

Detection of DNA of 'Candidatus Mycoplasma haemominutum' and Spiroplasma sp. in Unfed Ticks Collected from Vegetation in Japan

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(Received 25 May 2005/Accepted 19 August 2005)

ABSTRACT. DNA fragments of 'Candidatus Mycoplasma haemominutum', a feline haemobartonella pathogen, were detected from unfed *Ixodes ovatus* collected from vegetation in Hokkaido, Fukushima and Yamaguchi Prefectures, and unfed *Haemaphysalis flava* in Yamaguchi Prefecture. This finding suggests that ixodid tick is a possible vector of 'C. Mycoplasma haemominutum'. *Spiroplasma* DNA was also detected from unfed *I. ovatus* in Hokkaido, Fukushima and Yamaguchi Prefectures. The analysis of nucleotides sequence suggested that this *Spiroplasma* was distinct from registered species.

KEY WORDS: 'Candidatus Mycoplasma haemominutum', *Spiroplasma* sp., tick.

J. Vet. Med. Sci. 67(12): 1277-1279, 2005

The feline hemoplasmas, *Mycoplasma haemofelis* and 'Candidatus Mycoplasma haemominutum', were previously ascribed to *Haemobartonella felis* strains Ohio-Florida and California-Birmingham, respectively [11-13], which cause hemolytic anemia, thrombocytopenia, fever and jaundice [3, 4, 6]. Both species have been found in cats in Japan [9]. Transmission of feline hemoplasma has not been clearly demonstrated yet. It is thought that transmission may occur via cat-bite wounds [3]. Transmission of feline hemoplasma by blood-sucking arthropods is also considered by many to be the primary mode of transmission in cats [3], although such transmission has not been established experimentally. A canine hemoplasma, *M. haemocanis*, has been confirmed to be transmitted by *Rhipicephalus sanguineus* [14]; however, there have been no reports on the relationship between feline hemoplasma and ticks. The aim of the present study was to clarify the epidemiology of feline hemoplasma infection in Japan using tick samples. Accordingly, detection of *M. haemofelis* and 'C. M. haemominutum' from unfed-ticks recovered from vegetation in Japan was attempted using a species-specific nested PCR. Additionally, DNA of *Spiroplasma* sp., a potential zoonotic bacterium was also detected from Ixodid ticks in this study.

Unfed-adult ticks were collected from vegetation by flagging in Hokkaido, Fukushima and Yamaguchi Prefectures, Japan in March to June 2003. A total of 76 *Ixodes ovatus*, 10 *Haemaphysalis yeni*, 10 *Haemaphysalis flava* and 8 *Haemaphysalis longicornis* were subjected to DNA extraction. After identification, 5 to 16 ticks from vegetation were pooled for the further analysis (Table 1). Total DNA was extracted from each tick pool using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), adjusted to be 200 μ l of TE buffer and stored at minus 20°C until further use. The successfulness of DNA extraction was confirmed by

PCR with the primers 28SF and 28SR to detect the 28S rRNA gene of ticks as described previously [8]. The first PCR was performed in a 25- μ l reaction mixture containing 5 μ l of each DNA template with a primer set consisting of universal-fD1 [17] and Hemo-513R (5' ACG CCC AAT AAA TCC GAA TAA 3'), which were designed to amplify the 16S rRNA gene of hemoplasma and related species, including *Mycoplasma*, *Spiroplasma*, *Acholeplasma* and *Ureaplasma*. The amplification procedure was performed as reported previously [7]. The first PCR product from each sample was diluted 1 to 10 or 1 to 100 with distilled water and 5 μ l of each dilution was used as the template for the second PCR in a final volume of 25 μ l. For the second PCR, *M. haemofelis*-specific primers OH/FL-F (5' GGA TCT TGG TTT CGG CCA AG 3') and Hemo-457R (5' CAT AGT TTG CTG TCA CTT ATT C 3'), and 'C. Mycoplasma haemominutum'-specific primers CA/BM-F (5' GAA CGA AGA GGG TTT ACT CTC 3') and Hemo-457R were used. The amplification products were visualized on a 2% agarose gel after electrophoretic migration. To confirm the nested PCR results, the products with a positive reaction were purified using a QIAPCR purification kit (QIAGEN) and subjected to direct sequence analysis with a Perkin-Elmer ABI Prism 377 automated DNA sequencer at the DNA Core Facility of the Center for Gene Research, Yamaguchi University, as described previously. When a sample showed positive for the first PCR and negative for the second PCR, the first PCR product was also analyzed for sequence. The sequence data of the PCR products in both screening and confirmation were analyzed by the BLAST program at NCBI for homology. The GenBank accession numbers of 16S rRNA gene sequences used to analyze the percent identities and to construct a phylogenetic tree were as follows: *M. haemofelis*: U88563, 'Candidatus Mycoplasma haemominutum': AF271154, 'Candidatus Mycoplasma haemominutum': AY383241, *M. haemocanis*: AF197337, *M.*

