DEVELOPMENT OF MULTIPLEX POLYMERASE CHAIN REACTION FOR DETECTION OF EHRlichia canis, Babesia spp AND Hepatozoon canis IN Canine Blood

Kan Kledmanee, Sarin Suwanpakdee, Sakranmanee Krajangwong, Jarin Chatsiriwech, Parut Suksai, Pongpun Suwannachat, Ladawan Sariya, Ruangrat Buddhirongawatr, Phingphol Charoonrut and Kridsada Chaichoun

Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand

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
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>
<thead>
<tr>
<th>Pathogen</th>
<th>Primer</th>
<th>Sequences (5’ to 3’)</th>
<th>Length (bases)</th>
<th>Size of product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ehrlichia canis</td>
<td>Ehr1401F</td>
<td>CCATAAGCATAGCTGATAACCCTGTTACAA</td>
<td>30</td>
<td>380 bp</td>
</tr>
<tr>
<td></td>
<td>Ehr1780R</td>
<td>TGCTAAATAAAACCGCTTATATGTATGCTAG</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Babesia spp</td>
<td>Ba103F</td>
<td>CCAATCCTTGACACGAGGAGGTAGTGACA</td>
<td>28</td>
<td>619 bp</td>
</tr>
<tr>
<td></td>
<td>Ba721R</td>
<td>CCCAGAAGCCCAAAGCTTTAGATTCTCTCGAG</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Hepatozoon canis</td>
<td>Hep001F</td>
<td>CTCGAGTATCATACGACCAAATCTTCACAATT</td>
<td>30</td>
<td>737 bp</td>
</tr>
<tr>
<td></td>
<td>Hep737R</td>
<td>CCAACTGTCCCTATCAATCATTAAAGC</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

Table 1
Oligonucleotide primers used in multiplex PCR.
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 HotStarTaq DNA Polymerase (QIAGEN®, Germany), 1xPCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1.5 mM MgCl₂ 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 95°C followed by 30 cycles of 45 seconds at 94°C, 45 seconds at 65°C, and 90 seconds at 72°C with a final extension step of 10 minutes at 72°C. 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.

**ACKNOWLEDGEMENTS**

We would like to acknowledge the technicians of Molecular Biology Laboratory, Faculty of Veterinary Science, Mahidol University. We also thank Dr Piyanan Taweethavonsawat, Mr Kittipong Chaisiri and Mr Ekkaluck Kowong for their assistance.

**REFERENCES**

Adaszek L, Winiarczyk S. Molecular characterization of *Babesia canis* canis isolates from


