RESEARCH NOTE

DEVELOPMENT OF MULTIPLEX POLYMERASE CHAIN REACTION FOR DETECTION OF FELINE HEMOTROPIC MYCOPLASMA IN BLOOD AND TISSUE SPECIMENS

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Abstract. A multiplex polymerase chain reaction (PCR) was developed for the detection of feline hemotropic mycoplasmas which simultaneously differentiates infections of *Mycoplasma haemofelis* (Mhf), *Candidatus* Mycoplasma haemominutum (CMhm) and *Candidatus* Mycoplasma turicensis (CMtc) in feline blood and spleen. These organisms are responsible for the cause of various pathogenicity of feline infectious anemia. These infections are difficult to be detected by microscopic examination, the most commonly used method for general laboratory diagnoses. Specific primers were designed by selected consensus 16S rDNA sequences of three distinct species. The multiplex PCR assay developed in this study was sensitive and specific with detection limit 100 copies/µl DNA of Mhf and CMhm and 10 copies/µl DNA of CMtc. No amplicons could be amplified for other blood parasites or bacterial pathogens. This multiplex PCR will allow studies of pathogenicity and the monitoring of clinical treatment.

Key words: hemotropic mycoplasma, hemoplasma, multiplex PCR, feline

INTRODUCTION

Feline hemotropic mycoplasma or formally known as *Haemobartonella felis* are small (<1 µl), pleomorphic bacteria that attach to red blood cells (Willi *et al*, 2007). The inability to culture these agents outside the host has limited the possiblilities to investigate hemotropic mycoplasma. These agents were classified as rickettsial organism. Recently, the molecular characterization primarily based on 16S rDNA gene sequencing revealed a closer relationship to members of the class *Mollicutes* and the genus *Haemobartonella* was reclassified within the genus *Mycoplasma* (Willi *et al*, 2007). Three distinct species of feline hemotropic mycoplasma are recognized: *Mycoplasma haemofelis* (Mhf), *Candidatus* Mycoplasma haemominutum (CMhm) and *Candidatus* Mycoplasma turicensis (CMtc) (Gary *et al*, 2006; Peters *et al*, 2008).

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Mhf and CMhm infections in domestic cats have been diagnosed worldwide (Chungpivat *et al*, 2007; Willi *et al*, 2007; Bauer *et al*, 2008). CMtc was first identified in Switzerland. Subsequently, CMtc has also been reported in United Kingdom, USA, Canada, South Africa, Australia, Italy, Canada and Japan (Tasker *et al*, 2001; Kewish *et al*, 2004; Willi *et al*, 2005; Fujihara *et al*, 2007; Kamrani *et al*, 2008; Peters *et al*, 2008; Sykes *et al*, 2008; Gentilini *et al*, 2009; Sykes, 2010). In the later studies, CMtc prevalence is higher than Mhf but lower than the prevalence for CMhm (Willi *et al*, 2005, 2007). This organism is the cause of feline infectious anemia (FIA), lethargy, anorexia, weight loss, thrombocytopenia, fever and jaundice (Cooper *et al*, 1999; Tasker and Lappin, 2002; Inokuma *et al*, 2004; Chungpivat *et al*, 2007). These three mycoplasma species differ in pathogenicity. Mhf infection causes a severe hemolytic anemia but conversely CMhm infection does not usually induce severe anemia (Willi *et al*, 2006, 2007; Chungpivat *et al*, 2007; Peters *et al*, 2008). Although some reports have documented mild or moderate anemia from CMhm infection but co-infection with feline retrovirus may result in significant anemia (Tasker *et al*, 2004; Chungpivat *et al*, 2007). Pathogenicity of CMtc depends on immunosuppression or co-infection with other hemoplasma (Willi *et al*, 2007).

