

The Molecular Identification of *Mycoplasma haemofelis* and *Mycoplasma haemominutum* in Cats Suffering from Haemoplasmosis in Thailand

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Abstract

Haemotrophic *Mycoplasma* is the obligatory bacteria formerly classified to the genus *Haemobartonella*. This blood mycoplasma is responsible for the cause of a cat disease, haemoplasmosis or feline infectious anemia (FIA). The housed cats evidenced by cachexia, high fever and a certain degree of anemic condition have been reported with FIA worldwide. However, this disease is only a presumptive case in Thailand where the disease is presumably endemic. We examined two cats suffering from FIA tested with the standard microscopic examination. With the destruction of erythrocytes, one cat had only a mild clinical condition while the other was severe. The parasitized red cells of these cats were unconvincingly indicated by microscopy and the species of this pathogen was highly undefined. We used the molecular methods of detection and identification to confirm the presence of blood mycoplasma. The species differentiation of *Mycoplasma* was also performed. The standard PCR and DNA sequencing were able to elaborate the two most important species of FIA that were *Mycoplasma haemofelis* and *Mycoplasma haemominutum*.

Keywords : Feline, *Haemobartonella*, *Mycoplasma haemofelis*, *Mycoplasma haemominutum*, Thailand

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บทคัดย่อ

การตรวจทางอนุชีววิทยาของเชื้อ *Mycoplasma haemofelis* และ *Mycoplasma haemominutum* ในแมวที่ป่วยด้วยโรค haemoplasmosis ของประเทศไทย

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ฮีโมโทรฟิคไมโคพลาสมา (haemotrophic mycoplasma) คือ เชื้อในสกุล ไมโคพลาสมา เดิมเคยจัดอยู่ในสกุล ฮีโมบาโทเนลลา (*Haemobartonella*) เชื้อนี้ก่อให้เกิดโรคในแมว เรียกว่า ฮีโมพลาสโมซิส หรือ เฟไลน์อินเฟกเชียสแอนิเมีย (Haemoplasmosis, Feline infectious anemia, FIA) ในแมวที่เป็นสัตว์เลี้ยงนั้น พบอาการผอมแห้ง ขนหยอง มีไข้ และเลือดจาง โรคนี้มีรายงานแล้วในหลายประเทศทั่วโลก แต่สำหรับประเทศไทย โรคนี้เป็นเพียงสมมุติฐานของโรค และยังไม่พบรายงานด้วยว่า เกิดจากเชื้อชนิด (species) ใด ทั้งๆ ที่เชื้อนี้สามารถตรวจพบได้เสมอ จากการตรวจวินิจฉัยด้วยกล้องจุลทรรศน์กับฟิล์มเลือดบาง ย้อมลิยิมซา และเนื่องจากเชื้อนี้มีขนาดเล็กมาก เกือบขนาดด้านนอกของเม็ดเลือดแดงที่ติดเชื้อ จึงเป็นการยากที่จะจำแนกชนิดของ เชื้อนี้จากรูปร่างลักษณะทางกล้องจุลทรรศน์เท่านั้น ผู้วิจัยจึงใช้เทคนิคทางอนุชีววิทยาควบคู่กับการตรวจด้วยฟิล์มเลือดบาง เพื่อ ตรวจหาเชื้อและหาลำดับเบสของเชื้อที่ก่อโรคฮีโมพลาสโมซิส ในแมวจำนวน 2 ตัว พบเชื้ออย่างน้อย 2 ชนิดที่ก่อโรคในแมว ได้แก่ *Mycoplasma haemofelis* และ *Mycoplasma haemominutum*

คำสำคัญ : แมว ฮีโมบาโทเนลลา ไมโคพลาสมา ฮีโมเฟลิส ไมโคพลาสมา ฮีโมไมนุตัม ประเทศไทย

