

Prevalence of Hemoplasma Infection among Cattle in the Western Part of Japan

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(Received 5 June 2011/Accepted 15 July 2011/Published online in J-STAGE 29 July 2011)

ABSTRACT. We have examined for hemoplasma infection among cattle in the Hiroshima and Miyazaki prefectures by using a sensitive real-time PCR, with SYBR Green I and with melting curve analysis, which allow to distinguish the two bovine hemoplasma species, *Mycoplasma wenyonii* and ‘*Candidatus M. haemobos*’. We found 69.4% of 36 cattle in Hiroshima and 93.8% of 32 cattle in Miyazaki infected with either of these two hemoplasma species. High morbidity in western part of Japan may reflect the activity of arthropod vectors for hemoplasma transmission. We also demonstrated neonatal calves less than three months old affected with hemoplasmas without grazing in summer, suggesting a possibility of vertical transmission.

KEY WORDS: hemoplasma, mycoplasma, vertical transmission.

J. Vet. Med. Sci. 73(12): 1653–1655, 2011

Only two hemoplasma species, *Mycoplasma wenyonii* and ‘*Candidatus M. haemobos*’ (synonym for ‘*C. M. haemobovis*’) are currently recognized in cattle [6, 9]. Although *M. wenyonii* has been shown to exhibit worldwide geographical distribution, ‘*C. M. haemobos*’ has been solely reported from Switzerland, China, Germany and Japan [2, 3, 8, 9]. Both hemoplasma species are causative of infectious anemia in cattle to some extent and have been demonstrated in Hokkaido and Tohoku regions of this country. Incidences of *M. wenyonii* and ‘*C. M. haemobos*’ infections have been reported as 21.8 and 16.7%, respectively, in Hokkaido, and 40.3 and 56.8%, respectively, in the Miyagi prefecture [7, 9]. Infection has spread by transmission routes that we do not fully understand. Despite the relatively high incidence in these areas, little is known about the prevalence of bovine hemoplasma infections in western parts of Japan. Therefore, we examined for hemoplasma prevalence among cattle in the Hiroshima and Miyazaki prefectures by using a sensitive real-time PCR to detect the 16S rRNA gene [4], with SYBR Green I and with melting curve analysis, which allow to distinguish these two hemoplasma species.

EDTA-anticoagulated or heparinized blood samples from 68 cattle in different herds of the Hiroshima and Miyazaki prefectures were randomly collected between 2008 and 2011. Information on clinical diagnoses and age of all the cattle included in this study was obtained from the relevant veterinarians or owners. The ages of cattle were ranging from one month to 15 years old. Cattle included less than yearling in both the prefectures. Total DNA was extracted from 200 μ l whole blood samples by using the QIAamp

DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Negative controls consisting of 200 μ l phosphate-buffered saline solution were included with each batch. Extracted DNA samples were stored at –20°C prior to examination.

To detect the both hemoplasma species in real-time PCR, specific primers for the 16S rRNA gene were used as described previously [7]. Forward primer, 5’-ATATTC-CTACGGGAAGCAGC-3’, equivalent to nucleotide numbers 328 to 347 of *M. wenyonii* and reverse primer, 5’-ACCGCAGCTGCTGGCACATA-3’, equivalent to nucleotide numbers 503 to 522 of *M. wenyonii* amplified a 195 and 173 bp for *M. wenyonii* and ‘*C. M. haemobos*’, respectively. Nucleotide sequences and sizes bracketed by the primers are peculiar to each hemoplasma. The 22-bp gap in the PCR product from ‘*C. M. haemobos*’ attributes to a genetic marker to distinguish it from *M. wenyonii* in the real-time PCR [7].

Real-time PCR was performed in a SmartCycler instrument (Cepheid, Sunnyvale, CA, U.S.A.) with SYBR Premix Ex *Taq* (Code #RR041A, TaKaRa Bio., Shiga, Japan). The reaction mixture contained 1 μ l of each primer (10 pmol/ μ l), 12.5 μ l of 2X premix reaction buffer and water to volume of 23 μ l. Finally, 2 μ l of DNA samples as templates were added to this mixture. Amplification was achieved with 40 cycles of denaturation at 95°C for 5 sec, renaturation at 57°C for 20 sec, and elongation at 72°C for 15 sec, after the initial denaturation at 94°C for 30 sec. Positive cattle were found affected with each hemoplasma irrespective of the age.