The general laboratory method to diagnose feline hemoplasma is microscopic examination of blood smears, serology techniques and molecular biology methods. Microscopic examination is the most commonly used method to diagnose this organism on the surface of erythrocytes of a good quality blood sample. A diagnostic sensitivity of less than 20% has been reported and diagnostic specificity is often hampered by other organisms with stain precipitates or Howell-Jolly bodies (Tasker and Lappin, 2002; Willi *et al*, 2007). Furthermore, this method has so far not been able to identify species. False negative diagnoses are also a major problem when blood smears are the only technique used for the diagnosis of feline hemotropic mycoplasma infection. Although parasitemia can be extremely heavy during acute infection, the clearance of parasites from the blood can be rapid resulting in negative cytological findings within a few hours (Willi *et al*, 2007). Serology techniques have included Western immunobloting and immunofluorescent antibody tests; however no routine serological assays are available because the lack of hemoplasma culture has limited the establishment of protein-based serological assays and feline hemoplasma-specific antigen have so far not been established (Alleman *et al*, 1999).

Molecular biology methods provide another choice for diagnosis of this agent. Polymerase chain reaction (PCR) analysis can identify these three species of feline hemotropic mycoplasma (Tasker and Lappin, 2002; Willi *et al*, 2007; Peters *et al*, 2008). In this study, we have developed a one-tube multiplex PCR for species identification of feline hemotropic mycoplasma. It provides high sensitivity and diminishes time for routine feline blood parasite diagnosis.

MATERIALS AND METHODS

Specimen collection and DNA extraction

Blood and spleen specimens of domestic cats and wild felid species collected from the Veterinary Teaching Hospital, Faculty of Veterinary Science, Mahidol University, Thailand and The Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotics Animals

(MoZWE), Faculty of Veterinary Science, Mahidol University, Thailand, respectively. DNA from EDTA-blood samples were extracted using Genomic DNA Mini Kit for blood/cultured cells (Geneaid Biotech, Taiwan) and DNA from tissue specimens using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). DNA from both extraction kits was stored in 50 µl of elution buffer.

Primer design

Fifty-two sequences of Mhf, 74 sequences of CMhn and 29 sequences of CMtc 16S rRNA genes from GenBank were used for multiple alignments using BioEdit v7.0.4 software. The consensus 16S rRNA sequences for each feline hemotropic mycoplasma species were selected for primer design. All primers for Mhf, CMhm and CMtc were aligned with non-target nucleotide of others feline infected blood-parasites including *Dilofilaria immitis* 18S rRNA (accession no. AF217800.2, F217801.2, EU087700.1) and *Toxoplasma gondii* 16S rRNA (accession no. AM055943.1, DS984759.1, EQ967507.1, EQ970710.1, NW_002234567.1) to diminish non-specific binding. The sequences of the primers are listed in Table 1.

Multiplex PCR amplification

Using QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany), the reaction mixture contained 2 µl of template DNA, 25 µl of QIAGEN Multiplex PCR Master Mix, 0.2 µl of each primer (100 pmol/µl) and 21.8 µl of sterile nuclease-free water. The multiplex PCR thermocycling was performed in Thermocycle Mastercycler Gradient Thermocycler (Eppendorf, Germany) and consisted of a step of 15 minutes at 95ºC for initial denaturation, followed by 35 cycles of 30 seconds at 94ºC, 30 seconds at 58ºC and 30 seconds at 72ºC. The reactions were finally heated at 72ºC for 10 minutes. The specificity of multiplex PCR was verified by comparing with 10 blood samples received from healthy cats as negative controls and with parasite DNA of the *Trypanosome* spp, *Dilofilaria immitis*, *Hepatozoon canis*, *Ehrlichia canis*, *Babesia* spp and *Toxoplasma gondii*. The amplicons were separated by electrophoresis in 2.0% agarose gel, stained with GelRed™ (Biotium, USA) and recorded under UV light.

