RESEARCH NOTE

DEVELOPMENT OF MULTIPLEX POLYMERASE CHAIN REACTION FOR DETECTION OF *EHRLICHIA CANIS*, *BABESIA* SPP AND *HEPATOZOON CANIS* IN CANINE BLOOD

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Abstract. A multiplex polymerase chain reaction (PCR) has been developed for simultaneous detection of canine blood parasites, *Ehrlichia canis*, *Babesia* spp and *Hepatozoon canis*, from blood samples in a single reaction. The multiplex PCR primers were specific to *E. canis* VirB9, *Babesia* spp 16S rRNA and *H. canis* 16S rRNA genes. Specificity of the amplicons was confirmed by DNA sequencing. The assay was evaluated using normal canine and infected blood samples, which were detected by microscopic examination. This multiplex PCR offers scope for simultaneous detection of three important canine blood parasites and should be valuable in monitoring parasite infections in dogs and ticks.

INTRODUCTION

The geographical distribution of brown dog-tick, *Rhipicephalus sanguineus*, in tropical and semi-tropical regions can lead to many serious tick-borne diseases in dogs, such as ehrlichiosis, hepatozoonosis and babesiosis (Shaw *et al*, 2001). Canine ehrlichiosis is an important and potentially fatal disease caused by intracellular bacteria *Ehrlichia canis*, which parasitize monocytes and granulocytes. Chronic ehrlichiosis is associated with irreversible bone marrow destruction (Skotarczak, 2003). *Hepatozoon canis* causes hepatozoonosis and massive parasitemia imposes a toll on the host by consuming nutrients and causing direct injury to affected tissues, ultimate producing cachexia (Baneth *et al*, 2003). *Hepatozoon canis* commonly infects dogs in Asia and also is associated with co-infection of other diseases (Shaw *et al*, 2001). Babesiosis caused by hemoprotozoa, *Babesia* spp, *Babesia gibsoni* and *Babesia canis*, which infect dogs worldwide. The severity of babesiosis is related to the extent of parasite replication in the host's red blood cells with subsequent cell lysis.

Infection with multiple tick-transmitted pathogens can occur in animals. The same tick species can be a vector for several pathogens and co-infection of individual ticks can occur. In dogs, co-infection of *Ehrlichia*, *Babesia* and *Hepatozoon* occurs in endemic areas and could partially explain variations in clinical presentation, pathogenicity and response to therapy (Shaw *et al*, 2001).

Microscopic detection of blood parasites

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is more fruitful in an acute than in sub-clinical or chronic phase. The tendency for chronic and/or sub-clinical disease to be associated with cryptic infection reduces sensitivity of microscopic examination and does not allow intraspecies differentiation (Shaw *et al*, 2001). Serological assays that detect antibodies, such as indirect immunofluorescent antibody and dot-ELISA tests, are very sensitive in detecting the prevalence of exposure to parasites or cross reactivity with other species, but can be misleading especially in endemic areas of these diseases (Waner *et al*, 2001; Harrus *et al*, 2002; Mylonakis *et al*, 2003). However, they are not useful for determining current infection status or assessing clearance of parasites after antibiotic treatment (Wen *et al*, 1997).

Therefore, a more sensitive, specific and simple method to directly detect the organisms is desirable. That led to the development of multiplex PCR, a one-step PCR, to detect *E. canis*, *Babesia* spp and *H. canis*, which can be useful for the selection of specific treatment to each disease or co-infection and assessing clearance of parasites after treatment (Nelson and Couto, 2003).

MATERIALS AND METHODS

Blood samples

Parasite-positive blood and healthy dog blood samples were collected from the Veterinary Teaching Hospital, Faculty of Veterinary Science, Mahidol University, Thailand. Infected blood samples were identified by Wright-Giemsa staining and morphology examined under a light microscope.

