-time PCR detection. This assay is probe-free HRM and reduces a risk of PCR carryover. It does not require multiplex methods and DNA sequencing; therefore, HRM provides a new approach for genetic screening and rapid detection of closely related species in a clinical laboratory.*

Keywords: *Brugia, HhaI repetitive region, Small heat shock protein, Small subunit ribosomal DNA, Internal transcribed spacer, Tran-spliced leading Exon I gene, High- melting resolution, PCR, High resolution melting*

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The lymphatic filariasis is abundant and widely spreads in the tropical and subtropical areas. *Brugia malayi* is mainly distributed in Asian countries such as China, South Korea, Japan, India, Myanma, Indonesia, Malaysia, Bornaie islands, the Philippines, and Thailand. It has been reported that *B. malayi* has infected not only human, but also animals such as cats, monkeys, and dogs[1-3]. Hence, these animal reservoirs play an important role as the disease carriers which can lead to the problem of eradication in endemic area. *B. pahangi* is an important filarial parasite that can infect cats. It could coexist in many of the same hosts as the zoonotically transmitted subperiodic *B. malayi*[2-6]. Previously, *B. malayi* and *B. pahangi* were proven for their concomitance in cat reservoirs by using phylogenetic analysis of internal transcribed spacer region I nucleotide sequences[7]. Hence, the identification of the two filarial species is important for dynamic study of the parasites in cat reservoirs.

Basically, differentiation of *B. malayi* and *B. pahangi* microfilaria has been routinely attained by using traditional Giemsa staining. However, it cannot morphologically distinguish between those two species eventhough the technique is convenient and inexpensive. Instead, acid phosphatase staining is efficient, but it is not reproducible and the procedure is complicated. The PCR based methods such as PCR-RFLP have been established as a tool for discrimination of *B. malayi* and *B. pahangi*[8-12]. Despite the high sensitivity and specificity of PCR, this method

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cannot recognize mixed infection between closely related species. Additionally, PCR based techniques take about 2-3 hrs and the gel electrophoresis containing ethidium bromide, a carcinogenic agent, which is not appropriate for rapid detection.

HRM real-time PCR was first demonstrated by Wittwer and co-workers with the double-stranded DNA-specific dye SYBR Green I and has since seen widespread adoption in real-time PCR applications[13]. Melting curve analysis provides immediate practical benefits in real-time PCR, obviating the need for gel electrophoresis by providing a reproducible signature of the amplified DNA sequence that may be used for typing PCR products[14]. Typing is typically achieved by examining the first derivative of the melting curve and identifying the characteristic "melt peak" (*Tm*), which is the temperature at which the rate of fluorescence change (DNA denaturation) is highest and is observed in the raw data as a sudden decrease in fluorescence[14]. The melting curve assays can be applied for targeting single nucleotide polymorphisms. This assay is a probe-free HRM-real time PCR that does not require multiplex method and DNA sequencing providing a new approach for genetic screening and rapid detection of closely related species in a clinical laboratory.

This study has employed the HRM real-time PCR for identification of *Brugia* spp. in cat reservoirs based on several key genes/regions such as internal transcribed spacer region (ITS), trans-spliced leading Exon I gene (SLX1), H*ha*I repetitive region (HR), small heat shock protein (SHP), and small subunit ribosomal DNA (18S rDNA). The distinction in melting curve data could be practical and beneficial for dynamic survey and epidemiological studies of the parasites.

Material and method

Blood Samples

Blood samples of 5 naturally *Brugia* infected cats were collected from endemic areas of Thailand. Three feline blood samples were collected at the *B. malayi* endemic area, Narathiwas. Another two feline blood samples were collected at *B. malayi* non-endemic area, Lad Krabang district of Bangkok. The samples were previously screened by using the traditional Giemsa staining technique before undergoing the parasite isolation.

Parasite isolation

Five milliliters of microfilaria infected blood were taken from the host and transferred to a test tube containing 7 mg/ml of EDTA as an anticoagulant agent. The blood was diluted with equal volume of phosphate buffer saline (PBS), pH 7.0 (0.137 M NaCl, 10 mM Na_2HPO_4, 3.2 mM KH_2PO_4) and filtered through a 5-μm polycarbonate membrane (Millipore Corporation, Bedford, MA, USA). Microfilariae were then re-suspended in PBS and centrifuged at 1960 g for 10 min at 4°C. The pellet of microfilariae was washed with PBS for three times prior to storage at -70°C until use.