After real-time PCR, melting experiment was performed from 60 to 95°C at 0.2°C/sec with smooth curve setting averaging one point. Melting peaks were visualized by plotting the first derivative against the melting temperature (*T*_m) as described previously [1]. Melting curve analysis of the amplified products allowed differentiation of these two

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hemoplasma species, since nucleotide sequences and sizes bracketed by the primers are distinct between the species. Thus, the variations in the *Tm* may serve as a differential marker for hemoplasma species. The input amount of DNA, the copy number of the target as well as presence of co-infections with several targets did not influence the *Tm*. No melting peak was evident on negative cattle.

In the present study, we found overall 69.4% (25/36) and 93.8% (30/32) cattle infected with hemoplasmas in Hiroshima and Miyazaki, respectively (Fig. 1). This incidence was much higher than that in Hokkaido and Miyagi, suggesting factors in geographical latitude affected in infections. The geographical difference in morbidity may attribute to activity of arthropod vectors for hemoplasma transmission. 'C. M. haemobos' infection was predominant in both the prefectures. Thirty-six cattle in Hiroshima included 18 stores from yearling to two years old and 18 adult dairy cows. Despite missing exact age, no relationship to infections was evident among age and bleed of cattle in Hiroshima. In Miyazaki, although extremely high incidence of infections was demonstrated over all, relatively low incidence was found in elder cattle. More surprising result is that all the calves less than three months born in winter were unexpectedly infected with 'C. M. haemobos' (Table 1). It raises a question of whether vectors for the hemoplasma transmission are unnecessary, since ticks have been suspected as a vector for feline hemoplasma infection [10]. Given the absence of vectors in winter, it will turn out that

vertical transmission is most likely route of neonatal infection, since cattle may become infection while grazing in summer.

The hemoplasma-infected cattle in the present study did not exhibit clinical signs such as anemia attributable to hemoplasmosis, though hemoplasma infections in cattle were first recognized in Swiss dairy cows with hemolytic anemia [3]. In our study, no significant association was found between the infection status and anemic syndromes. One possibility is that there might be distinct strains in virulence or anemia might be caused by opportunistic infections. As none of the PCR-positive cattle caused clinical manifestations of severe anemia or all cattle had been presented for reasons unrelated to hemoplasmosis, laboratory parameters were not subjected to in-depth investigation. Although our results indicate wide distribution of *M. wenyonii* and 'C. M. haemobos' among cattle population in this country without developing anemic syndromes, infected animals probably remain chronic carriers after clinical signs have resolved. Thus, the persistent infections with hemoplasmas may contribute to the progression of retroviral, neoplastic, or immune-mediated diseases [5]. Collectively, our findings suggest possibility of vertical transmission because of neonatal infections and monitoring of hemoplasma infection may help to maintain hygienic conditions for cattle production.

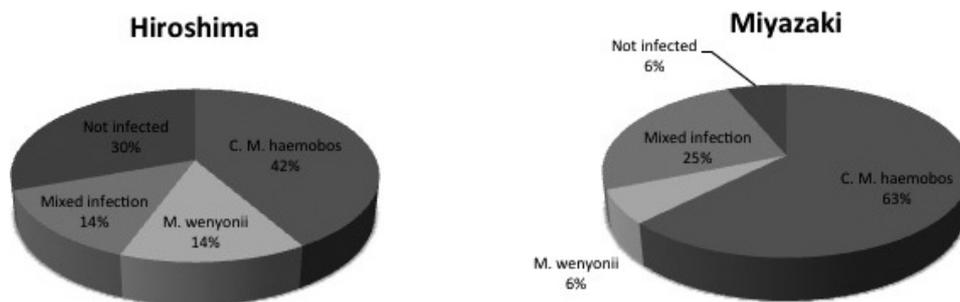


Fig. 1. Hemoplasma infections among cattle in Hiroshima and Miyazaki prefectures. Percentages were shown in round figures.

Table 1. Incidence of hemoplasma infections in cattle of the Miyazaki Prefecture

Age	No. of cattle examined	<i>M. wenyonii</i>	'C. M. haemobos'
Less than 3 months	4	0	4
Yearling	23**	7*	21*
2 years old	1	0	1
7 years old	1	0	0
11 years old	1	1*	1*
14 years old	1	1	0
15 years old	1	1*	1*
Total	32	10 (31.3%)	28 (87.5%)

* Numbers include mixed infections. ** One of the yearlings was not infected with either of the hemoplasma species.

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