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Table 1. Detection of hemoplasma and *Spiroplasma* from unfed-ticks collected from vegetation

Pool ID	Tick species	Numbers of ticks		Results of PCR and sequences
		Male	Female	
Hokkaido IO-1	<i>Ixodes ovatus</i>	6	0	<i>Spiroplasma</i> sp.
Hokkaido IO-2	<i>I. ovatus</i>	0	11	'C. M. haemominutum'
Fukushima IO-1	<i>I. ovatus</i>	7	0	'C. M. haemominutum'
Fukushima IO-2	<i>I. ovatus</i>	0	9	<i>Spiroplasma</i> sp.
Fukushima IO-3	<i>I. ovatus</i>	7	0	<i>Spiroplasma</i> sp.
Fukushima IO-4	<i>I. ovatus</i>	0	16	—
Yamaguchi IO-1	<i>I. ovatus</i>	2	2	'C. M. haemominutum'
Yamaguchi IO-2	<i>I. ovatus</i>	5	11	<i>Spiroplasma</i> sp.
Yamaguchi HY-1	<i>Haemaphysalis yeni</i>	0	10	—
Yamaguchi HF-1	<i>H. flava</i>	4	6	'C. M. haemominutum'
Yamaguchi HL-1	<i>H. longicornis</i>	0	8	—

haemomuris: U82963, *M. suis*: U88565, *M. ovis*: AF338268, *M. fastidiosum*: AF125878, *Spiroplasma* spp. from various hosts: AB030022, AB048263, AJ006775, AJ132412, AJ245996, AY189126, AY771927, M24476, M24477, M24662, *Acholeplasma laidlawii*: M23932, *Ureaplasma urealyticum*: L08642, *Anaplasma marginale*: M60313. The accession numbers of the partial sequences of the 16S rRNA gene of 'C. Mycoplasma haemominutum' and *Spiroplasma* sp. determined in this study were as follows: 'C. Mycoplasma haemominutum' Hokkaido IO-2: DQ059994, Fukushima IO-1: DQ059995, Yamaguchi IO-1: DQ0599916, Yamaguchi HF-1: DQ059997, *Spiroplasma* sp. Hokkaido IO-1: DQ059993; Fukushima IO-2, DQ059991, Fukushima IO-3: DQ059992, Yamaguchi IO-2: DQ059990.

A total of 11 pools of unfed ticks from vegetation were examined for hemoplasma infection (Table 1). Among 8 pools of *I. ovatus*, 3 were positive for 'C. Mycoplasma haemominutum', but none for *M. haemofelis*. A female pool of *I. ovatus* in Hokkaido (Hokkaido IO-2) was positive, while another male pool in Fukushima (Fukushima IO-1) also showed positive. It is possible that both males and females may carry 'C. Mycoplasma haemominutum'. This hemoplasma was also detected in pools from *I. ovatus* (Yamaguchi IO-1) and *H. flava* (Yamaguchi HF-1) in Yamaguchi. Both *I. ovatus* and *H. flava* are common tick species of feline hosts in Japan [15]. Results of the nested PCR were confirmed by subsequent sequence analysis. The nucleotide sequences of the PCR products showed 99.8 to 100% homology with 'C. Mycoplasma haemominutum' (Fig. 1). The present data suggest that these common tick species found in Japan are possible vectors of feline hemoplasma. As all the ticks examined in this study were adult stage, the ixodid ticks might be infected with hemoplasma by feeding blood from animals infected with the pathogen at nymphal stage. Thus it was thought that transstadial transmission can be occurred among ixodid ticks. This is the first detection of feline hemoplasma in unfed ticks; however, more direct evidence, including experimental transmission, will be required in the future to confirm these findings.

