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**Differential Detection of Hemotropic Mycoplasma Species in Cattle by Melting Curve Analysis of PCR Products**

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**ABSTRACT.** We developed a real-time PCR procedure followed by melting curve analysis using the green fluorescence dye SYBR Green I for rapid detection and differentiation of hemplasmas in cattle. Analysis of the melting temperature (Tm) of the PCR products allowed for differentiation of the 2 bovine hemplasmas, *Mycoplasma wenyonii* and a provisional species, ‘*Candidatus* Mycoplasma haemobos’ (a synonym of ‘*Candidatus* M. haemobovis’). The Tm (mean ± S.E.) of the PCR products from the bovine hemplasmas were 86.98 ± 0.12°C for ‘*M. wenyonii*’ and 82.04 ± 0.27°C for ‘*Candidatus* M. haemobos’ in the melting experiments. The protocol described in the present study can decrease the time to results by simultaneous detection and differentiation of the two hemplasmas in cattle. By using this protocol, we examined hemplasma prevalence in 109 cattle in Miyagi Prefecture and found that 67 (61.5%) were infected with *M. wenyonii*, 25 (22.9%) were infected with ‘*Candidatus* M. haemobos’ and 14 (12.8%) were infected with both.

**KEY WORDS:** hemplasma, Mycoplasma haemobos, *Mycoplasma wenyonii*. 

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In cattle (*Bos taurus*), two distinct hemotropic mycoplasmas (also known as hemplasmas) have been identified, *Mycoplasma wenyonii* (formerly *Eperythrozoon wenyonii*) [4] and a provisional species, ‘*Candidatus* Mycoplasma haemobos’ (synonym: ‘*Candidatus* M. haemobovis’) [6, 7]. Hemplasmas are tiny erythrocytic bacterial parasites that lack a cell wall like other mycoplasmas and are susceptible to tetracyclines, but have not been cultured in vitro. Infection may lead to hemolytic anemia in cattle, but veterinary investigation has been hampered by the lack of appropriate diagnostic procedures. Although most studies have relied on cytological identification of the organisms on blood smears, this method has a low diagnostic sensitivity and cannot distinguish the different species [3]. Furthermore, this diagnostic method may misidentify the hemplasmas as Howell-Jolly bodies, since they both appear frequently after splenectomy, are associated with anemia and contain DNA. Only recently have real-time PCR-based assays been applied for detection and identification of feline and canine hemplasma species [8, 9], though no report has been appeared concerning bovine hemplasmas until now. Distinguishing between these 2 hemplasmas is necessary because etiological significance has only been established for *M. wenyonii* as a mild anemia in cattle.

There is still little knowledge of the epidemiology of the hemotropic mycoplasmas in cattle [2]. Although *M. wenyonii* in cattle has been shown to exhibit a worldwide geographical distribution, ‘*Candidatus* M. haemobos’ has only been reported in Switzerland (accession numbers EF616467 and EF616468), China (accession number EF460765) and Japan (accession number EU367965).

In the present study, we demonstrated a rapid method of detecting and distinguishing between the two hemplasmas in cattle using sensitive real-time PCR with SYBR Green I and melting curve analysis. By using this method, we examined the prevalence and clinical importance of bovine hemplasma infections in Miyagi Prefecture, Japan.

EDTA-anticoagulated blood samples from 109 cattle in dairy and beef herds in Miyagi Prefecture, Japan, were collected in January 2009 and stored at −80°C for three weeks prior to examination. Information on the clinical diagnoses and ages of all the cattle included in this study were obtained from the Research Unit for Food Animal Internal Medicine & Production Medicine of Iwate University, Japan. The ages of the cattle ranged from seven months to 14 years old.

Total DNA was extracted from 200-μl EDTA-anticoagulated blood samples collected from cattle using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Negative controls consisting of 200 μl phosphate-buffered saline were prepared for each batch. Extracted DNA samples were stored at −20°C prior to use.

