The Journal of Veterinary Medical Science

NOTE

Parasitology

Theileria-free grazing of dairy heifers on grassland in Kyushu, Japan where *T. orientalis* was epidemic before a 7-year vacancy

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J. Vet. Med. Sci.
84(5): 628–633, 2022
doi: 10.1292/jvms.21-0666

Received: 23 December 2021
Accepted: 27 February 2022
Advanced Epub:
31 March 2022

ABSTRACT. This study aimed to produce a *Theileria*-free grazing system for Holstein heifers reared on a dairy farm in the Hita area, Kyushu, Japan. In the grazing area, spreading of *T. orientalis* infection was confirmed in 2009. To eradicate the *T. orientalis* infection, four measures were conducted: 1) 7-year deferred grazing; 2) grazing only *T. orientalis*-uninfected heifers; 3) anemia check by red blood cell parameters at least once per month; and 4) protecting heifers from blood-sucking *T. orientalis*-infected ticks. Grazing was restarted in 2017 in the same area and continued to 2021. During last 2 years of pasturing (2020–2021) all of the 129 heifers were confirmed to be *T. orientalis*-free. In summary, it is possible to establish a *T. orientalis*-free grazing system by conducting appropriate measures.

KEYWORDS: dairy cattle, pasture, *Theileria orientalis, Theileria orientalis*-free grazing

Theileria orientalis is an important blood parasite of cattle, especially grazing animals. The parasite causes mild to severe anemia and malnutrition in cattle [16]. *T. orientalis* is present in many countries, including Australia, Britain, Iran, Japan, the USA, Korea, and Russia [15]. In Japan, economic losses due to *T. orientalis* infection have been mainly investigated in cattle grazed in the Hokkaido region, where the infection easily spreads in grazing areas [6, 8, 11]. In addition to the Hokkaido region, our previous study conducted in Kyushu, which is located in southern Japan and has a temperate climate with hot, humid summers and cold winters, found that *T. orientalis* infection was widely observed and caused severe anemia in cattle [1]. In Kyushu, there is a wide swath of grassland, which is suitable for grazing or pasture, and many beef cattle such as Japanese Black and Brown are pastured to reduce rearing cost. However, few dairy cattle are grazed in this region because Holstein cattle are more susceptible to *T. orientalis* infection than beef cattle [13, 14]. Therefore, a *Theileria*-free grazing area should be established to raise dairy heifers on pasture.

The objective of this study was to describe the process of establishing *Theileria*-free grazing and to investigate the advantage of *Theileria*-free grazing by comparing blood cell (mainly red blood cell) parameters of heifers grazed in *Theileria*-free pasture with those of heifers grazed in *T. orientalis*-contaminated pasture.

This study focused on Holstein heifers grazed on grasslands in a large commercial dairy farm in Kyushu. These heifers normally grazed from 10 months to 16 months of age on the mountainous grasslands (400–800 m altitude) located in the Hita area. Grazing was stopped in 2010 because serious *T. orientalis* infection was confirmed in 2009 [1]. Grazing was restarted in 2017 in the same area, again with dairy heifers from 10–12 months to 14–16 months of age, from March to November. During the 7-year period of deferred grazing, grass harvesting was performed every year. In this study, blood samples were collected from heifers before deferred grazing, defined as the *T. orientalis*-spreading period, and after restarting grazing, defined as the re-pasturing period. Blood sampling was performed once or twice a month during the grazing period. From 2018, the number of ticks which adhered to the heifers was also counted during blood sampling. In the *T. orientalis*-spreading period, a total of 1,748 blood samples were collected from 689 heifers grazed in 2009, and in the re-pasturing period, 1,872 blood samples were collected from 358 heifers grazed between 2017 and 2021.

