Prevalence and Molecular Analyses of Hemotrophic Mycoplasma spp. (Hemoplasmas) Detected in Sika Deer (Cervus nippon yesoensis) in Japan

Michihito TAGAWA¹,²,³, Kotaro MATSUMOTO¹, Naoaki YOKOYAMA⁴ and Hisashi INOKUMA¹)*

¹Department of Clinical Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido, 080–8555, Japan
²United Graduate School of Veterinary Sciences, Gifu University, Gifu 501–1193, Japan
³Research Fellow of the Japan Society for the Promotion of Science
⁴National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido, 080–8555, Japan

(Received 3 October 2013/Accepted 8 November 2013/Published online in J-STAGE 22 November 2013)

ABSTRACT. Hemotropic mycoplasmas (hemoplasmas) are cell-wall deficient, erythrocytic bacteria that cause infectious anemia in several mammalian species. The prevalence of hemoplasma species was examined by screening and species-specific PCR using blood samples collected from 51 sika deer in Hokkaido, Japan. Molecular analyses were performed for the 16S rRNA, 23S rRNA and RNase P RNA (rnpB) gene sequences. A total of 23/51 (45%) deer DNA samples were positive for hemoplasmas in the screening PCR. Using species-specific PCR, 12 and 17 samples were positive for ‘Candidatus Mycoplasma haemocervae’ and ‘Candidatus M. erythrocervae’, respectively. Sequencing and phylogenetic trees of those three genes indicate that the ‘Candidatus M. haemocervae’ and ‘Candidatus M. erythrocervae’ detected in Japanese deer are potentially different species from the cervine hemoplasma found in deer from America and Brazil.

KEY WORDS: Cervus nippon yesoensis, hemoplasma, Japan, phylogeny.

doi: 10.1292/jvms.13-0486; J. Vet. Med. Sci. 76(3): 401–407, 2014

Hemotropic mycoplasmas or hemoplasmas (formerly Haemobartonella and Eperythrozoon spp.) are cell-wall deficient, erythrocytic bacteria that have a worldwide distribution and cause infectious anemia in several mammalian species [7]. To date, all attempts to cultivate these organisms in vitro have failed [11], and the sensitivity and specificity of microscopic examination of blood smears are low [6]. PCR-based assays have been used to detect and diagnose hemoplasma infection [13]. A few reports exist regarding hemoplasma infections in the family Cervidae. Stoffregen revealed that at least two species of hemoplasma are found in reindeer (Rangifer tarandus) [12]. Sequences from one species were closely related to Mycoplasma ovis, and the other species, which was most closely related to Mycoplasma haemofelis and Mycoplasma haemocanis, was named ‘Candidatus Mycoplasma haemotarandirangiferis’. Variant strains of Mycoplasma ovis (M. ovis-like sp.) were detected in Dwarf Brocket deer (Mazama nana), Red Brocket deer (Mazama americana) and Marsh deer (Blastocerus dichotomus) in Brazil [3] and in White-tailed deer (Odocoileus virginianus) in America [1]. In addition, three species including an M. ovis-like sp. were found in Pampas deer (O. bezoarticus) and Marsh deer in Brazil and were deemed to be distinct based on 16S and 23S rRNA gene sequences [2]. A recent study has shown that M. ovis-like sp. and other two novel hemotropic Mycoplasma species were found in White-tailed deer in America based on 16S rRNA and RNase P RNA (rnpB) genes [5]. On the other hand, 2 distinct hemoplasma species have been identified in sika deer (Cervus nippon centralis) in Japan [16]. Although there is no rnpB gene sequence available for ‘Candidatus M. erythrocervae’, the report called these 2 pathogens ‘Candidatus M. haemocervae’ and ‘Candidatus M. erythrocervae’ based on sequence results of their 16S rRNA and rnpB genes [16]. In short, there is no report using those 3 genes for the comparison of phylogenetic position of various hemoplasma strains which were found in the family Cervidae.

Hokkaido sika deer (Cervus nippon yesoensis) is the largest subspecies of sika deer that inhabits Hokkaido, the northern island of Japan. Hemoplasma infection in Hokkaido sika deer is poorly characterized. Thus, the aim of this study was to determine the prevalence and phylogenetic positions of hemoplasma in Hokkaido sika deer in Japan using 16S rRNA, 23S rRNA and rnpB genes.

