DETECTION OF *EHRLICHIA CANIS* IN CANINE BLOOD SAMPLES BY REAL-TIME FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) PCR AND MELTING CURVE ANALYSIS

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Abstract. *Ehrlichia canis* is a small pleomorphic gram-negative, coccoid, obligatory intracellular bacterium and the cause of canine monocytic ehrlichiosis. A real-time fluorescence resonance energy transfer polymerase chain reaction (real-time FRET PCR) coupled with melting curve analysis was established for detection of *E. canis* infection in canine blood samples. The *VirB9* gene was amplified using one pair of primers and the melting curve analysis was generated by heating the hybridizing probes and amplified products. Eight *E. canis*-infected dog blood samples were initially identified using the Giemsa staining/microscopic method followed by conventional PCR (cPCR)/Sanger sequencing for confirmation. The sensitivity and specificity of the real-time FRET PCR detection were 87.5% and 100%, respectively and the limit of detection was 6.6 x 10^3 copies of positive *E. canis* control plasmids. The real-time FRET PCR with melting curve analysis reported here is better than microscopic visualization or cPCR because the method is not affected by the false bias inherent in the microscopic method. Furthermore, many samples can be processed rapidly at the same time. This convenient tool is beneficial as an alternative assay for the epidemiologic study of canine ehrlichiosis as well as for eradication of these organisms in prevention and control programs in endemic areas.

Keywords: *Ehrlichia canis*, detection, real-time FRET PCR, melting curve analysis

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INTRODUCTION

Ehrlichia canis (Alphaproteobacteria, order Rickettsiales, family Anaplasmataceae) is a small pleomorphic gram-

negative, coccoid, obligatory intracellular bacterium, which parasitizes circulating monocytes, forming clusters of individuals (morulae) in the cytoplasm. It is the causative agent of canine monocytic ehrlichiosis (CME) and is transmitted by the brown dog tick (*Rhipicephalus sanguineus*) (Groves *et al*, 1975; Dumler *et al*, 2001; Skotarczak, 2003; Bowman, 2011). *Ehrlichia canis* may also be a cause of human ehrlichiosis (Perez *et al*, 2006). Microscopic visualization of the typical cytoplasmic *E. canis*-morulae within monocytes in blood smears strongly supports a diagnosis of CME (Harrus and Waner, 2011). In addition, blood smear analysis also has low sensitivity due to the low level of the bacteremia and provides only supportive evidence of infection because of the potential for misinterpreting toxic granulation, various cytoplasmic inclusions and staining artifacts (Thomas *et al*, 2009; Schotthoefer *et al*, 2013). Serological tests can assist in reaching a diagnosis of *E. canis* infection. However, the presence of antibodies may be indicative of past exposure to the pathogen and does not reveal the duration of infection and nor the severity of disease (Neer *et al*, 2002). Conventional polymerase chain reaction (cPCR) using primers based on 16S ribosomal RNA (rRNA) (Suksawat *et al*, 2001) or the *VirB9* gene (Kledmanee *et al*, 2009) has been reported as a sensitive assay for detection of acute monocytic ehrlichiosis in dogs. Quantitative real-time PCR (qPCR) is also a reliable, sensitive and specific method for the detection of *Ehrlichia* spp in the blood or in tissue culture (Branger *et al*, 2004; Bell and Patel, 2005; Sirigireddy and Ganta, 2005). A real-time fluorescence resonance energy transfer (FRET) PCR-based technique, was developed for detection and differentiation of a heat shock protein (hsp) operon *groEL* genes of *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in human blood samples. The analytical sensitivity was high and melting curve analysis can be used for species differentiation (Bell and Patel, 2005). The applicability of the real-time FRET PCR for detection of *E. canis* in dog blood samples, however, has not yet been demonstrated.

In this study, the real-time FRET PCR followed by melting curve analysis was established for the detection of *E. canis* infection in dog blood samples. The method used one pair of specific primers and probes, designed from the *VirB9* gene of *E. canis* (GenBank accession number AY205342). The performance was evaluated with other defined genomic DNA controls and blood samples from healthy dogs. The aim was to develop an accurate, sensitive and specific real-time FRET PCR method for detection of *E. canis*.

