

First evidence of *Mycoplasma haemocanis* in China

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Received 17 February 2017; received in revised form 22 March 2017; accepted 23 March 2017

Abstract. Previous studies show hemotropic *Mycoplasma* infection frequently occurs among splenectomized, immuno-suppressive or co-infected dog populations. However, in our study, the detection of 162 blood samples from dogs found 3 healthy, female dogs infected with *Mycoplasma haemocanis* in southeastern China. These infected dogs were grown in dog breeding center and had a history of tick infestation. This is the first molecular report of *M. haemocanis* in dogs from China. The 16S rRNA gene was partially sequenced and a phylogenetic tree constructed. *Mycoplasma* spp. was 99.9%–100% identical to the corresponding gene sequences of *M. haemocanis* and *M. haemofelis* available in GenBank. In this study, *Mycoplasma* spp. was identified as *M. haemocanis* because the bacterium was obtained from dogs.

INTRODUCTION

Hemotropic Mycoplasmas are characterized as cell wall-deficient bacteria that are found in a variety of feline, canine, bovine, ovine and wild animal hosts (Aquino *et al.*, 2014; Hampel *et al.*, 2014; Novacco *et al.*, 2010; Santos *et al.*, 2013; Sashida *et al.*, 2013; Tagawa *et al.*, 2012). The spherical, rod-shaped, or ring-shaped microorganisms formerly named as Haemobartonella are uncultivable in culture until today. They attach to the outside surface of red blood cells and grow upon them (Messick, 2004). Currently, three hemoplasma species, *Mycoplasma haemocanis*, *Candidatus M. haemominutum* and *C. M. haematoparvum* are recognized in dogs (Obara *et al.*, 2011; Roura *et al.*, 2010; Sykes *et al.*, 2005; Zhuang *et al.*, 2009). *M. haemocanis*, a dominant canine hemotropic *Mycoplasma*, infects erythrocyte and usually leads to asymptomatic manifestation, but sometimes

causes subclinical symptoms in dogs such as fever, anemia, anorexia and thrombocytopenia when dogs are splenectomized, immuno-compressive or have coexistent pathogens. In more severe cases acute hemolytic anemia maybe occur and frequently recover. However, in some dogs this may cause death (Chalker, 2005; Kemming *et al.*, 2004b; Messick, 2004).

M. haemocanis is widely distributed around the globe including the US, Italy, France, Portugal, Spain, Brazil, Japan, Australia, Ivory Coast and Gabon (Barker *et al.*, 2012; Compton *et al.*, 2012; Marie *et al.*, 2009; Novacco *et al.*, 2010; Sasaki *et al.*, 2008; Soares *et al.*, 2016). The current dog population in China is estimated to be between 150 and 200 million (Ma *et al.*, 2012). The brown dog ticks (*Rhipicephalus sanguineus*) usually live in the crack and crevice of kennels that seriously infest dogs in China and are threat to dogs' health. These ticks potentially transmit *M. haemocanis*

to dogs during the process of blood sucking (Seneviratna & Ariyadasa, 1973). However, no data were available on the prevalence of *M. haemocanis* infection in Chinese dogs until today. Therefore, this study aimed to achieve a understanding of this etiological agent in China. Molecular techniques including conventional PCR and sequencing were used to examine and characterize *M. haemocanis* in dogs from China.

MATERIALS AND METHODS

Study area

The study was conducted in two districts: Changbei and Xinjian of Jiangxi province, southeastern part of China (Fig. 1). Bio-environment in Jiangxi remarkably facilitates tick development and tick-borne pathogen transmission. The current dog population in Jiangxi province is estimated to be between 5 and 6 millions. Most of them comprise police dogs, guard dogs and pet dogs.

Ethical clearance

This work involved animal sampling and interviewing of farmers. All the procedures were carried out according to ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Animal experiment access num: 28-100; DNA experiment approval num:1219-3). The dog owners were duly informed about the study and their written consent was sought prior to the start of data collection.