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Introduction

The haemotrophic mycoplasma found on the surface of erythrocytes was previously described to the genus *Haemobartonella* responsible for haemoplasmosis or feline infectious anemia (FIA). Haemoplasmosis was commonly found in numbers of mammals such as felidae, canidae, cattle and swine containing a diverse group of more than five species as reported previously (Foley and Pedersen, 2001; Neimark et al., 2001, 2002; Willi, et al., 2005). For a certain degree of clinical signs, the virulent form of FIA was responsible by *Mycoplasma haemofelis*. This severe form of FIA is likely to be one of the most important causes of death in cats. Meanwhile, *Mycoplasma haemominutum*, another species of blood mycoplasma, is causing a mild symptom of anemia. Being characterized by the biochemical property, these organisms are the obligatory bacteria forming a minute colony as spots or chains on the surface of affected erythrocytes. The 0.2-0.6 μm of acidophilic dots of bacteria varying in sizes which depend on species, are a

certain cause of hemolytic anemia. The similar disease was also found in cattle and swine infected with a related group of organisms previously classified to a genus *Eperythrozoon* (Messick et al., 1999; Lappin et al., 2006).

Among all known species of haemotrophic mycoplasma, both *M. haemofelis* and *M. haemominutum* are reported throughout the US, Canada, the UK, many countries in Europe and in the countries of Asia such as Japan (Jensen et al., 2001; Criado-Fornelio et al., 2003; Tasker et al., 2003; Inokuma et al., 2004; Kewish et al., 2004). However, these two species were yet reported in Thailand. The tentative diagnosis was based on the conventional blood examination. In this study, we are presenting the alternative laboratory technique to examine cats with FIA responsible for a severe anemia (haematocrit < 10%) or a mild condition ($\geq 25\%$). The aim of this study is to search for the etiological species of FIA. The molecular methods using PCR detection and the analysis of the partial 16S rRNA gene sequences of *Mycoplasma* were performed.

Materials and Methods

The patients

The affected cats having cachexia, fever, loss of appetite and 10% dehydration were admitted to the veterinary teaching hospital. Blood collection was performed followed by the determination of hematology and blood chemistry profiles which were routinely determined. In addition, aliquot of EDTA- containing blood samples were sent to the unit of Parasitology for the screening test of blood parasites using Giemsa staining technique. The remaining portion of blood around 200 µl was subjected to the consequence molecular process.

The molecular methods of DNA extraction, PCR and DNA sequencing

The 200 µl of EDTA preserved blood was taken to proceed for the DNA purification using the mini-blood kit for DNA extraction (Qiagen®, USA). Five µl of purified DNA was added to the subsequent Polymerase Chain Reaction (PCR). Primers used in the reaction were to amplify the target of 16S rRNA gene of *Mycoplasma*. According to this procedure the reaction can produce an amplicon of 170 bp from *M. haemofelis* and a 193 bp from *M. haemominutum* (Forward primer, 5'- ACG AAA GTC TGA TGG AGC AAT A-3' and reverse primer 5'- ACG CCC AAT AAA TCC GRA TAA T-3') (Kewish et al., 2004). The template DNA (5 ul) was added to 25 µl of reaction mixture containing sterile ultrapure water, 1x PCR buffer, 3.5 mM MgCl₂, 0.25 mM dNTPs, 0.4 µM each primer and 2.5 unit of Taq polymerase (Invitrogen®, USA). Based on the previous procedures and some conditions were adjusted, the PCR reaction was assigned to the initial denaturation step for 4 min at 94°C, followed by 35 cycles of 1 min: denaturation at 94°C, 30 sec: annealing step at 60°C, and 30 sec: extension at 72°C. In the last cycle, the extension was hold at 72°C for 10 min. (Jensen et al., 2001; Inokuma et al., 2004; Kewish et al., 2004). To ensure that our PCR products were directly amplified from *Mycoplasma* genome, not an artifact from other contaminated pathogens, the product

was cloned into pGEM-T plasmid (Promega®, USA) and the bidirectional sequencing procedure was preformed. Four DNA sequences from each plasmid clone were denoted to be the unknown clones 1, 2, 10 and 24. Sequencing reactions were carried out in an ABI machine (Applied Biosystems Inc.) and automated sequencer was performed (Macrogen, Korea). DNA sequences were analyzed using MEGA3 software (Kumar et al., 2001).