Other four pools, Hokkaido IO-1, Fukushima IO-1 and 2,

and Yamaguchi IO-2 showed positive reaction in the screening PCR, but negative for the hemoplasma specific-PCR. Analysis of the partial 16S rRNA gene indicated that the organism was closely related to *Spiroplasma* spp. found in insects and ticks (Fig. 1). However, *Spiroplasma* sp. detected in this study showed a distinct clade from other species with higher bootstraps values. The partial 16S rRNA gene sequences of the PCR products showed 97.5 to 98.1% homology with that of *Spiroplasma ixodetis*, that was isolated from *Ixodes pacificus* in the U.S.A. [16]. These findings suggest that *Spiroplasma* sp. detected in this study might be distinct from known species. More studies including isolation and gene analysis to characterize this organism are required. Spiroplasmas are symbiotic bacteria associated with insects, ticks, and plant, but some species are known to be pathogenic for their hosts; for example, sex ratio trait of *Drosophila melanogaster* is affected by spiroplasmas [1]. Recently, spiroplasmas were also reported to be pathogenic to both human and animals. They are related with diseases such as cataract, Creutzfeldt-Jakob disease and scrapie [2, 10]. At this moment, the effects of spiroplasmas on ticks and animals are not known at all.

ACKNOWLEDGMENTS. The authors would like to acknowledge Drs. Kenji Miyamoto and Hiromi Fujita for tick collection. They are thankful for the technical expertise of the DNA Core Facility of the Center for Gene Research, Yamaguchi University, supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan. This work was supported in part by Merial Japan Ltd. and Nippon Zenyaku Kogyo Co., Ltd., and a Grant-in-Aid for Scientific Research from JSPS.

REFERENCES

1. Anbutsu, H. and Fukatsu, T. 2003. *Appl. Environ. Bacteriol.* **69**: 1428–1434.
2. Bastian, F. O. and Foster, J. W. 2001. *J. Neuropathol. Exp. Neurol.* **60**: 613–620.
3. Breitschwerdt, E. B. 2000. pp. 400–408. *In: Textbook of Veterinary Internal Medicine*, 5th ed. (Ettinger, S. J. and Feldman, E. C. eds.), W. B. Saunders, Philadelphia.