Sampling of dogs

One hundred and sixty two dogs were selected for blood collection. Blood sampling was conducted during the peak period of tick infestation in dogs between March and April 2016. Approximately 5 ml aliquot of blood was collected from the cephalic vein by using sterile vacutainer tubes with EDTA. The samples were temporarily kept in a sealed cool box with ice pack and transported to the laboratory where they were stored at 4°C for later use. Demographic data, age, previous

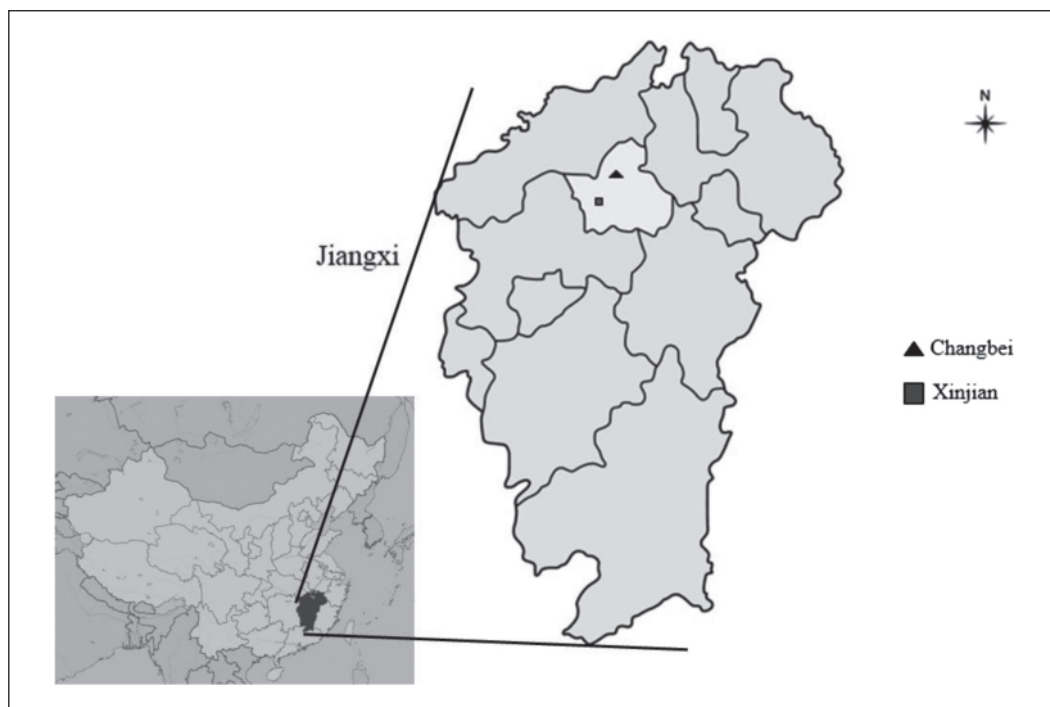


Figure 1. Geographical location of Changbei and Xinjian where dog blood samples were collected. Legends indicate the location of the sampling sites. ■, all samples collected from police dog base or veterinary hospital. ▲, samples obtained from pet dog owners or in pet dog breeding centers.

history of tick infestation and health status were recorded for each dog. Previous history of tick infestation was investigated by two routes, namely, questionnaire distribution to dog owners and administrators, and visual inspection in the field. All participants filled in a questionnaire concerning tick infestation on dogs that are whether there were ticks, where ticks most attached and remained, and how infestation intensity was.

DNA extraction from blood samples

Genomic DNA was extracted from a volume of 200 µl of whole blood using the QIAamp® DNA blood Mini Kit (QIAGEN, Germany) according to the manufacturer's recommendation. 100µl eluted DNA samples (30 ng/µl -90 ng/µl) were harvested and stored at -30°C for subsequent PCR analysis. DNA concentrations and purities were determined by measuring the absorbance at 230 nm, 260 nm and 280 nm with a NanoDrop Spectrophotometer (Thermo Scientific, USA).

Oligonucleotide primers and PCR amplification

A PCR to target the 16S rRNA gene of *Mycoplasma* spp. was done with forward primer (ATACGGCCCATATTCCTACG) and reverse primer (TGCTCCACCACTTGTTCA). The PCR reaction was carried out in a total volume of 10 µl including the primers (Sigma-Aldrich, Japan) at 1 µM, 1 µl template DNA, 250 µM dNTP, 1×PCR buffer, 0.5 U Taq-polymerase (EX-Taq DNA polymerase, Takara, Japan) and distilled water added up to the final volume. The PCR amplification program performed by thermocycler (BioRad, USA) included an initial denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1 min, and final elongation at 72°C for 7 min. Non-template controls and positive controls were included in each PCR assay. Plasmid containing target gene was used as positive control. The PCR products were checked by electrophoresis on 1.5% tris-acetate-EDTA buffer (1×TAE) agarose gels, ethidium bromide-stained, visualized under an UV transilluminator and photographed.