MATERIALS AND METHODS

Samples: EDTA-anticoagulated blood samples were collected from 51 sika deer (Cervus nippon yesoensis) that were maintained in captivity for the purpose of meat processing in the Kushiro District (Hokkaido, Japan) in June and July 2011. DNA was extracted from 200 µl of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), eluted with 200 µl of buffer AE according to the
manufacturer’s instructions, and stored at −30°C until use.

Screening and species-specific PCR: We screened all DNA samples for the presence of hemoplasma using a previously described protocol [13]. The F2/R2 primer set amplifies the samples for the presence of hemoplasma using a previously described primer set [13].

The 16S rRNA gene sequences of hemoplasma, 2 PCRs were performed using the following 2 primer sets: 23S Fw and Deerhemo R, and Deerhemo F and 23S Rv [3]. PCR conditions were as previously described, except that the annealing temperatures were 55°C.

The screening PCR was performed for all deer samples and cooling to 4°C. All amplicons were electrophoresed on a 15% polyacrylamide gel at 72°C for 90 sec; and a final extension at 72°C for 5 min. Cycling conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 90 sec; and a final extension at 72°C for 5 min and cooling to 4°C. All amplicons were electrophoresed on a 2.0% agarose gel in TBE buffer and visualized under UV light.

To distinguish between ‘Candidatus M. haemocervae’ and ‘Candidatus M. erythrocytum’, species-specific PCR method was developed for the 16S rRNA gene. Primers for the species-specific PCR were designed based on the 16S rRNA gene sequences of ‘Candidatus M. haemocervae’ (AB558899) and ‘Candidatus M. erythrocytum’ (AB558897 and AB558898). The primer sets were CMhc F (5′-CCGC

\[ \text{AB836744 (No. 13), AB836745 (No. 16), AB836746 (No. 33), AB836747 (No. 34) and AB836748 (No. 49).} \]

RESULTS

Prevalence of hemoplasma infection: A total of 23/51 (45%) deer DNA samples were positive for hemoplasmas in the screening PCR. The species-specific PCR was performed using DNA samples from deer which were confirmed as ‘Candidatus M. haemocervae’ and ‘Candidatus M. erythrocytum’ by sequence analysis in this study. Two PCR were used to amplify each gene, and 439 and 363 bp amplicons were observed in ‘Candidatus M. haemocervae’ and ‘Candidatus M. erythrocytum’ positive samples, respectively (Fig. 1). When the species-specific PCR was performed to screen PCR-positive samples, 12 and 17 samples were found to be positive for ‘Candidatus M. haemocervae’ and ‘Candidatus M. erythrocytum’, respectively. Six samples were found to be dual infections (Table 1).

Molecular characterization of hemoplasmas: The complete 16S rRNA gene sequences were successfully determined for the 6 randomly chosen hemoplasma-positive samples. Among cervine hemoplasma isolates, the 16S rRNA gene sequences obtained from deer Nos. 12, 16, 33 and 34
were most closely related to ‘Candidatus M. haemocervae’ (AB558899) with a percent identity of 99.86 to 99.32%. Isolate No. 16 which was representative sequence of ‘Candidatus M. haemocervae’ matched with a Mycoplasma sp. from White-tailed deer in America (FJ824847) with a percent identity of 97.61%. Hemoplasma species in deer No. 49 were most closely related to Mycoplasma sp. (HQ634381) of Marsh deer in Brazil with a percent identity of 95.70% (Table 2).

The 16S rRNA gene-based phylogenetic tree revealed that ‘Candidatus M. haemocervae’ from Hokkaido sika deer Nos. 12, 16, 33 and 34 was on a cluster separate from the other family Cervidae in parenthesis.