Conventional PCR was carried out with 50-μl reaction mixtures containing 1 μl of DNA solution, 0.5 μl of TaKaRa LA Taq™ (5 units/μl), 5 μl of 10X LA PCR™ Buffer II, 8 μl of 25 mM MgCl₂ (final 4.0 mM), 8 μl of dNTP mixture (2.5 mM each), 0.2 μl of forward primer (5'-ATCTAA-CATGCCCCCTCTGTAA-3’, equivalent to nucleotide numbers 109 to 128 of ‘*Candidatus* M. haemobos’, or 5'-ACTTTTACCGAGGAGGATAGC-3’, equivalent to nucleotide numbers 124 to 143 of *M. wenyonii*), reverse primer (5'-GTAGTATCCGGTCACAA-3’, equivalent to nucleotide numbers 589 to 608 of ‘*Candidatus* M. haemobos’, or 5'-TGATTTAATGAAAAAGCCG-3’, equivalent to nucleotide numbers 634 to 653 of *M. wenyonii*) (50 pmol/μl each) and water to a final volume of 50 μl. After the
bp and 173 bp product for otide numbers 503 to 522 of ACCGCAGCTGCTGGCACATA-3', equivalent to nucleotide numbers 328 to 347 of TCCTACGGGAAGCAGC-3', equivalent to nucleotide were originally designed. The forward primer, 5'-ATAT-primers for the 16S rRNA gene of bovine hemoplasmas polymorphism was evident in the nucleotide sequences. The cattle were affected with each hemoplasma irrespective of age. The input amount of DNA, copy number of the target and presence of co-infections with several targets did not influence the Tm.

After real-time PCR, a melting experiment was performed from 60 to 95°C at 0.2°C/sec with a smooth curve setting averaging one point. Melting peaks were visualized by plotting the first derivative against the melting temperature (Tm) as described previously [1]. The Tm was defined as a peak of the curve, and if the highest point was a plateau, then the mid-point was identified as the Tm. Since the nucleotide sequences and sizes bracketed by the primers are different between the two species, melting curve analysis of the amplified products allowed for differentiation of these two hemoplasmas. Thus, the variations in the Tm depend on sequence variations in the PCR products, which may serve as a differential marker for hemoplasma speciation. No melting peak was evident in the negative cattle.

The Tm (mean ± S.E.) of the PCR products from the bovine hemoplasmas were estimated to be 86.98 ± 0.12°C for M. wenyonii and 82.04 ± 0.27°C for ‘Candidatus M. haemobos’ in the melting experiments (Fig. 1). We identified the strains showing a Tm above 86.75°C as M. wenyonii and a Tm below 82.50°C as ‘Candidatus M. haemobos’. Of the 109 cattle, 67 (61.5%) were infected with M. wenyonii, 25 (22.9%) were infected with ‘Candidatus M. haemobos’ and 14 (12.8%) were infected with both. There were a few samples showing a Tm between the ranges for M. wenyonii and ‘Candidatus M. haemobos’. Positive controls were prepared by mixing positive blood samples for the two different hemoplasmas.

The hemoplasma-infected cattle in the present study did not exhibit clinical signs, such as anemia, attributable to hemoplasmosis. The hematocrit values ranged from 22 to 55% in the PCR-positive cattle. Hemoplasma infections in cattle were first recognized in Swiss dairy cattle with hemolytic anemia [2]. In our study, no significant association was found between the infection status and anemic syndrome. This may be due to low level infections, which were
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suggested by the poor intensity in PCR. As none of the PCR-positive cattle showed signs of severe anemia and all cattle had been presented for reasons unrelated to hemoplasmosis, laboratory parameters were not analyzed in more detail. Although our results indicate a wide distribution of *M. wenyonii* among the cattle population in Miyagi, Japan, without the development of anemic signs, infected animals probably remain chronic carriers after clinical signs have resolved. Thus, persistent infections with hemoplasmas may contribute to the progression of retroviral, neoplastic or immune-mediated diseases [5].

In the present study, we demonstrated a rapid diagnosis procedure for hemoplasma infections in cattle that allows for distinguishing between *M. wenyonii* and ‘Candidatus *M. haemobos*’ infections by using real-time PCR with melting curve analysis of the PCR products.

REFERENCES