Extraction of parasite DNA

Parasite DNA was extracted by proteinase K digestion and phenol/chloroform/isoamyl (25:24:1) extraction as previously described (Sambrook and Russell, 2001). In brief, 600 µl of lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% w/v SDS) were added to 300 µl of whole blood and the solution was incubated with proteinase K (200 µg/ml) for 4 hours at 56ºC. Samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) solution and DNA was precipitated with ethanol. DNA was dissolved in 20 µl of 10 mM Tris-HCl pH 8.0 containing 1mM EDTA. The quality of DNA preparation was assessed by electrophoresis in 0.8% agarose gel.

Primers design

Six sequences of virB9 protein gene of *E. canis* (accession number AF546158, AY205339, AY 205340, AY205341, AY205342, AY205343), 8 sequences of the 18S rRNA gene of *Hepatozoon* spp (accession number AF176835, AY461377, AY461378, AY864678, AF176835, AF130361, AF110244, AY252105) and 10 sequences of the 18S rRNA gene of *Babesia* spp (accession number AF158702,

Table 1
Oligonucleotide primers used in multiplex PCR.

Pathogen	Primer	Sequences (5´ to 3´)	Length (bases)	Size of product
Ehrlichia canis	Ehr1401F	CCATAAGCATAGCTGATAACCCTGTTACAA	30	380 bp
	Ehr1780R	TGGATAATAAAACCGTACTATGTATGCTAG	30	
Babesia spp	Ba103F	CCAATCCTGACACAGGGAGGTAGTGACA	28	619 bp
	Ba721R	CCCCAGAACCCAAAGACTTTGATTTCTCTCAAG	33	
Hepatozoon canis	Hep001F	CCTGGCTATACATGAGCAAAATCTCAACTT	30	737 bp
	Hep737R	CCAACTGTCCCTATCAATCATTAAAGC	27	

AY027815, AY048113, AY072926, AY102164, AY278443, AY527063, AY534602, AY572457, AY649326) from GenBank were used for multiple alignments by using the BioEdit v7.0.4 software. The consensus regions of these genes were selected for primer designing. All primer pairs were aligned with non-target nucleotides including *Dilofilaria immitis* (accession number AB192887, AJ537512, AB004253, AR581080) and *Haemobartonella felis* (accession number AY529629, AY150987, AY150977, AY150986) to diminish the nonspecific-binding. The sequences of the primers are listed in Table 1.

Multiplex PCR amplification

Multiplex PCR was carried out in a solution containing 5 µl of extracted DNA and 45 µl of 0.4 pmol of each primer, 300 µM of each dNTP (QIAGEN®, Germany), 4 units of HotStar*Taq* DNA Polymerase (QIAGEN®, Germany), 1xPCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$ and ultrapure sterile water. Amplification was performed in a thermocycler (PTC-200, MJ Research, Water Town, MA) and thermocycling consisted of one step of 15 minutes at 95ºC followed by 30 cycles of 45 seconds at 94ºC, 45 seconds at 65ºC, and 90 seconds at 72ºC with a final extension step of 10 minutes at 72ºC. The amplicons were separated by electrophoresis in 2.5% agarose gel in 40 mM Tris-acetic acetate pH 8.4, 1 mM EDTA, stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

RESULTS

We have developed the multiplex PCR for simultaneously detection of three blood parasites (*Ehrlichia canis*, *Babesia* spp and *Hepatozoon canis*) in canine blood sample. Representative results of the multiplex PCR are shown in Fig 1. To confirm the multiplex