---

**Table 2.** Sequence identities (%) among (a) 16S rRNA, (b) 23S rRNA and (c) rnpB genes of ‘Candidatus Mycoplasma haemocervae’ (isolate 16) and ‘Candidatus M. erythrocervae’ (isolate 49) detected in Hokkaido sika deer. Isolates 16 and 49 are one representative sequence of ‘Candidatus M. haemocervae’ and ‘Candidatus M. erythrocervae’. Genbank accession numbers of each hemoplasma detected in the other family Cervidae are in parenthesis.

| Sample No. | M. ovis-like sp. | Mycoplasma sp. | Mycoplasma sp. | Mycoplasma sp. | Mycoplasma sp. |
|------------|-----------------|----------------|----------------|----------------|----------------|
| (a) 16S rRNA | (FJ824847) | (HQ634379) | (HQ634380) | (KC512403) | (KC512402) |
| Isolate 16 | 98.32 | 95.91 | 95.1 | 96.3 | 95.77 |
| Isolate 49 | 95.92 | 97.75 | 93.58 | 97.79 | 97.8 |
| (b) 23S rRNA | (HQ197752) | (HQ634381) | (HQ634382) | NA | NA |
| Isolate 16 | 97.61 | 93.08 | 90.83 | - | - |
| Isolate 49 | 92.64 | 95.7 | 90.83 | - | - |
| (c) rnpB gene | (JQ610624)* | NA | NA | (JQ610626) | (JQ610628) |
| Isolate 16 | 92.73 | - | - | 92.78 | 88.89 |
| Isolate 49 | 92.59 | - | - | 91.84 | 92.59 |

NA: Not available. *: Mycoplasma sp. isolate from White-tailed deer in America (Group A) (5).
Canidatus M. erythrocervae' from deer Nos. 13 and 49 was also on a cluster separate from the unclassified hemoplasma (HQ634379) from Marsh deer (Fig. 2). The 23S rRNA gene-based phylogenetic tree revealed a similar result, wherein hemoplasmas from Nos. 16 and 33 and from No. 49 were separated from the M. ovis-like spp. and the unclassified Mycoplasma sp. (HQ634381) from Marsh deer, respectively (Fig. 3). The rnpB phylogenetic tree showed that hemoplasma from Nos. 16, 33 and 49 formed a separate cluster from Mycoplasma sp. (JQ610624) which are closely related to a M. ovis-like spp. (Fig. 4).

**DISCUSSION**

In the present study, hemoplasma species were detected from 45% (23/51) of the blood samples from sika deer in Hokkaido, Japan, by screening PCR. A past study revealed that hemoplasma infections were found in only 9% (13/147) of free-ranging sika deer in Iwate prefecture in the Tohoku region.
Hemoplasmas of Hokkaido Sika Deer

405

Region of Honshu island, Japan [16]. However, hemoplasma infections were found in 89.0% (65/73) of white-tailed deer in America [5] and 87.1% (27/31) of Brazilian deer [3], both groups of which were maintained in captivity or confined area. Given that the sika deer in the present study had also been maintained in captivity, it seems that the high prevalence of hemoplasma infections is attributable to situations in which animals are in close contact with one another.

As a result of the comparison of phylogenetic position of various hemoplasma strains which were found in the family Cervidae using 16S, 23S rRNA and rnpB genes, ‘Candidatus M. haemocervae’ obtained from Hokkaido sika deer was closely related to a previously reported species which are so-called M. ovis-like spp. on 16S rRNA (FJ824847) and 23S rRNA (HQ634381; HQ197750; HQ634382), M. wenyonii (NR076982), M. suis (NC015153), M. haemocanis (NR076944), M. haemofelis isolates Ohio2, Langford1 (NC017520; NC014970) and ‘Candidatus M. haemomolae’ (NR076983) are shown. M. pneumonia (NR077056) was used as an outgroup.

Fig. 3. Phylogenetic relationship of deer hemoplasma isolates (Nos. 16, 33 and 49) and the other hemoplasma species based on 23S rRNA gene using a neighbor-joining method. Mycoplasma ovis-like sp. isolates 0221, B86-1, Deer-Fawn (HQ197751; HQ634383; HQ197752), Mycoplasma sp. isolates B62-2, 1585, B88-3 (HQ634381; HQ197750; HQ634382), M. wenyonii (NR076982), M. suis (NC015153), M. haemocanis (NR076944), M. haemofelis isolates Ohio2, Langford1 (NC017520; NC014970) and ‘Candidatus M. haemomolae’ (NR076983) are shown. M. pneumonia (NR077056) was used as an outgroup.