Results

Five and ten years old male and female cats were admitted to the emergency wards at a time period when both were suffering from anemia with pale mucous membrane and undulant febrile. However, the cause of these symptoms was not declared. At first, the blood of the male cat was examined and the condition of kidney failure was diagnosed. Tests were subsequently conducted for FIV/FeLV, which were negatively found in these cats and no ectoparasites were detected. Both cats were then retained to receive further diagnosis and a substantial treatment.

The haematology and blood biochemistry data were presented in Table 1 as a referral source of information. The retrospective observation of haemoplasmosis or FIA-like symptom was determined as approximate to 10% of the regular cases (disclosed by the hospital records). The male cat which was diagnosed with kidney failure was also diagnosed microscopically with a small form FIA. After being under supportives, the condition of kidney failure was improved by the reduction in BUN and creatinine. Being treated with 10 mg/kg doxycycline for 28 days, the anemic condition was recovered indicated the clearance of pathogen (between 15/2/06 to 27/3/06, 40 days later) (Table 1). The haematocrit was also rising from <25% to 31% after treatment. However, the female cat suffering from the severe FIA was unfortunate to survive and later died of anemia.

Table 1 The parasitological findings, hematology and biochemistry profiles of a cat suffering from FIA.

The 5 years old male	Parasitology		Hematology										Blood Biochemistry				
	9/2/06	ND	RBC	Hb	Hct	Platelets	WBC	PMNC	Bands	Eosinophils	Lymphocytes	Monocytes	AST	ALT	AP	BUN	Creatinine
			10 ⁶ /μl	g%	%	X 10 ³ /μl	/μl	%	%	%	%	%	U/L	U/L	U/L	mg%	mg%
9/2/06	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	16	20	109	258	13.1
Receiving treatment for kidney failure																	
15/2/06	<i>M. haemominutum</i>		6.16	8	25	ND	22260	71	<	10	17	2	21	ND	281	38	2.2
Receiving 10 mg/kg doxycycline for FIA																	
27/3/06	Negative		6.63	9	31	609	14230	63	<	11	33	2	128	ND	482	64	2.8

ND : not determined, RBC : Red blood cells, Hb : Hemoglobin, Hct : Haematocrit, WBC : White blood cells, PMNC : Polymorphonuclear cells, Bands : Band Neutrophils, AST : Aspartate aminotransferase, ALT : Alanine aminotransferase, AP : Alkaline phosphatase and BUN : Blood urea nitrogen.

The parasitological method was routinely applied as a presumptive diagnosis resulting in the finding of haemotrophic *Mycoplasma*. Figure 1 showed the microscopic parasitized erythrocytes. One could hardly differentiate a group of these organisms based on their morphology, although the pathogenicity of *M. haemofelis* and *M. haemominutum* was different. The larger form of haemoplasmosis is more virulent causing more critical conditions of decreasing in haematocrit than that of a small form (Foley and Pedersen, 2001). Comparatively, Figure 1A showed a large form FIA on the erythrocytes in the female cat and Figure 1B showed a small form of pathogen.

The results of PCR detection and DNA sequences had declared that the female cat that died of a severe anemia was identified with *Mycoplasma haemofelis* (Figure 2, Lane 2, 170 bp). In addition, the male cat was suffering from *M. haemominutum* (Figure 2, Lane 1, 193 bp). The species of mycoplasma were confirmed by DNA sequencing methods. Fragments of PCR products were cloned and designated as clones; 1, 2, 10 and 24. Figure 3 showed the result of the multiple alignments of the DNA sequences from these plasmid clones resulting in *Mycoplasma haemofelis* as found in clones 1 and 2 and *M. haemominutum* in clones 10 and 24.

