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Short communication

Interference between *Theileria orientalis* and hemotropic *Mycoplasma* spp. (hemoplasmas) in grazing cattle

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ABSTRACT

Theileria orientalis and hemotropic *Mycoplasma* spp. (hemoplasmas) can cause anemia in cattle. Cattle infected with one of these two pathogens tend to resist infection by the other pathogen. This is called the "interference phenomenon". However, the detailed investigation of this phenomenon using molecular techniques has not been performed until now. We used PCR to analyze blood samples from cattle grazing in two pastures, and investigated interference between *T. orientalis* and hemoplasmas. Results indicate that single infection with *T. orientalis* or hemoplasmas was more common than co-infections. The degree of anemia observed in co-infected animals was significantly milder compared to those only infected with *T. orientalis*. These findings revealed that the interaction between these two pathogens in cattle demonstrated interference.

1. Introduction

Theileria orientalis is a non-lymphoproliferative, tick-transmitted protozoan that infects a broad range of domestic and wild animals, particularly ruminants (Gubbels et al., 2000). The parasite has been reported in several countries and is a member of the relatively benign *Theileria* group (*Theileria sergenti*/*buffeli*/*orientalis*), but infected cattle sometimes show clinical signs including fever, anemia, and anorexia (Yokoyama et al., 2012). Hemotropic mycoplasmas, also known as hemoplasmas, are unculturable bacteria that have a worldwide distribution and cause infectious anemia in several mammalian species (Messick, 2004). Originally known as *Eperythrozoon* species, they were reclassified as *Mycoplasma* based on 16S rRNA gene sequences and morphological similarities (Neimark et al., 2001). Two distinct species have been identified that infect cattle: *Mycoplasma wenyonii* (formerly, *Eperythrozoon wenyonii*) (Neimark and Kocan, 1997), and a provisional species, "*Candidatus* Mycoplasma haemobos" (synonym, "*Candidatus* M. haemobovis") (Tagawa et al., 2008). Clinical signs of acute infection in cattle include anemia, transient fever and lymphadenopathy (Smith et al., 1990). However, chronic infection in most animals is subclinical, and the parasites almost disappear (Messick, 2004). The mode of transmission is unknown, but arthropods and transplacental route have been suggested (Hornok et al., 2011).

Interestingly, past studies using experimental animals showed that animals infected with one of two pathogens tend to resist infection by another pathogen. The observation was called "interference phenomenon". Foote et al. (1957) found that an acute relapse of *E. wenyonii* in splenectomised calves may prevent a subsequent relapse of *Anaplasma marginale*. Raynaud (1962) also found that these parasites may interfere with each other after splenectomy. On the other hand, a similar phenomenon was observed in cattle co-infected with *T. orientalis* and *A. marginale* (Gale

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et al., 1997). Although *T. orientalis* and bovine hemoplasma infections are very common in pasturelands of endemic areas, the clinical pathological background of co-infection with these two pathogens has not yet been described. Ishihara (1962) described that cattle infected with one of these two pathogens tend to resist infection by the other. However, the phenomenon was only described in experimental infections, and molecular techniques were not utilized to detect these pathogens. In addition, it is unknown whether *T. orientalis* infection is affected by chronic phase of bovine hemoplasma in which the organism is no longer detectable. The present study aimed to demonstrate interference between *T. orientalis* and bovine hemoplasma infections in field conditions using molecular techniques.

2. Materials and methods

2.1. Pastures

The two pastures (A and B) were located in Hokkaido, Japan, and were known to be endemic for *T. orientalis*. Approximately 400 heifers were grazed on each pasture annually. Cattle were physically checked before grazing to ensure that only healthy cattle were grazed. We collected cattle from farmers near the pastures, allowed them to graze from May to September, and then returned them to their respective farmers. Furthermore, we periodically treated the two pastures with flumethrin pour-on (1 mg/100 kg, Bayticol®, Bayer Japan) to control ticks approximately every two weeks from June to August.