Hemoplasmas of Hokkaido Sika Deer

405

region of Honshu island, Japan [16]. However, hemoplasma infections were found in 89.0% (65/73) of white-tailed deer in America [5] and 87.1% (27/31) of Brazilian deer [3], both groups of which were maintained in captivity or confined area. Given that the sika deer in the present study had also been maintained in captivity, it seems that the high prevalence of hemoplasma infections is attributable to situations in which animals are in close contact with one another.

As a result of the comparison of phylogenetic position of various hemoplasma strains which were found in the family Cervidae using 16S, 23S rRNA and rnpB genes, ‘Candidatus M. haemocervae’ obtained from Hokkaido sika deer was closely related to a previously reported species which are so-called M. ovis-like spp. on 16S rRNA (FJ824847) and 23S rRNA (HQ634381) gene sequences, while low identity was shown on rnpB gene between those species. In addition, ‘Candidatus M. erythrocervae’ was closely related to a Mycoplasma sp. (HQ634379) detected from Marsh deer in Brazil on 16S rRNA gene, while homology of 23S rRNA gene sequence between those two species seemed to be low because the threshold of the 23S rRNA gene sequence for identification between different bacterial species was 1.15% [8]. Moreover, phylogenetic trees based on the three genes also indicate that ‘Candidatus M. haemocervae’ and ‘Candidatus M. erythrocervae’ are independent from other cervine hemoplasma found in America and Brazil.

The phylogeny of hemoplasma species has been investigated based on the rnpB gene sequences, a more suitable target for phylogenetic discrimination of closely related taxa when compared with 16S rRNA sequences [9]. The RNA polymerase beta subunit (rpoB) and the 16S-23S rRNA intergenic transcribed spacer (ITS) region were found to be reliable and useful taxonomic tools for species differentiation within the family Mycoplasmataceae [15]. Further research is required to compare those genes among unclassified hemoplasma in the family Cervidae, including ‘Candidatus M. haemocervae’, ‘Candidatus M. erythrocervae’, M. ovis-like sp. and other hemoplasma of free-ranging deer in America and Brazil.

In this study, hemoplasma infections are common in Hokkaido sika deer. However, a further study using free-ranging Hokkaido sika deer is necessary, because animals using in this study were maintained in captivity. Lower percent identities and divergent phylogenetic position support the notion that ‘Candidatus M. haemocervae’ and ‘Candidatus M. erythrocervae’ are potentially different species from the other cervine hemoplasma found in America and Brazil. Further investigation is needed to clarify phylogenetic position, molecular characterization and pathogenicity of deer hemoplasma in Japan.
ACKNOWLEDGMENTS. We thank Ms. Hiroko Yamamoto for her excellent technical assistance. This study was supported by a Grant-in-Aid for JSPS Fellows (Grant Number 24.1401) from the Japan Society for the Promotion of Science, Japan.

REFERENCES

1. Boes, K. M., Goncarovs, K. O., Thompson, C. A., Halik, L. A., Santos, A. P., Guimaraes, A. M., Feutz, M. M., Holman, P. J., Vemulapalli, R. and Messick, J. B. 2012. Identification of a Mycoplasma ovis-like organism in a herd of farmed white-tailed deer (Odocoileus virginianus) in rural Indiana. Vet. Clin. Pathol. 41: 77–83. [Medline] [CrossRef]

2. Grazziotin, A. L., Duarte, J. M., Szabó, M. P., Santos, A. P., Guimarães, A. M., Mohamed, A., Vieira, R. F., de Barros Filho, I. R., Biondo, A. W. and Messick, J. B. 2011. Prevalence and molecular characterization of Mycoplasma ovis in selected free-ranging Brazilian deer populations. J. Wildl. Dis. 47: 1005–1011. [Medline] [CrossRef]

3. Grazziotin, A. L., Santos, A. P., Guimaraes, A. M., Mohamed, A., Cubas, Z. S., de Oliveira, M. J., dos Santos, L. C., de Moraes, W., Vieira, R. F., Donati, L., de Barros Filho, I. R., Biondo, A. W. and Messick, J. B. 2011. Mycoplasma ovis in captive cervids: prevalence, molecular characterization and phylogeny. Vet. Microbiol. 152: 415–419. [Medline] [CrossRef]