Specificity and sensitivity test

In order to confirm the specificity of the multiplex PCR results, amplicons were purified using QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany) and sent to a reference laboratory (Bio Basic, Amherst, NY) for nucleotide sequencing. The sequences were aligned with BioEdit software and sequence similarity was then checked against sequences deposited in GenBank using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

To determine the sensitivity of the test, PCR amplicons from parasite 16S rDNA were inserted in pGEM-T plasmids and used as templates for multiplex PCR. In brief, each PCR product of Mhf, CMhm and CMtc was gel purified and ligated into the pGEM-T easy vector (Promega, Madison, WI), according to instructions from the supplier, to generate positive control pMhf, pCMhm and pCMtc. Then, the plasmids were tranfected into competent *Escherichia coli* Top10F' (Invitrogen, Carlsbad, CA) using the calcium chloride method. Transformants were selected and propagated in LB-broth containing 100 µg/ml of ampicillin. Purified plasmids were prepared using MiniPrep DNA preparation kit (QIAGEN, Hilden, Germany). DNA concentration was determined by measuring absorbance at 260 and 280 nm. Copy numbers of pMhf, pCMhm and pCMtc

Table 1
Oligonuecleotide primers used in multiplex PCR.

Pathogen	Primer	Sequence (5′ to 3′)	Size of amplicon
Mycoplasma haemofelis	Mhf-F Mhf-R	GAGGGATAATTATGATAGTACTTCGTG CAATCTAGACATGTAGTATTCGGTG	190 bp
Candidatus Mycoplasma haemominutum	CMhm-F CMhm-R	TCTGAT TGTAAAGTTCTTTTATTTAG TAATTCTAGACAAACGATATCTATC	241 bp
Candidatus Mycoplasma turicensis	CMtc-F CMtc-R	GAAAAATTTGATGGTACCCTC GCCGAAACACAAATCCCGAC	359 bp

Table 2
Results of Mhf, CMhm and CMtc multiplex PCR detection of feline blood and spleen samples.

Sample	Number of samples	Number of feline hemoplasma infection							
		Mhf	CMhm	CMtc	Mhf + CMhm	Mhf + CMtc	CMhm + CMtc	Mhf + CMhm + CMtc	Negative
Wild felid species									
Blood sample	67	0	1	0	2	0	0	0	64
Spleen tissue	8	0	1	0	0	0	0	0	7
Domestic cat									
Blood sample	47	1	9	3	0	1	0	0	33
Total	122	1	11	3	2	1	0	0	104

Mhf, *Mycoplasma haemofelis*; CMhm, *Candidatus* Mycoplasma haemominutum; CMtc, *Candidatus* Mycoplasma turicensis

were calculated as mole multiplies of Avogadro's number. Each recombinant plasmid was 10-fold serially diluted from 10^6 to 10^{-2} molecules and used as a template for multiplex PCR to analyze the sensitivity. Amplification condition of the multiplex PCR used was as described above.

RESULTS

Multiplex PCR was developed for the simultaneous detection and identification of the 3 feline hemotropic mycoplasmas in feline blood or tissue specimens. Amplicons were separated by gel-electrophoresis and visualized under UV light. The expected size of amplicon specific for Mhf, CMhm and CMtc is 190, 241 and 359 bp, respectively. Representative results are shown in Fig 1. No amplicons were obtained using DNA from *Trypanosome* spp, *Dilofilaria immitis*, *Hepatozoon canis*, *Ehrlichia canis*, *Babesia* spp and *Toxoplasma gondii* (data not shown).