During the re-pasturing period, the grasslands were separated using electric fences to conduct rotational grazing and heifers

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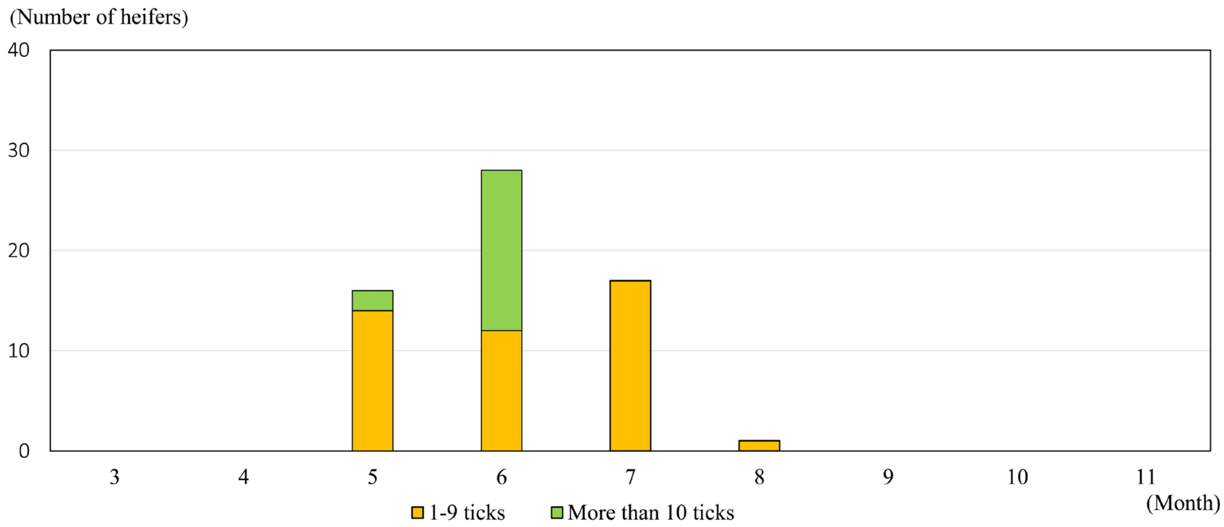

Fig. 1. The number of heifers that adhered by ticks (*Haemaphysalis longicornis*) with 1–9 or more than 10 in each month for 4-years observation.

were reared as herd level. A total of 3–6 kg TMR (Total Mixed Ration) was fed daily divided into two feeds. During pasturing, flumethrin (Bayticol®, Bayer, Osaka, Japan) was administered once every 2–3 weeks and blood testing was conducted once every month. In 2017, PCR analysis for *T. orientalis* was conducted for all heifers at the end of grazing. From 2018, this PCR analysis was also performed for all heifers prior to the beginning of grazing. Only uninfected heifers were grazed from 2018. In the grasslands, some tabanids, stable flies and black flies were found between the rainy season and summer. Sting sites were found in grazing heifers, however, it is unclear whether or not such blood suckling insects had roles for transmitting *T. orientalis* among grazing heifers.

Collected blood samples were used to assess the following red blood cell (RBC) parameters: RBC count; hemoglobin concentration (HGB); hematocrit (HCT); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); and mean corpuscular hemoglobin concentration (MCHC). White blood cell (WBC) and platelet (PLT) counts were also assessed. These variables were determined using a particle counter (PCE-210N, ERMA Inc., Tokyo, Japan).

Heifers grazed during the *T. orientalis*-spreading period were defined as *T. orientalis*-positive if the parasite was detected in 10^4 RBC counted on blood smears by light microscopy. The method is described in our previous report [1]. Regarding heifers grazed during the re-pasturing period, *T. orientalis* infection was assessed using PCR on blood samples to detect the *T. orientalis* p23 gene. The method is described in our previous report [7].