Cloning and sequencing

Mycoplasma spp. positive blood DNA samples were randomly selected for cloning and sequencing of amplicons. PCR products were extracted from agarose gel using QIAquick Gel Extraction Kit (QIAGEN, Germany) ligated into pGEM-T Easy Vector (Promega, USA) and then transformed into *Escherichia coli* DH5α-competent cells. For each isolate, at least two clones confirmed positive by colony PCR were multiplied and purified using NucleoSpin® Plasmid QuickPure (MACHERY-NAGEL, Germany). Sequencing reactions were performed using ABI BigDye™ Terminator Kit (ABI Applied Biosystems, USA). DNA fragment sequences were determined using an ABI PRISM 3100 DNA sequencer (ABI Applied Biosystems, USA). Identities and similarities of the nucleotide sequences were analyzed using online GenBank BLASTn.

Phylogenetic analysis

Phylogenetic analysis was performed using the sequences of this study and those reported from other regions. Both SeqMan and MEGA 7 programs were used to build the alignment of all selected sequences and then a neighbor-joining phylogenetic tree was constructed using MEGA 7 program. The confidence of internal branches was estimated by bootstrapping with 1000 replications.

RESULTS

Animals and samples

A total of 162 blood samples were randomly gathered from 3 selected sites in China. 56 samples collected from pet dogs and 25 samples collected in a pet dog breeding center were obtained. Meanwhile, 81 samples were gathered in a police dog base. Most (76.54%) of the dogs were adult. At the time of sampling, 46.3% of dogs sampled were female and 53.7% were male. The dominant breeds of the study samples were German shepherd (20.37%), Rotterman (12.96%), and Samoyed (8.64%). 40.74% of the sample dogs were infested by ticks before. Compared with

the 76.54% of healthy dogs, 23.46% of the dogs were diseased with leptospirosis, back leg twitches, fungi infection, ascites and /or cough (Table 1).

Pathogen detection and identification

Of the 162 blood samples tested, 3 (1.85%) samples were detected with *Mycoplasma* spp. infection by using the 16S rRNA primers (Fig. 2). Pathogens were examined in three healthy adult dogs with history of tick infestation. All the dogs positive for *Mycoplasma* spp. were either female Samoyed (2 individuals) or Golden Retriever (1 individual) and they had been grown in a dog breeding base (Table 1).

Sequences comparison and phylogenetic analysis

595 bp products were isolated from the amplicon of the dogs containing *Mycoplasma* spp.. The 16S rRNA fragment of *Mycoplasma* China isolates (KX519722, KX519723) were 99.99%–100% identical to the sequences of *M. haemocanis* and *M. Haemofelis* available in GenBank. The tree established in this study based on 16S rRNA fragment of *Mycoplasma* spp. presented *M. haemofelis* and *M. haemocanis* in the same clade with the China *Mycoplasma* spp. isolate. In view of the fact that the bacterium was obtained from dogs, *M. haemocanis* was identified in this study (Fig. 3).

Table 1. Distribution of *Mycoplasma* spp. among dog population studied

Variables	No. (%) detected	No. (%) positive for <i>Mycoplasma</i> spp.
Gender		
Male	87(53.7)	0
Female	75(46.3)	3(1.85)
Age group		
Young	38(23.46)	0
Adult	124(76.54)	3(1.85)
Breed		
German shepherd	33(20.37)	0
Rotterman	21(12.96)	0
Samoyed	14(8.64)	2(1.23)
Golden Retriever	13(8.02)	1(0.62)
Rottweiler	12(7.41)	0
Springer	11(6.79)	0
Other breeds	58(35.8)	0
Health status		
Healthy	124(76.54)	3(1.85)
Disease	38(23.46)	0
Sampling site		
Police dog base	81(50)	0
Pet dog breeding center	25(15.43)	3(1.85)
Companion dog owners	56(34.57)	0
History of tick infestation		
Presence	66(40.74)	3(1.85)
Absence	96(59.26)	0
Total	162 (100)	3(1.85)