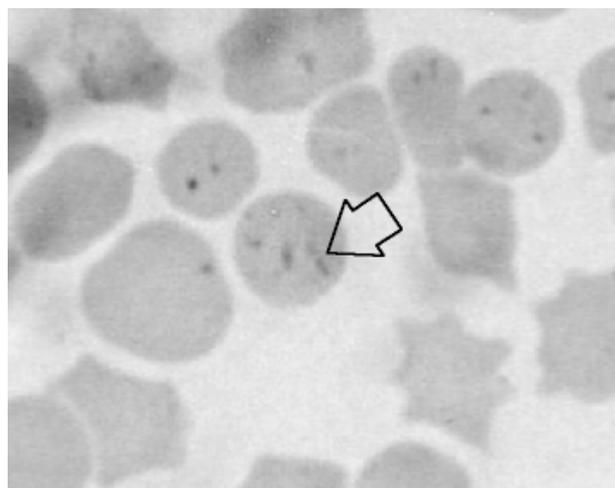
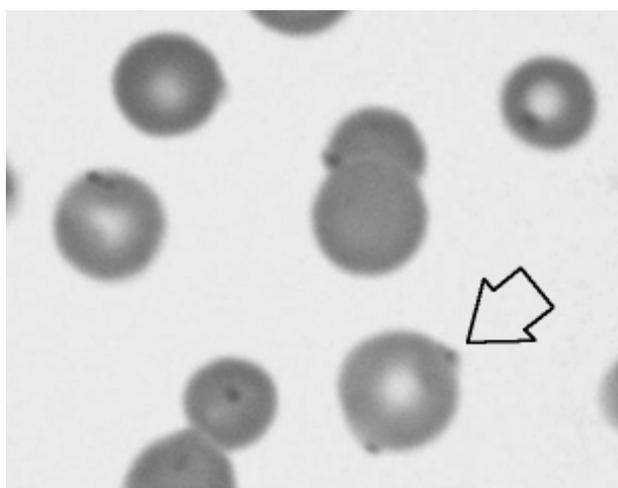


Figure 1 Microscopic examination of blood film from the hospitalized cats at 1000 x magnification. (A) The surface of red blood cells was found with large form haemotrophic *Mycoplasma* (arrow). (B) The cell infected with a small form mycoplasma (arrow).

Discussion

The hematology and the routine blood examination for parasitological findings were previously proposed as a method of choice to diagnose FIA (Fig.1A and B). The significantly low level of haematocrit was a hallmark of FIA (Table 1). When the level of haematocrit went below 25% and a large form of parasites was detected, the disease could be assumed as a severe FIA (Foley and Pedersen, 2001). On the other hand, the small form FIA seems to be an opportunistic organism simply interfering with the clinical status of this cat. In both cases, however, these clinical factors had resulted in the undefined species of FIA. In this study, PCR and DNA sequencing had helped confirming the presence of the blood parasites in these cats and the sequences of the 16S rRNA gene were able to identify the species of this pathogen.

The technique of PCR detection was specifically developed to amplify the fragment of 16S rRNA gene as described (Kewish et al., 2004). The species identification of haemotrophic *Mycoplasma* was based on their DNA sequences of the highly polymorphic region on the gene. It has been used widely to classify the species of *Mycoplasma* (Messick et al., 1999; Neimark, et al., 2001). The cloned PCR fragments were carefully sequenced by bidirectional method. The total of 197 nucleotide sites were