All statistical analyses were performed using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). Model 1 was built to compare RBC parameters between *T. orientalis*-positive heifers and *T. orientalis*-negative heifers during the *T. orientalis*-spreading period and heifers during the re-pasturing period, using the mixed-effects linear model. The unit of observation was the blood sample. The dependent variables were the above-mentioned RBC parameters and the independent variables were the three groups of heifers (*T. orientalis*-positive heifers and *T. orientalis*-negative heifers during the *T. orientalis*-spreading period and heifers during the re-pasturing period). The month of blood draw and heifers were included as random effects. Model 2 was constructed to assess the temporal change in RBC parameters in heifers grazed during the re-pasturing period using the mixed-effects linear model. The unit of observation was the blood sample. The dependent variables were the above-mentioned RBC parameters and the independent variable was the week of blood draw in relation to grazing (before grazing, 4, 8, 12, and 16 weeks after grazing). The month of blood draw and heifers were included as random effects. In this model, 210 heifers that had records before grazing, 4, 8, 12, and 16 weeks after grazing were included. In each model, *P*-values <0.05 were considered significant.

This study included 1,748 blood samples from 689 heifers grazed during the *T. orientalis*-spreading period and 1,872 blood samples from 358 heifers grazed during the re-pasturing period. In the 689 heifers grazed during the *T. orientalis*-spreading period, the prevalence of *T. orientalis* was 42.2% (291/689). In the 358 heifers grazed during the re-pasturing period, only three heifers grazed in 2017 and one heifer grazed in 2019 were infected with *T. orientalis* (The prevalence of *T. orientalis* was 1.1% (4/358) during the re-pasturing period). Figure 1 shows the number of heifers that adhered by ticks (*Haemaphysalis longicornis*) with 1–9 or more than 10 in each month for 4-years observation. Adherent of ticks are checked at blood sampling. The most frequent number of heifers adhered by ticks was found in June, and no heifer was adhered from March to April and from September to November. The proportion of heifers with 1–9 ticks in 2018, 2019, 2020, and 2021 were 0% (0/369), 0.004% (2/452), 2.1% (8/382), and 14.2% (34/239), respectively, and those having more than 10 ticks were 0% (0/369), 0% (0/452), 3.6% (14/382), and 1.7% (4/239), respectively.

Comparison of RBC parameters between *T. orientalis*-positive and *T. orientalis*-negative heifers during the *T. orientalis*-spreading period (Fig. 2a) and heifers during the re-pasturing period are shown in Fig. 2b. Heifers during the re-pasturing period