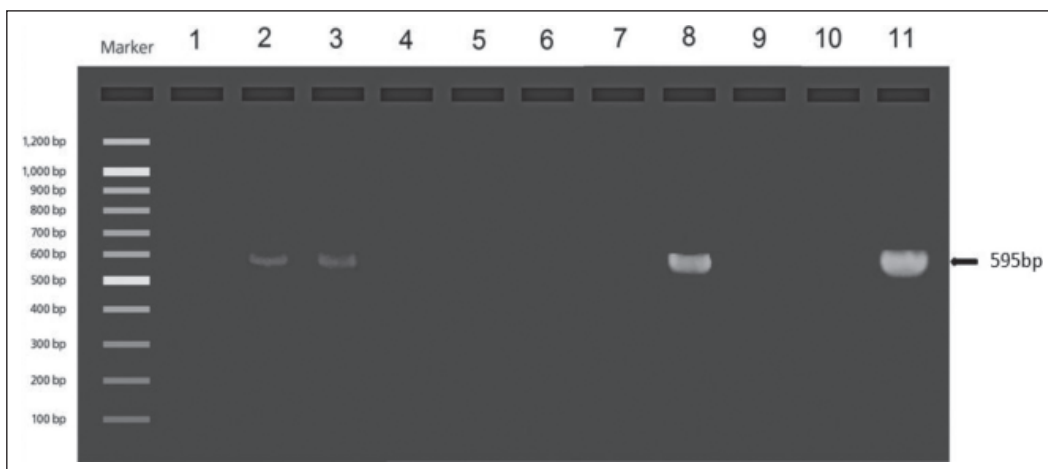


Figure 2. PCR amplification of *Mycoplasma* spp. 16s ribosomal RNA gene. Lanes 1,4,5-6, 9-10: negative samples, lanes 2-3, 8: positive samples, lane 11: positive control, lane 7: negative control.

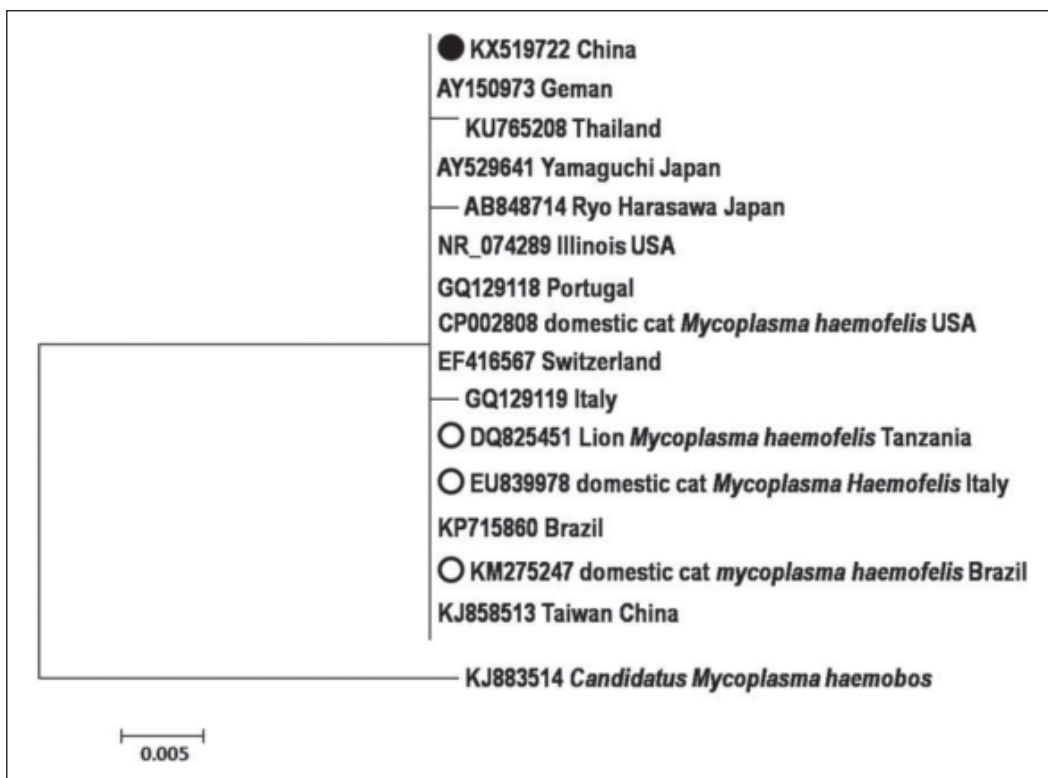


Figure 3. Phylogenetic analysis of *M. haemocanis* based on 595bp of 16S ribosomal RNA gene. *C. M. haemobos* was employed as the out group. ●, sequences obtained in this study. The marker “○” signifies partial 16S rRNA gene fragment from *M. haemofelis*.