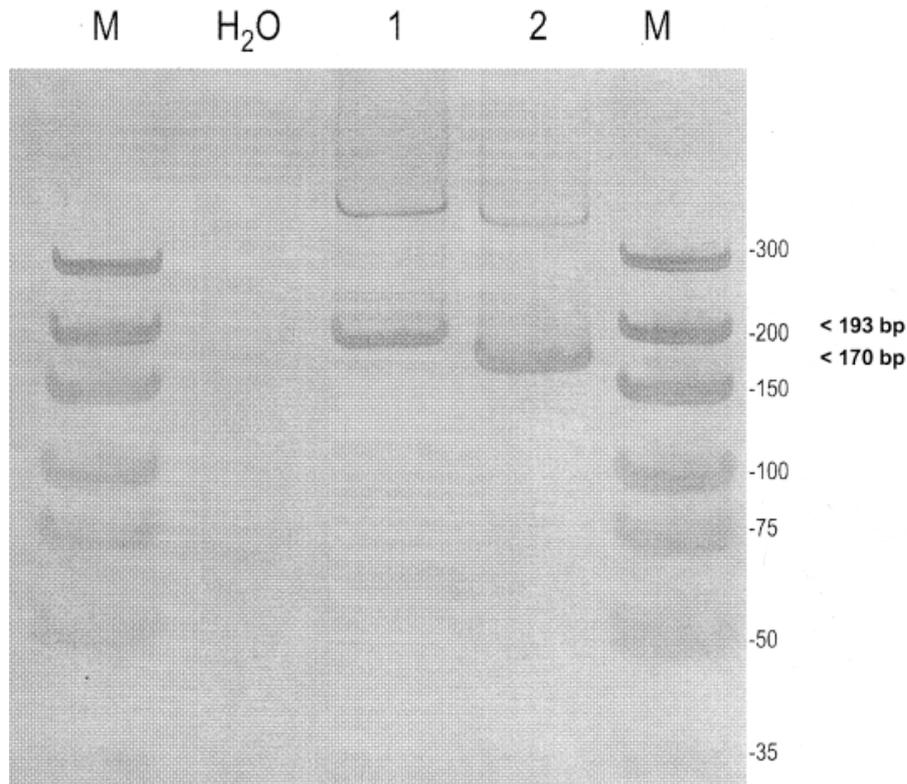


Figure 2 PCR detection of haemotrophic *Mycoplasma*. Lane 1 shows the 193 bp product specific to *M. haemominutum* found in male cat with a mild condition of anemia. Lane 2 shows the specific amplicon of 170 bp found in female cat infected with *M. haemofelis*.

compared to the sequences retrieved from Genbank (Figure 3). This molecular procedure had ensured that clones 1 and 2 were closely related to *M. haemofelis* (with 100% identity in clone 1 and 98.9% in clone 2). DNA sequences of clones 10 and 24 were to *M. haemominutum* (with 100% identity in clone 10 and 99.5% in clone 24, Fig. 3). It was noticed that variations in the DNA sequences of the plasmid clones retrieved from one isolate of mycoplasma were less than 1.5%. This is due to the erroneous in the sequencing method interfered by the secondary structure of rRNA gene (Messick, et al., 1998). However, the differences in sequences occurred between strains were due to the strain variations among isolates (Inokuma et al., 2004).

The deletion of twenty-three bp found on the 16S rRNA gene is useful in classification of the large form FIA as *M. haemofelis* while a small form,

M. haemominutum has no such region (Fig. 1 and Fig. 3). The variations occurred on this gene will also be useful to classify other species of mycoplasma once found in cats. Furthermore, the prevalence tests and the mode of transmission of FIA could be defined by the molecular methods since the epidemiology of this disease is not known (Foley and Pedersen, 2001). Another advantage of PCR detection is to indicate the health condition of cats intended to be the blood donors. It is important to prevent the unintentional infection of cats via blood transfusions while asymptomatic carrier cats with FIA usually exist. However, this method has never been used in our veterinary teaching hospital before but according to its powerful application, the method is therefore requested as a routine blood examination at the Faculty of Veterinary Science, Chulalongkorn University, Thailand.