MATERIALS AND METHODS

Source of blood specimens and control DNAs for sensitivity and specificity

A total of 13 EDTA dog blood samples infected with *E. canis* (*n*=8), *Hepatozoon canis* (*n*=1), *Babesia canis vogeli* (*n*=1), *Anaplasma platys* (*n*=1), *Brugia pahangi* (*n*=1) or *Dirofilaria immitis* (*n*=1) were obtained from a private animal hospital, Bangkok, Thailand. Control blood samples (from one healthy dog and one healthy cat) were obtained from the same source. In addition, extracted DNAs of *Plasmodium falciparum* (*n*=1), *Trypanosoma evansi* (*n*=1), *Leishmania siamensis* (*n*=1), *Wuchereria bancrofti* (*n*=1), *Brugia malayi* (*n*=1) and human white blood cells (*n*=1) were received from the frozen sample bank at the Department of Parasitology, Faculty of Medicine, Khon Kaen University and were also used as specificity controls.

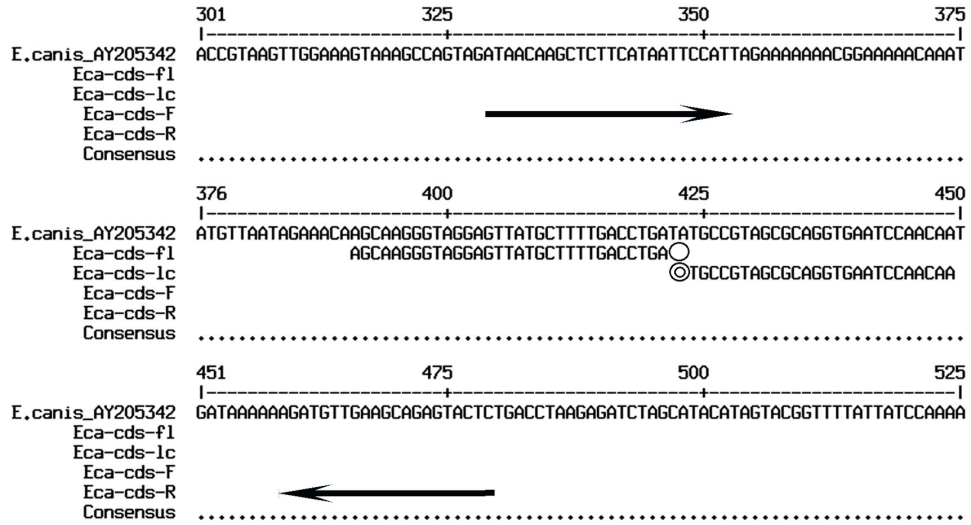

Fig 1–Schematic illustration of the hybridization sites of the PCR primers (Eca-cds F and Eca-cds R primer, arrows) and anchor and sensor probes for the *VirB9* gene from *E. canis*. The Eca-cds-fl probe was labeled with fluorescein at the 3′ end and served as the anchor probe for the sensor Eca-cds-lc probe that was labeled with LightCycler Red 640 fluorophore (LC red 640) at the 5′ end. Circle, fluorescein; double circle, LC red 640.

All *E. canis*-infected dog blood samples had been initially identified using the Giemsa staining/microscopic method and the diagnosis confirmed by cPCR/Sanger sequencing. The nucleotide sequences obtained are available in the GenBank database under the accession numbers: KJ459913-KJ459920.

Preparation of specimens for real-time PCR

Of each blood specimen, 200 µl was put into 1.5-ml microcentrifuge tube, homogenized with disposable polypropylene pestles (Bellco Glass, Vineland, NJ) and extracted using the Nucleospin Tissue kit (Macherey-Nagel, Duren, Germany). The DNAs were eluted in 50 µl of 5 mM Tris/HCl, pH 8.5 of which 5 µl were used in the PCR reaction.

LightCycler-based PCR assay

The LightCycler PCR and detection system (Roche Applied Science, Mannheim, Germany) was used for amplification and quantification. The PCR was performed in glass capillaries. The specific primer pairs for *E. canis* detection are Eca-cds-F (5′ ATA ACA AGC TCT TCA TAA TTC CAT 3′) and Eca-cds-R (5′ GAG TAC TCT GCT TCA ACA TCT 3′) (Sigma-Proligo, Singapore). For amplification detection, the LightCycler FastStart DNA Master HybProbe Kit (Roche Applied Science) was used as recommended by the manufacturer. Briefly, a pair of adjacent oligoprobes was hybridized with the *E. canis VirB9* gene. One probe had been labeled at the 5′ end with the LightCycler Red 640 fluorophore (5′ Red 640 TGC CGT AGC GCA GGT GAA TCC AAC AA -Phosphate 3′; Eca-cds-lc) and the other probe had been labeled at the 3′ end with a 530 fluorescein (5′ AGC AAG GGT AGG AGT TAT GCT TTT GAC CTG A -Fluo 530 3′; Eca-cds-fl) (TIB Molbiol GmbH, Berlin, Germany). Probes and primers were designed using the LC probe design software (Roche Applied Science). A schematic diagram of primers and probes is shown in Fig 1.