Real-time PCR and high resolution melting (HRM)

Five sets of primers specific to region of H*ha*I repetitive region (HR), small heat shock protein (SHP), small subunit ribosomal DNA (18S rDNA), internal transcribed spacer region (ITS), and trans-spliced leading Exon I gene (SLX1) were designed (Table 1). The two closely related sequences used in this study were amplified by typical PCR, then cloned into a plasmid vector and finally determined by DNA

Table 1. Primer sequences, melting temperatures, and amplicon size for High resolution melting analysis

Genes/regions	Primers	Nucleotide sequences (5'→ 3')	*Tm* (°C)	Amplicon size (bp)
ITS	ITS1F	GGT GAA CCT GCG GAA GGA TC	75	~1200
	ITS2R	AGC GGG TAA TCA CGA CTG	45	
SHP	Bmshsh/F	ATG GCT CGA CGT TCG CTA ATT T	70	439
	Bmshsh/R	GTA TTG TCC TTG TTT GCG CCT C	68	
18S rDNA	Bmsr/F	AAT ACA TGC ACC AAA GCT CCG A	69	1641
	Bmsr/R	CCT TCC GCA GGT TCA CCT ACG	71	
SLX1	SLX1	GTC TAC GAC CAT ACC ACG TTG	64	294
	SLX2	GAA ACA TTC AAT TAC CTC AAA C	58	
HR	BM1	GCG CAT AAA TTC ATC AGC AA	64	280
	BM2	ATG ACA ACA CAA TAC ACG AC	64	

ITS, internal transcribed spacer region: SHP, small heat shock protein: 18S rDNA, small subunit ribosomal RNA: SLX, Trans-spliced leader exon 1 gene: HR, H*ha*I repetitive regions

sequencing (Macrogen, Korea). Real-time PCR mixture was prepared using 50 ng of DNA, 10 iL of 2X QuantiMix Probe (Biotools, Germany), 2 µM of SYTO 9 (1:100), and primers were mixed to the final concentration of 20 µM. The intercalating dye was SYTO 9 (Invitrogen, USA). Real-time PCR reactions and HRM analysis were performed on Rotor-Gene 6000™ (Corbett Research, Cybeles, Thailand). The Real-time PCR profile comprised of one initial cycle of 95°C for 2 min and followed by 40 cycles of 95°C for 10 sec, 58°C for 15 sec, 72°C for 20 sec. After real-time PCR amplification, HRM was performed using melting profile from 72 to 95°C rising at 0.2°C per sec. The melting curves were normalized by the software provided by the company[15].

The specificity of primers used in the experiment was confirmed by a single amplicon melting analysis. Data form HRM analysis was determined using Rotor-Gene 6000 series 1.7 software. All of the analyzed data was achieved according to the manufacturer's protocol.

Results

The HRM analysis of the PCR products was examined and used the melting profiles as molecular finger prints for distinction of *B. malayi* and *B. pahangi*. Within 30 min after real-time PCR detection, the two species could be distinguished. The HRM of *B. malayi* (Cats 1, 2, 4) and *B. pahangi* (Cats 6 and 7) based on internal transcribed spacer region (ITS), and trans-spliced leading Exon I gene (SLX1) clearly generated 2 profiles (Fig. 1a, b) while those of small subunit ribosomal DNA (18S rDNA), small heat shock protein (SHP), H*ha*I repetitive region (HR) could classify *B. pahangi* profile from the others (Fig. 1c-f). The melting profiles were consistent with minimal variability in each test. The data were corresponded to the sequencing analysis of each tested genes/regions with nucleotide