4. Jensen, W. A., Lappin, M. R., Kamkar, S. and Reagen, W. J. 2001. Use of a polymerase chain reaction assay to detect and differentiate two strains of Haemobartonella felis in naturally infected cats. Am. J. Vet. Res. 62: 604–608. [Medline] [CrossRef]

5. Maggi, R. G., Chitwood, M. C., Kennedy-Stoskopf, S. and DePerno, C. S. 2013. Novel hemotropic Mycoplasma species in white-tailed deer (Odocoileus virginianus). Comp. Immunol. Microbiol. Infect. Dis. (in press). [Medline] [CrossRef]

6. McAuliffe, L., Lawes, J., Bell, S., Barlow, A., Ayling, R. and Nicholas, R. 2006. The detection of Mycoplasma (formerly Eperythrozoon) wenyonii by 16S rRNA PCR and denaturing gradient gel electrophoresis. Vet. Microbiol. 117: 292–296. [Medline] [CrossRef]

7. Messick, J. B. 2004. Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. Vet. Clin. Pathol. 33: 2–13. [Medline] [CrossRef]

8. Pei, A., Nossa, C. W., Chokshi, P., Blaser, M. J., Yang, L., Rosmarin, D. M. and Pei, Z. 2009. Diversity of 23S rRNA genes within individual prokaryotic genomes. PLoS One 4: e5437. [Medline] [CrossRef]

9. Peters, I. R., Helps, C. R., McAuliffe, L., Neimark, H., Lappin,
HEMOPLASMAS OF HOKKAIDO SIKA DEER

M. R., Gruffydd-Jones, T. J., Day, M. J., Hoelzle, L. E., Willi, B., Meli, M., Hofmann-Lehmann, R. and Tasker, S. 2008. RNase P RNA gene (rnpB) phylogeny of Hemoplasmas and other Mycoplasma species. J. Clin. Microbiol. 46: 1873–1877. [Medline] [CrossRef]

10. Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425. [Medline]

11. Schreiner, S. A., Hoelzle, K., Hofmann-Lehmann, R., Hamburg,er, A., Wittenbrink, M. M., Kramer, M. M., Sokoli, A., Felder, K. M., Groebel, K. and Hoelzle, L. E. 2012. Nanotransformation of the haemotrophic Mycoplasma suis during in vitro cultivation attempts using modified cell free Mycoplasma media. Vet. Microbiol. 160: 227–232. [Medline] [CrossRef]

12. Stoffregen, W. C., Alt, D. P., Palmer, M. V., Olsen, S. C., Waters, W. R. and Stasko, J. A. 2006. Identification of a haemomyco-plasma species in anemic reindeer (Rangifer tarandus). J. Wild. Dis. 42: 249–258. [Medline] [CrossRef]

13. Tagawa, M., Ybañez, A. P., Matsumoto, K., Yokoyama, N. and Inokuma, H. 2012. Prevalence and risk factor analysis of bovine hemoplasma infection by direct PCR in eastern Hokkaido, Japan. J. Vet. Med. Sci. 74: 1171–1176. [Medline] [CrossRef]

14. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731–2739. [Medline] [CrossRef]

15. Volokhov, D. V., Simonyan, V., Davidson, M. K. and Chizhikov, V. E. 2012. RNA polymerase beta subunit (rpoB) gene and the 16S-23S rRNA intergenic transcribed spacer region (ITS) as complementary molecular markers in addition to the 16S rRNA gene for phylogenetic analysis and identification of the species of the family Mycoplasmataceae. Mol. Phylogenet. Evol. 62: 515–528. [Medline] [CrossRef]

16. Watanabe, Y., Fujihara, M., Obara, H., Matsubara, K., Yamauchi, K. and Harasawa, R. 2010. Novel hemoplasma species detected in free-rangign sika deer (Cervus nippon). J. Vet. Med. Sci. 72: 1527–1530. [Medline] [CrossRef]

17. Weisburg, W. G., Burns, S. M., Pelletier, D. A. and Lane, D. J. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697–703. [Medline]