When hybridized to the same DNA strand internal to the PCR primers, the probes are in close proximity and produce a FRET. The FRET occurs when the donor fluorophore on the 3′ end of the anchor probe (Eca-cds-fl) is excited by an external energy source and transfers its energy to the adjacent acceptor fluorophore on the 5′ end of the second hybridization sensor probe (Eca-cds-lc) which then emits fluorescence light. This energy transfer results in the emission of a detectable signal by the acceptor fluorophore molecule when the 2 probes are annealed in adjacent positions to target DNA. The probes are displaced from the template during denaturation, thus changing fluorescence is directly proportional to the quantity of PCR product generated during the PCR process.

The PCR mixture contained 1X LightCycler Faststart DNA Master HybProbe (Roche Applied Science), 1 mM $MgCl_2$, 0.2 μM Eca-cds-F, 0.2 μM Eca-cds-R, 0.2 μM Eca-cds-fl and 0.2 μM Eca-cds-lc probes. The total reaction volume was 20 μl. The samples were pre-incubated for 10 minutes at 95°C, then subjected to 45 cycles of successive denaturation (10 seconds at 95°C), annealing (30 seconds at 50°C), and extension (10 seconds at 72°C). After amplification, a melting curve was produced by heating the reaction mixture to 95°C at 20°C/second, cooling it to 65°C, keeping it at 65°C for 30 seconds and then slowly heating it at 0.1°C/second to 80°C. The fluorescence intensity change was measured throughout the slow heating phase.

In order to determine the specificity of the oligonucleotide hybridization based on the FRET technique, DNA extracted from control samples, other than *E. canis*-infected dog blood samples, were analyzed separately. Each run contained at least one negative control consisting of 5 μl distilled water. For improved visualization of the melting temperatures (*Tm*) and melting peaks they were derived as previously described (Intapan *et al*, 2008).

Positive control plasmids

A positive control plasmid was constructed by cloning a PCR product of the *E. canis* target gene (obtained using the primers Eca-cds-F and Eca-cds-R) into the pGEM-T Easy vector (Promega, Madison, WI) according to the protocol of the manufacturer. The plasmids were propagated in *Escherichia coli* and the identity of the inserted gene confirmed by sequencing in both directions.

RESULTS

Standardization of the real-time PCR

The sensitivity of real-time FRET PCR was evaluated using 5 μl of 10-fold serial dilutions of *E. canis* (ranging from 6.6×10^8 to 6.6 copies/reaction) in distilled water. The lower limit of detection was 6.6×10^3 copies of the *E. canis* positive control plasmid (Fig 2), when considering 40 cycles as the cut-off. Furthermore, no fluorescence signal was detected when purified DNA was tested from *H. canis*, *B. canis vogeli*, *A. platys*, *B. pahangi*, *D. immitis*, *P. falciparum*, *T. evansi*, *L. siamensis*, *W. bancrofti*, *B. malayi*, the non-infected dog and cat blood samples and human white blood cells (Fig 3).

Real-time FRET PCR and melting curve analysis to differential detect *E. canis* in dogs

Seven of the eight *E. canis*-infected dog blood samples were positive. The real-time FRET PCR successfully amplified a predicted 151-bp product from the DNA of the organism (Fig 4). The melting curve analyses are shown (Fig 3). The mean ± SD of *Tm* value of *E. canis* infected