2.2. Tick collection

For the tick survey, we collected unfed ticks by flagging the grass in and around the two pastures during May and June of 2011, before the beginning of pasturage. Following tick identification, we extracted DNA from each tick using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and stored it at −30 °C until use.

2.3. Blood sampling and hematology

We randomly selected 120 cattle from each pasture before pasturage for blood sampling to screen for *T. orientalis* infection by PCR. We included 90 cattle (40 for pastureland A and 50 for pastureland B) that were negative for *T. orientalis* in this study. All cattle were Holstein-Friesian aged between 6 and 21 months. We then collected a total of five peripheral blood samples using EDTA anticoagulant tubes in alternate weeks from May to July in 2011. Additionally, packed cell volume (PCV) was obtained for all samples using a Celltac α MEK-6350 (Nihon Kohden, Japan). For detection of *T. orientalis*, we extracted total DNA from each 200-μL blood sample as described above. The remainder of the whole blood samples was stored until detection of bovine hemoplasma.

2.4. PCR assays

We screened all DNA samples, including blood and ticks, for the presence of *T. orientalis* using a previously described MPSP-PCR assay that detects all genotypes of the parasite (Ota et al., 2009). Furthermore, we tested whole blood samples for bovine hemoplasmas using a previously described direct PCR (Tagawa et al., 2012). The F2/R2 primer set amplifies the 16S rRNA genes of most hemoplasmas, including *M. wenyonii*, and "*Candidatus* M. haemobos" (Jensen et al., 2001). All amplicons were electrophoresed on a 2.0% agarose gel in TBE buffer and visualized under UV light.

2.5. Statistical analysis

Results from PCR assays and PCV values were compiled and analyzed. For the categorical variables of *T. orientalis* and hemoplasma prevalence rates, we used the chi-square test. The continuous variables of the PCV was analyzed by the Mann–Whitney *U*-test (for 2 groups) and the Kruskal–Wallis test, followed by Sheffe's *F*-test (for >2 groups). We considered differences to be statistically significant if $P < 0.05$.

3. Results and discussion

The PCR assays were able to specifically detect *T. orientalis* and bovine hemoplasma infections. *T. orientalis* infections increased rapidly in pasture A, with 34 of 36 (94.4%) cattle infected at the 5th sampling. On the other hand, the prevalence rate of hemoplasma infection was relatively low and remained constant throughout the study period (Fig. 1). In pasture A, two and four cattle were excluded at the 3rd and 5th samplings, respectively, because they were returned to their respective farmers for treatment of diseases other than *T. orientalis* infection. We observed only a slight increase in *T. orientalis* infection from the 2nd to 5th sampling in Pasture B, while hemoplasma prevalence remained high until the last sampling (Fig. 1). Results of the chi-square test revealed that single infection with *T. orientalis* or hemoplasma was more common than co-infections at the 4th and 5th samplings (Table 1). Previous studies performed in Hokkaido, Japan, suggested that grazing increases *T. orientalis* infection and ticks play an important role as transmission vectors of this pathogen (Ota et al., 2009; Yokoyama et al., 2012). We collected 202 and 570 ticks from pastures A and B, respectively (Table 2). Three tick species, *Ixodes ovatus* ($n = 303$), *Haemaphysalis douglasi* ($n = 252$), and *Ixodes persulcatus* ($n = 217$), were identified among the collected ticks with similar distributions in the two pastures. *T. orientalis* was detected in all three tick species, and positive rates in pastures A and B were 6.9% and 6.0%, respectively. The chi-square test revealed no significant difference in the prevalence of the parasite in ticks between pastures. In addition, both pastures underwent the same tick control program although a long-term evaluation is necessary. It was suggested that other factors might be associated with the increase in *T. orientalis* infection. In contrast to *T. orientalis*, ticks are considered to be unlikely vectors of hemoplasma in