To confirm the multiplex PCR results, each amplicon obtained from multiplex

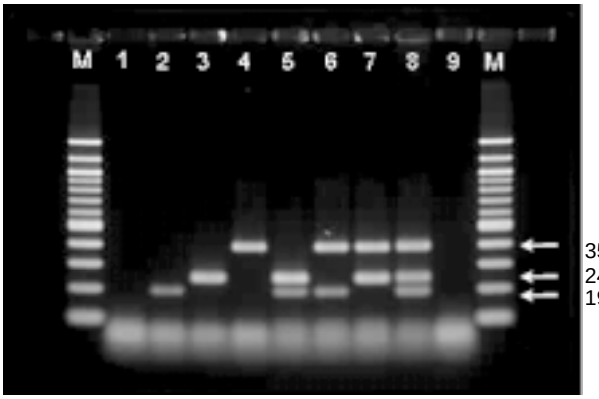

Fig 1–Electophoretogram demonstrating multiplex PCR amplicons of feline hemotropic mycoplasmas. 18S rDNA was amplified using specific feline hemotropic mycoplasma primers as described in Materials and Methods. Expected size of amplicons specific to Mhf, CMhm and CMtc are 190, 241 and 359 base pairs, respectively. Lane M: 100 base pairs ladder (New England Biolab™, USA); lane 1: healthy feline blood sample; lane 2: Mhf positive sample; lane 3: CMhm positive sample; lane 4: CMtc positive sample; lane 5: Mhf and CMhm positive sample; lane 6: Mhf and CMtc positive sample; lane 7: CMhm and CMtc positive sample; lane 8: Mhf, CMhm and CMtc positive control; lane 9: reagent negative control.

PCR was purified and sequenced. Each sequence was then checked against sequences deposited in GenBank using BLAST software. Sequences from each specific DNA band of feline hemotropic mycoplasma in this study showed 100% identity to Mhf and CMhm sequences and 99% identity to those of CMtc. The specificity of multiplex PCR was also verified by comparing the PCR results of 10 blood specimens from healthy domestic felids as negative controls. Overall, the results obtained using 16S rDNA of feline hemotropic mycoplasma indicated a high specificity of the primers used for amplification. As for the sensitivity of multiplex PCR, the threshold concentration for detecting feline hemotropic mycoplasma was 3 fg of DNA(data not shown). The results showed high sensitivity of detection.

One hundred and twenty-two blood and spleen samples randomly collected from 47 domestic cats and 75 captive wild felids [53 tigers (*Panthera tigris*), 4 lions (*Panthera leo*), 1 leopard (*Panthera pardus*), 15 fishing cats (*Prionailurus viverrinus*), 1 flat-headed cat (*Prionailurus planiceps*) and 1 Puma (*Puma concolor*)] were tested by this multiplex system. One fishing cat was infected with CMhm without clinical signs; co-infections of Mhf and CMhm were found in blood samples received from 2 fishing cats; and 1, 9 and 3 domestic cats were positive for Mhf, CMhm and CMtc, respectively (Table 2). Furthermore, co-infection of Mhf and CMtc was also found in blood sample of a domestic cat. These blood samples were also examined by cytopathology and the results were negative for all blood samples.

DISCUSSION

Feline hemotropic mycoplasmas are the cause of infectious anemia in felid worldwide. Routine detection for feline hemotropic mycoplasma by microscope examination of blood smears has low diagnostic sensitivity. An accurate diagnosis is important for proper treatment. Westfall *et al* (2001) found that only 37.5% of samples were positive on cytopathology examination after experimental infection compared to 100% positivity with PCR. Therefore, we have designed a multiplex PCR for detecting feline hemoplasma. This method is the first report of detecting

multi-species of feline hemotropic mycoplasmas. Diagnostic specificity of this protocol was based on primers specific to each 16S rDNA species sequence. This method is highly specific and allows diagnosis of co-infections. Furthermore, this multiplex PCR can be used to confirm, monitor infection with feline hemoplasma during treatment and to screen feline blood donors for feline infection prior to blood transfusion (Reine, 2004).

In summary, this is the first report that describes a simple, multi-species detection protocol for feline hemotropic mycoplasmas. This multiplex PCR technique is suitable for processing a large number of blood specimens and can diminish time, cost and personnel required.

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