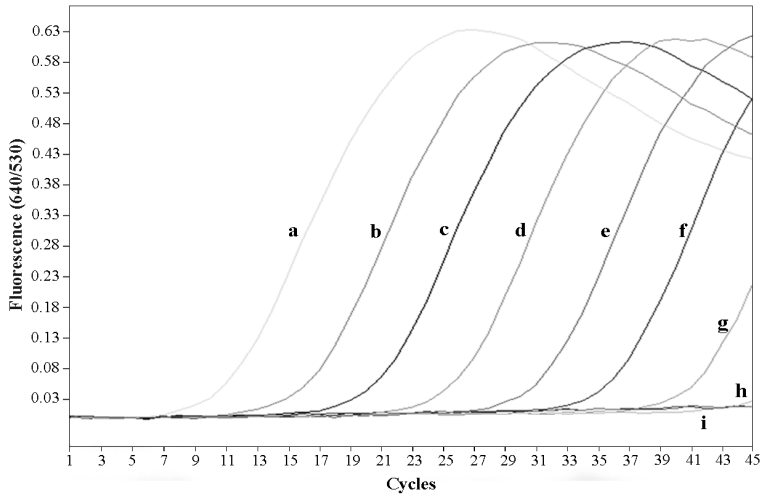

Fig 2–Amplification curves: the y-axis indicates fluorescence and the x-axis shows number of PCR cycles. These curves show the analytical sensitivity of real-time FRET PCR for detecting *E. canis* control plasmid DNA in tenfold dilutions from 6.6 x 10^8 to 6.6 copies/reaction (a-i, respectively); distilled water (h).

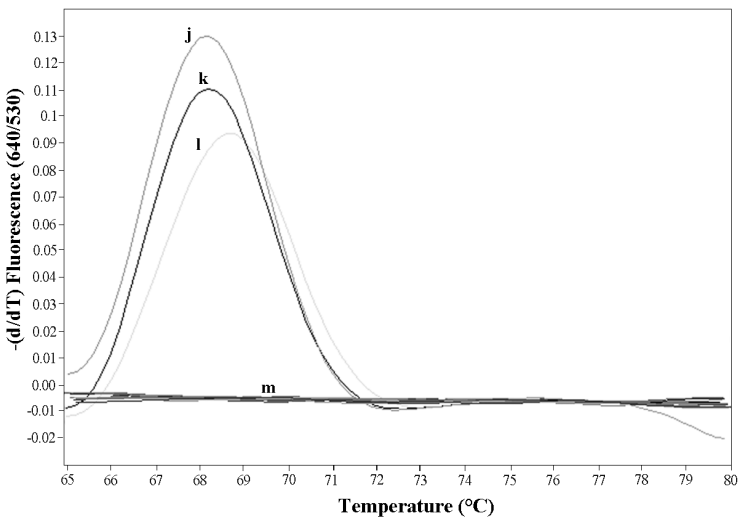

Fig 3–Representative melting curve analyses of fluorophore-labeled probes hybridized to the amplification products of *E. canis VirB9* gene. The melting peak of *E. canis* (j, k), *E. canis* positive control plasmid (l) and DNAs of *Hepatozoon canis*, *Babesia canis vogeli*, *Anaplasma platys*, *Brugia pahangi*, *Dirofilaria immitis*, *Plasmodium falciparum*, *Trypanosoma evansi*, *Leishmania siamensis*, *Wuchereria bancrofti*, and *Brugia malayi*, negative dog and cat blood samples, human white blood cells and distilled water (m).

dog blood samples was 68.34 ± 0.38 (n=7). No *Tm* value was shown in negative blood samples or other control DNAs. The diagnostic values were 87.5% sensitivity and 100% specificity.

DISCUSSION

Real-time PCR has progressively superseded cPCR for detection of infectious diseases because of its highly improved