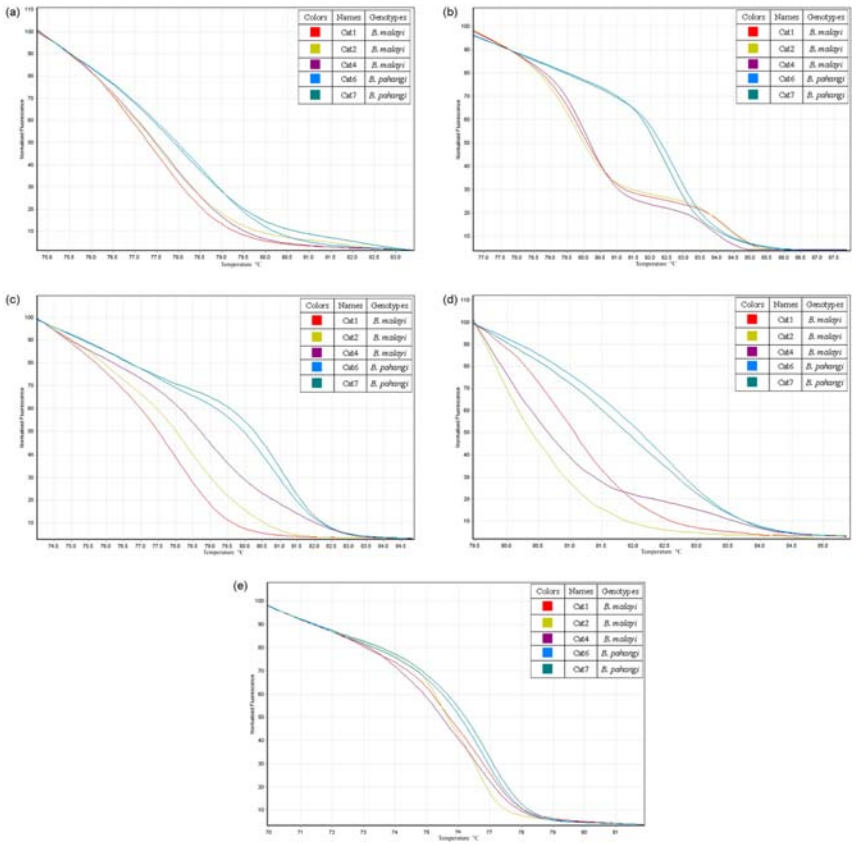

Fig. 1 HRM profiles of 5 key genes/regions (a) ITS, (b) SLX, (c) 18S rDNA, (d) SHP, and (e) HR using 5 sets of specific primers for real-time PCR amplification

sequences sharing greater than 95% identity. Hence, an HRM database and a working protocol were created for the differentiation of these two parasites.

Discussion

It has been known that *B. malayi* and *B. pahangi* are morphologically similar. Species discrimination by using traditional Giemsa staining may not be adequate, even if it is inexpensive to carry out. Nucleotide sequence comparison of the coding and non-coding genes between these two species revealed that they are highly homologous. For example, the 18S rDNA nucleotide sequences of both species showed high similarity up to more than 95%. Similarly, the cuticle, heat protein**,** heat shock protein, trans-spliced leading exon 1, the H*ha*I repetitive region, and glutathione exhibited few differences in nucleotide sequences between the two *Brugia* species.

The HRM is a simple and rapid scanning method that can dramatically reduce the amount of sequencing and requires no multiplexing or hybridization probes. The distinction in melting curve between *B. malayi* and *B. pahangi* could be effective for dynamic survey and epidemiological studies as well as genetic inspection of these two closely related species.

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เทคนิค high resolution melting real-time PCR ในการตรวจสอบเชื้อ Brugia malayi และ Brugia pahangi ได้อย่างรวดเร็ว ในแมวที่เป็นโรค

สุพัตรา อารีกิจ, พรพิมล กาญจนวาศ, อาชว์ดาม์ ภาคพิธเจริญ, ไพศาล ขาวสัก, สินทวี คู่เจริญถาวร, ทายาท ศรียาภัย, โกสุม จันทร์ศิริ

วัตถุประสงค์: *เพื่อตรวจวิเคราะห์ความแตกต่างของเชื้อ พยาธิเท้าช้างชนิด Brugia malayi และ B. pahangi ในแมวรังโรค โดยเทคนิค high resolution melting real-time PCR (HRM real-time PCR)*

วัสดุและวิธีการ: *ใช้เทคนิคการวิเคราะห์ HRM โดยเครื่อง Corbett Rotor-Gene 6000 มาใช้ตรวจสอบพยาธิเท้าช้างชนิดบรูเกีย โดยใช้ไพร์เมอร์ที่แตกต่างกันทั้งหมด 5 คู่ ที่มีความจำเพาะในแต่ละบริเวณคือ HhaI repetitive region (HR), small heat shock protein (SHP), small subunit ribosomal DNA (18S rDNA), internal transcribed spacer region (ITS) และ trans-spliced leading Exon I gene (SLX1)*

ผลการศึกษา: *จากการวิเคราะห์ ด้วยวิธี HRM พบว่าไพร์เมอร์ที่จำเพาะต่อจีน ITS และ SLX1 สามารถนำมาใช้แยกกลุ่มของเชื้อ B. malayi และ B. pahangi ได้อย่างชัดเจน ในขณะที่ไพร์เมอร์ที่จำเพาะต่อจีน HR, 18S rDNA, และ SHP สามารถแยกกลุ่มของ B. pahangi ได้*

สรุป: *โดยเทคนิค HRM เป็นวิธีที่ง่าย และรวดเร็ว สามารถนำมาใช้วิเคราะห์ความแตกต่างของ พยาธิเท้าช้างชนิด Brugia malayi และ B. pahangi ในแมวรังโรคได้ ซึ่งเทคนิค HRM ใช้เวลาในการวิเคราะห์ 30 นาทีหลังจากการทำ real-time PCR อีกทั้งยังลดการปนเปื้อนในการทำ PCR และวิธีการนี้ไม่ต้องทำ multiplexing และ DNA sequencing จึงเป็นวิธีใหม่ในการวิเคราะห์ genetic screening และวิเคราะห์ species ที่มีความใกล้เคียงกันได้อย่างรวดเร็ว*