DISCUSSION

The result of this study provides molecular evidence for the emergence of *Mycoplasma* spp. in dogs, which was identified as *M. haemocanis*. In fact, this is the first report of *M. haemocanis* in dogs from China. Previously *C. M. haemominutum* has been detected in Chinese dogs (Zhuang *et al.*, 2009). Our study showed 1.85% infection rate with *M. haemocanis* in 162 dogs, higher than that in North Carolina of US, but lower than that in Aomori Prefecture, Japan and Mato Grosso do Sul, Brazil (Compton *et al.*, 2012; Sasaki *et al.*, 2008; Soares *et al.*, 2016).

Despite *M. haemocanis* infection founded in Samoyed and Golden Retriever dogs, our result is insufficient to contradict the infection in other dog breeds in this region due to sample size. Furthermore, a previous study conducted in Spain has clearly confirmed insignificant relationship between dog breeds and the infection (Roura *et al.*, 2010).

Many previous studies have reported that *M. haemocanis* infection in dogs is associated with concurrent infection, splenectomy and immunosuppression. However, a study performed by Kemming *et al.* showed that healthy kennel-raised dogs could also be seriously affected by *M. haemocanis* infection (Kemming *et al.*, 2004a). Kennel-raised dogs living in the conditions that facilitate brown dog tick growth are susceptible to this tick bite, that potentially transmit *M. haemocanis*. In this study, three clinically healthy dogs positive for *M. haemocanis* infection were grown in a dog breeding base, where 1 to 10 dogs were restricted within a kennel depending on their body size and age, and hygiene was not at an optimal level.

M. haemocanis is not a single contributor to canine hemolytic anemia. In a study of dogs with hemolytic anemia collected in England, results showed no *M. haemocanis* presence in dog populations (Warman *et al.*, 2010). Previous studies partially explained this phenomenon by noting that other agents including *Babesia gibsoni* and *B. canis* subspecies can induce

hemolytic anemia (Brown *et al.*, 2006; Mandal *et al.*, 2016). Therefore, veterinary clinician should pay more attention to confirm pathogens responsible for hemolytic anemia and accordingly choose effective chemicals to alleviate the symptom and treat the disease. The current technique available for haemotropic *Mycoplasma* treatment includes the use of antibiotics such as tetracycline, oxytetracycline, and doxycycline for three weeks (Chalker, 2005).

The phylogenetic tree constructed in this study based on partial 16S rRNA difficultly differentiated *M. haemocanis* from *M. haemofelis*. *M. haemocanis* and *M. haemofelis* were before regarded as the same species due to the high similarity 99% of the corresponding 16S rRNA fragment. However, the evidence that the hemotropic *Mycoplasma* can survive in cats inoculated with the blood from infected dogs, while dogs receiving infected blood from cats do not acquire a *Mycoplasma* infection obviously refuted this hypothesis. Further studies showed a potential presence of *M. haemocanis* in feline animals but impossible occurrence of *M. haemofelis* in canine animals (do Nascimento *et al.*, 2012). Recent full genome sequencing of *M. haemocanis* showed several differences with that of *M. haemofelis*. Up to this day, the RNase P gene is popularly considered as an efficacious bio-molecular marker to differentiate *M. haemocanis* from *M. haemofelis* with an identity of 94.3-95.5% to each other (do Nascimento *et al.*, 2012). Sequences in our study isolated from dogs and forming a clade with both *M. haemocanis* and *M. haemofelis* substantially supported *M. haemocanis* infection in the dog. *M. haemocanis* in this study appeared to be remarkably similar to previously described species with 99%-100% homology to the reference sequences from around the world.

Three healthy, female dogs were molecularly found to carry *M. haemocanis* in 162 dogs in southeastern China. These infected dogs were grown in pet dog breeding center and had a history of tick infestation. This is the first molecular report of *M. haemocanis* in dogs from China.

Acknowledgements. This study was supported financially by grant 2016BBG70005, funded by the Jiangxi Provincial Department of Science and Technology, and grant 20162007, funded by the Health and Family Planning Commission of Jiangxi Province. Weiqing Zheng was partially supported by Sasakawa Medical Fellowship through Japan-China Medical Association. Meanwhile, the authors also wish to acknowledge Yuanping Deng (the director of Anyi Center for Disease Control and Prevention) and Jianping He (the director of Xinjian Center for Disease Control and Prevention) for technical assistance during the collection of samples.

Authors' contribution

XX, CH and ZW were involved in the conception and design of the study. ZW drafted the manuscript. LM supervised the study. ZW and LM were involved in the provision of study material and data acquisition. MPFA and ZW were involved in data analysis and interpretation. ZW and LZ participated in design and field sampling. XX, MPFA and AE helped to review and revise the manuscript. All authors read and approved the final version of the manuscript.

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