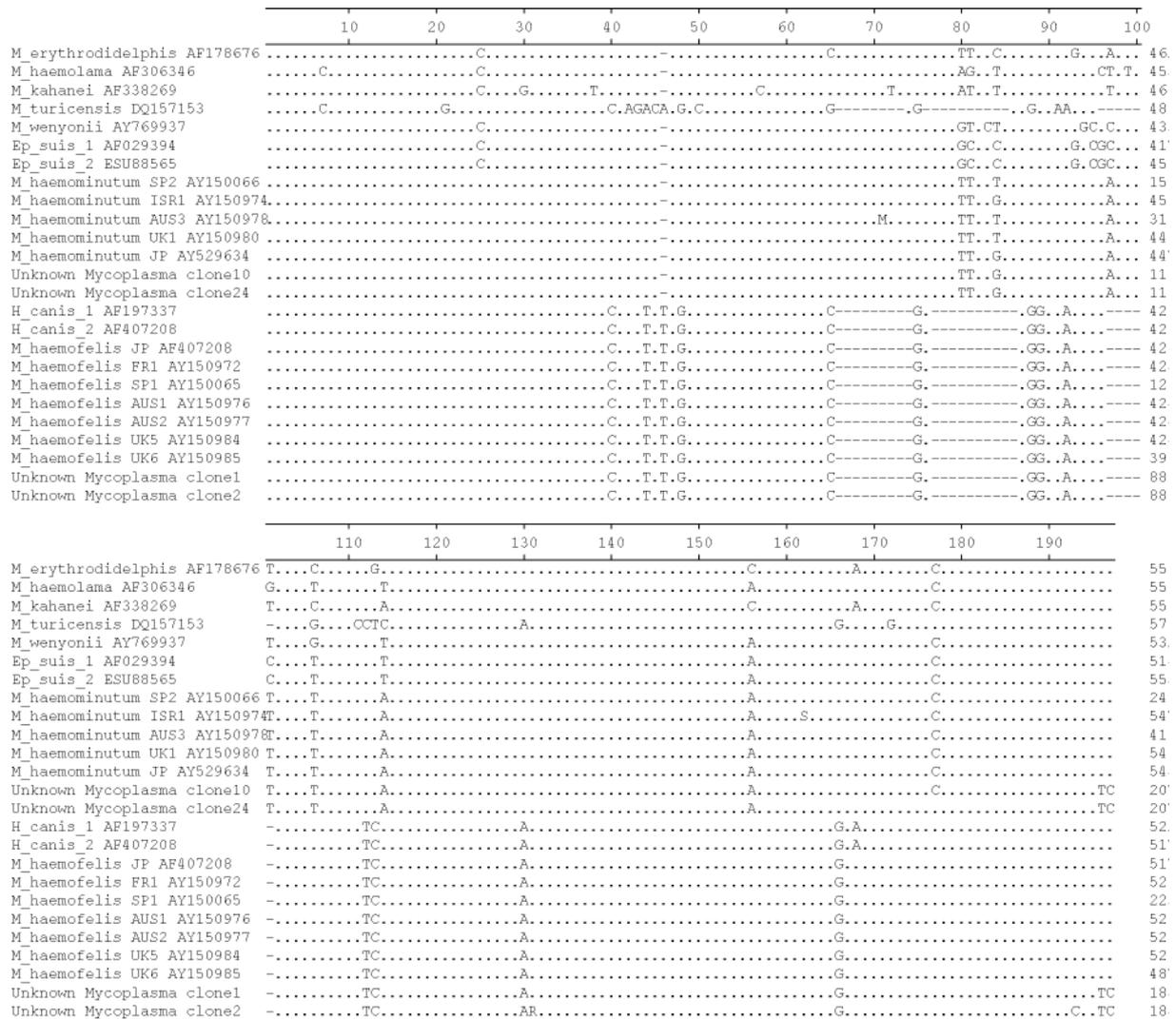


Figure 3 The multiple alignments of 27 partial 16S rRNA gene sequences showing the nearly perfect match of *Mycoplasma* strains found in Thailand and the sequences from Genbank. Genbank accession numbers referred to the genes from 10 species of mycoplasma were indicated. When compared, the unknown clones 1 and 2 are related to *M. haemofelis* while clone 10 and 24 are to *M. haemominutum*.

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