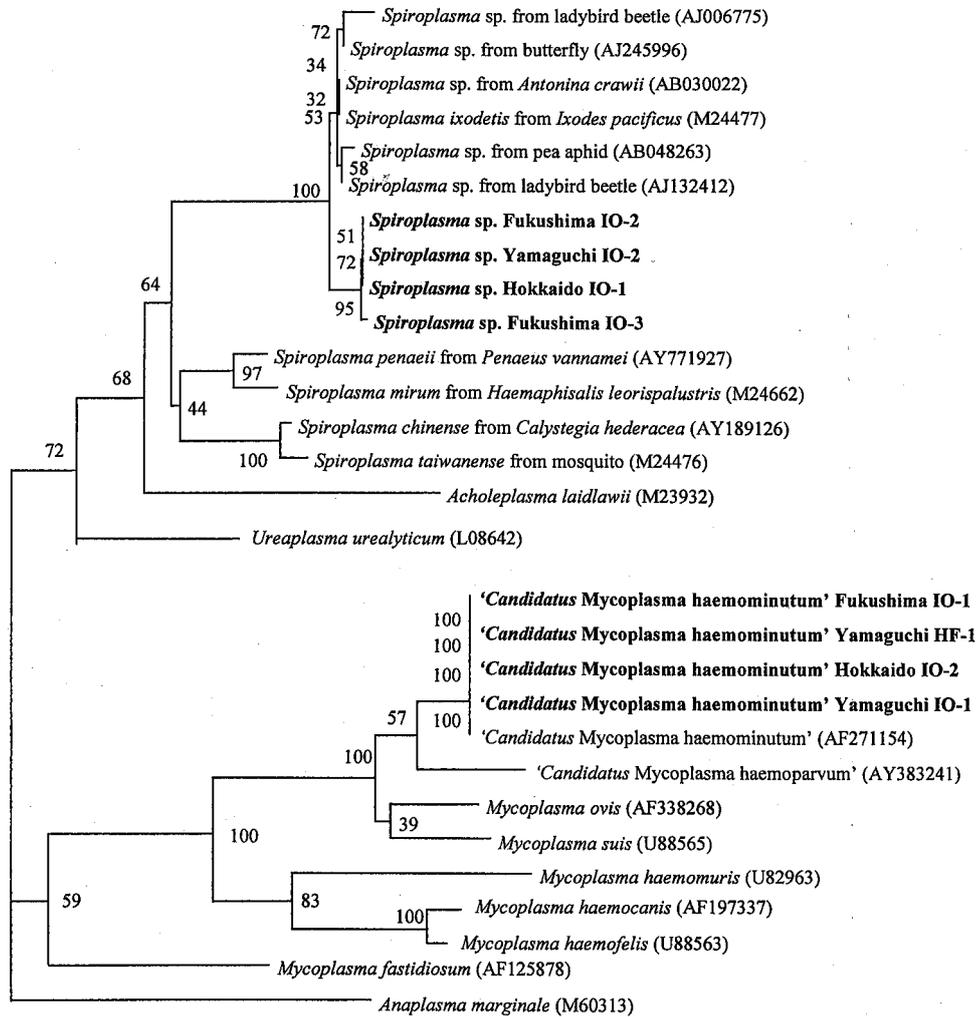


Fig. 1. Phylogenetic relationship of various hemoplasmas, spiroplasmas and related species based on partial sequences of the 16S rRNA gene. The neighbor-joining method was used to construct the phylogenetic tree with the Clustal W program. The scale bar represents 10% divergence. The numbers at nodes are the proportions of 100 bootstraps resamplings that support the topology shown.

- Flint, J. C. and Moss, L. C. 1953. *J. Am. Vet. Med. Assoc.* **122**: 45–48.
- Fukatsu, T., Tsuchida, T., Nikoh, N. and Koga, R. 2001. *Appl. Environ. Bacteriol.* **67**: 1284–1291.
- Harvey, J. W. and Gaskin, J. M. 1977. *J. Am. Anim. Hosp. Assoc.* **13**: 28–38.
- Inokuma, H., Parola, P., Raoult, D. and Brouqui, P. 2001. *Vet. Parasitol.* **99**: 335–339.
- Inokuma, H., Beppu, T., Okuda, M., Shimada, Y. and Sakata, Y. 2003. *Vet. Parasitol.* **115**: 343–348.
- Inokuma, H., Taroura, S., Okuda, M., Hisasue, M., Itamoto, K., Une, S., Nakaichi, M. and Taura, Y. 2004. *J. Vet. Med. Sci.* **66**: 1017–1020.
- Lorenz, B., Schroeder, J. and Reischl, U. 2002. *Graefe's Arch. Clin. Exp. Ophthalmol.* **240**: 348–353.
- Messick, J. B., Walker, P. G., Raphael, W., Bernt, L. and Shi, X. 2002. *Int. J. Syst. Evol. Microbiol.* **52**: 693–698.
- Neimark, H., Johansson, K. E., Rikihisa, Y. and Tully, J. G. 2001. *Int. J. Syst. Evol. Microbiol.* **51**: 891–899.
- Rikihisa, Y., Kawahara, M., Wen, B., Kochiba, G., Fuerst, P., Kawamori, F., Suto, C., Shibata, S. and Futohashi, M. 1997. *J. Clin. Microbiol.* **35**: 823–829.
- Seneviratna P, Weerasinghe and Ariyadas, S. 1973. *Res. Vet. Sci.* **14**: 112–114.
- Shimada, Y., Inokuma, H., Beppu, T., Okuda, M. and Onishi, T. 2003. *Vet. Parasitol.* **111**: 231–239.
- Tully, J. G., Rose, D. L., Yunker, C. E., Carle, P., Bove, J. M., Williamson, D. L. and Whitcomb, R. F. 1995. *Int. J. Syst. Bacteriol.* **45**: 23–28.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. L. 1991. *J. Bacteriol.* **173**: 697–703.