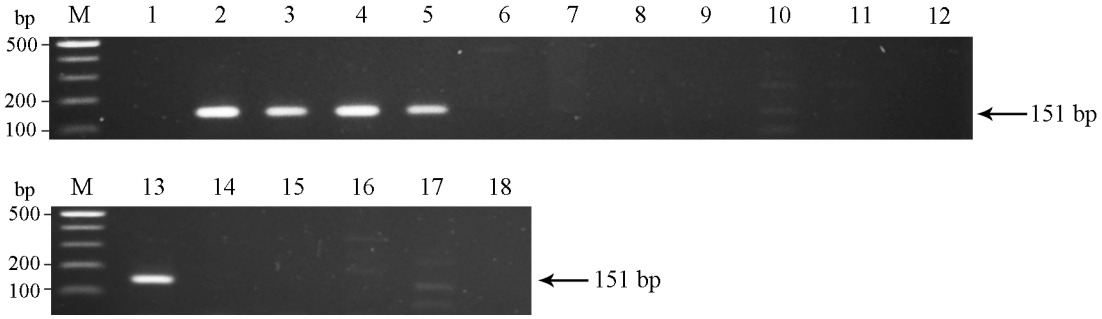

Fig 4–Amplification products visualized in a 1% agarose gel stained with ethidium bromide. The arrows indicate the 151-bp *Ehrlichia canis*-specific band. All DNA samples (1 ng each) were evaluated by the real-time fluorescence resonance energy transfer (FRET) PCR. Lane "M" shows a 1 kb-plus DNA ladder; negative controls containing no DNA (lane 1); *E. canis* control plasmid (lanes 2 and 13; Cn=20.01), *E. canis*-infected dog blood samples (lanes 3-5; Cn=39.29, 32.63, 35.42, respectively), *Brugia malayi* (lane 6: Cn>40), *Brugia pahangi* (lane 7: Cn>40), *Wuchereria bancrofti* (lane 8: Cn>40), *Dirofilaria immitis* (lane 9: Cn>40), *Babesia canis vogeli* (lane 10: Cn>40), *Hepatozoon canis* (lane 11: Cn>40), *Trypanosoma evansi* (lane 12: Cn>40), *Leishmania siamensis* (lane 14: Cn>40), *Plasmodium falciparum* infected human (lane 15: Cn>40), healthy cat (lane 16: Cn>40), healthy dog (lane 17: Cn>40) and human genomic DNA (lane 18: Cn>40).

capability of molecular detection. The method not only allows for differentiating species or strains of several pathogenic agents by melting curve analysis, but is also rapid, accurate and can measure the specific DNA quantities in samples (Lyon and Wittwer, 2009). Here, a real-time PCR strategy is proposed that can be used not only for the diagnosis of canine monocytic ehrlichiosis, but also for epidemiological study (Socolovschi *et al*, 2012). Recently, qPCR techniques for detection of *Ehrlichia* spp in infected ticks, humans, dogs and capybaras, using genus-specific primers from the *dsb* gene of *Ehrlichia* species, have been reported and gave high sensitivity and specificity (Labruna *et al*, 2007). In addition, a qPCR assays were developed for diagnosis of *E. canis* DNA in hard ticks (Ionita *et al*, 2013), cats (Sasaki *et al*, 2012) and dogs (Peleg *et al*, 2010). The *Taqman* minor grove binding based real-time PCR assay for detection of the *E. canis p30* gene in infected ticks revealed high efficiency and specificity (Ionita *et al*, 2013). Moreover, a dual-labeled probe-based multiplex real-time PCR for the simultaneous detection of *E. canis* and *B. canis vogeli* was recently demonstrated in infected dogs by using different target genes, 16S rRNA and *hsp70*. The detection limit was 1-10 copies/µl for plasmid internal controls and for parasites and low interference was observed (Peleg *et al*, 2010). However, the method when used for differential detection of the *E. canis* 16S rRNA gene in infected cats had low specificity and sensitivity (Sasaki *et al*, 2012).

Kledmanee and others (2009) developed multiplex cPCR for detection of canine blood parasites, using primers based on the *E. canis VirB9* gene. The study found that the samples which were positive for *Ehrlichia* spp by inspection of stained blood films were also positive by multiplex cPCR. Here, it was developed

for the rapid real-time FRET PCR to detect it using specifically designed *VirB9* gene primers and probes. The lower limit of detection of *E. canis* positive control plasmid was acceptable (6.6 x 10^3 copies/reaction) when comparison with the real-time FRET PCR laboratory setting of previous report (Intapan *et al*, 2008). The method also had 100% specificity. No fluorescence was detected from other parasites. The sensitivity of this method, however, is also quite similar to previous reports (Peleg *et al*, 2010; Ionita *et al*, 2013). The real-time PCR has been used for the quantification of ehrlichial load in experimentally and naturally infected dogs (Baneth *et al*, 2009). It is less prone to contamination than cPCR.

In conclusion, the real-time FRET PCR with melting curve analysis reported here is better than microscopic visualization because this method is not affected by bias inherent in the microscopic method. Furthermore, many samples can be done rapidly at the same time. This convenient tool may be beneficial as an alternative assay for epidemiologic studies of canine ehrlichiosis as well as for a monitoring tool in eradication of these organisms in endemic areas.

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