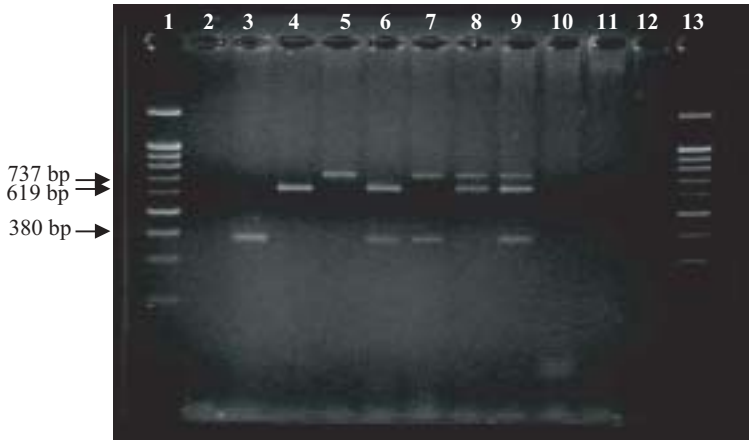

Fig 1–Agarose gel-electrophoresis of amplicons of *E. canis* VirB9, *Babesia* spp 16S rRNA and *H. canis* 16S rRNA genes. DNA extracted from healthy blood samples or parasite-positive blood samples were analyzed by multiplex PCR using specific primers for *E. canis*, *Babesia* spp, and *H. canis*. Lane 1 and 13: 100-basepair molecular size marker (New England Biolab™, USA); lane 2: healthy canine blood sample; lane 3: *E. canis*-positive blood sample; lane 4: *Babesia* spp-positive blood sample; lane 5: *H. canis*-positive blood sample; lane 6: *E. canis*- and *Babesia* spp-positive blood sample; lane 7: *E. canis*- and *H. canis*-positive blood sample; lane 8: *Babesia* spp- and *H. canis*-positive blood sample; lane 9: *E. canis*-, *Babesia* spp- and *H. canis*-positive blood sample; lane 10: *Dilofilaria immitis*-positive blood sample; lane 11: *Haemobartonella felis*-positive (feline) blood sample; lane 12: negative control (containing ultrapure sterile water).

PCR results, amplicons obtained from multiplex PCR were purified using QIAquick PCR Purification Kit (QIAGEN®, Germany) and sequenced using the Automated Capillary DNA Sequencer (ABI PRISM 310, Perkin Elmer, USA). Each sequence was analyzed with the BioEdit software and the sequence similarity was then checked against sequences deposited in the GenBank using the BLAST software. We found that sequences from the specific DNA bands of *E. canis*, *Babesia* spp and *H. canis* from the multiplex PCR showed 100% identity to the sequences of *E. canis*, *Babesia* spp and *H. canis*, respectively. The specificity of the multiplex PCR was also verified by comparing the PCR results of 20 clinical blood specimens with morphology examination using Wright-Giemsa staining. Blood samples from 10 healthy dogs as negative control showed negative results by both multiplex PCR and morphology examination. All five blood samples that were positive for *Ehrlichia canis* by multiplex PCR were also positive for *Ehrlichia* spp by morphology detection. Similarly, all six blood samples that were positive for *Hepatozoon canis* by multiplex PCR showed positive result for *Hepatozoon* spp by morphology examination. On the other hand, from nine blood samples that were positive for *Babesia* spp by multiplex PCR, only six samples showed positive result for *Babesia* spp by Wright-Giemsa staining. However, sequencing analysis of the 3 negative samples from Wright-Giemsa staining showed 100% identity to the sequences of *Babesia* spp suggesting more sensitivity of the multiplex PCR for this pathogen detection.

DISCUSSION

Canine blood parasites are fatal diseases in dog worldwide. Three blood parasites (*E. canis*, *H. canis* and *Babesia* spp) are commonly found in canine blood. Each infection requires a specific treatment, so accurate diagnosis is necessary for the correct treatment.

Many PCR-based methods to detect infections with *Ehrlichia*, *Hepatozoon* and *Babesia* species are described (Sacchini *et al*, 2007; Adaszek and Winiarczyk, 2008; Rubini *et al*, 2008). However, reported methods are designed to detect only one pathogen at a time. This is the first report that describes a simplified multipathogen detection protocol for *E. canis*, *Babesia* spp and *H. canis* infections. This multiplex PCR technique is suitable for processing of a large number of blood specimens. It can diminish time consumption, cost and personnel required. Specificity of amplicons for each blood parasites was based on primer sequences derived from the specific genome sequence of each species.

In summary, we describe the use of multiplex PCR to detect three blood parasites in canine blood samples. This method may provide a highly specific tool for diagnosis of tick-borne parasitic co-infections. Clinical applications for this multiplex PCR not only include the detection of blood parasites that are difficult to diagnose by morphology examination but also to monitor the status and clearance of blood parasite infection during treatment.

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