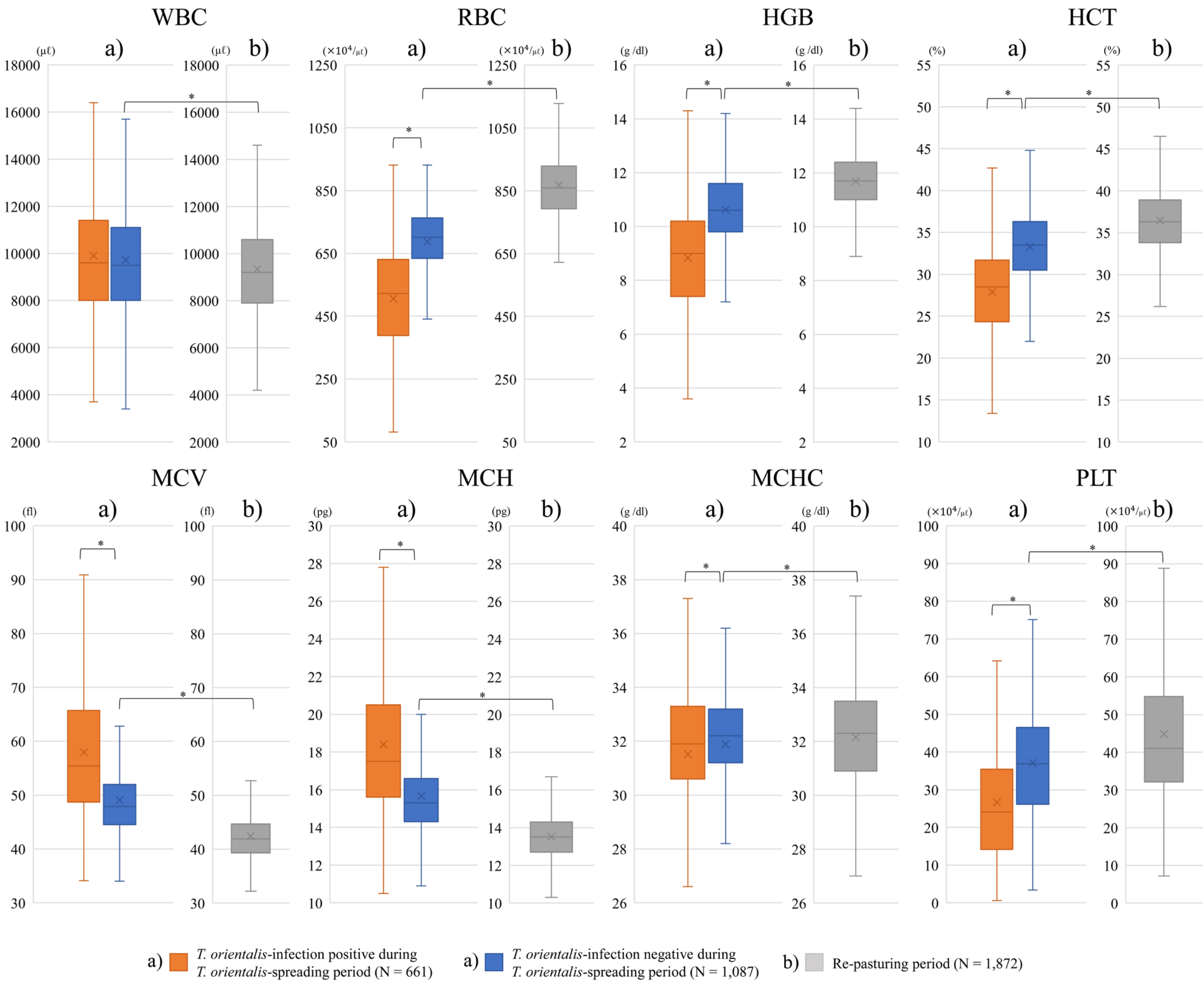

Fig. 2. Comparison of red blood cell (RBC) parameters between *Theileria orientalis*-positive and *T. orientalis*-negative heifers during the *T. orientalis*-spreading period (Fig. 2a). Infection was diagnosed by finding of piroplasm in a 10^4 RBC on the thin blood smear. Infection of heifers during the re-pasturing period (Fig. 2b) was diagnosed by PCR. RBC parameters included RBC count, hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). White blood cell (WBC) and platelet (PLT) counts were also assessed. * Indicates statistical significance of differences between groups (P<0.05).

had significantly different RBC parameters (P<0.05) than the *T. orientalis*-negative heifers during the *T. orientalis*-spreading period, i.e. higher RBC count, HGB, HCT, MCHC, and PLT and lower WBC, MCV, and MCH.

The serial changes in RBC parameters in heifers grazed during the re-pasturing period are shown in Fig. 3. All measured RBC parameters changed significantly during the grazing period (P<0.05) except for MCV (P=0.19). Heifers had the highest WBC, HGB, HCT, and PLT before grazing and those parameters gradually decreased with time during the grazing period (P<0.05). Although RBC levels decreased compared with those before grazing (P<0.05), the RBC remained at a higher level during the re-pasturing period than during the *T. orientalis*-spreading period.

This study shows that it is possible to establish a *T. orientalis*-free grazing system by conducting four measures: 7-year deferred grazing; grazing only *T. orientalis*-uninfected heifers; anemia check by red blood cell parameters at least once per month; and protecting heifers from blood-sucking *T. orientalis*-infected ticks, for example by regular administration of flumethrin. Few studies have focused on *T. orientalis*-free grazing around the world. Although it is difficult to evaluate which one of the four measures was most important, the combination of these measures was effective in establishing *T. orientalis*-free grazing. Deferred grazing is useful to stop the life cycle of *T. orientalis*-infected ticks, which are the main causative agent of horizontal transmission of *T. orientalis* in Japan [17]. However, the minimum required period of no grazing is still uncertain. Moreover, administration of flumethrin (pour on), a fast-acting acaricide, rapidly kills ticks and therefore, is useful to prevent horizontal transmission of

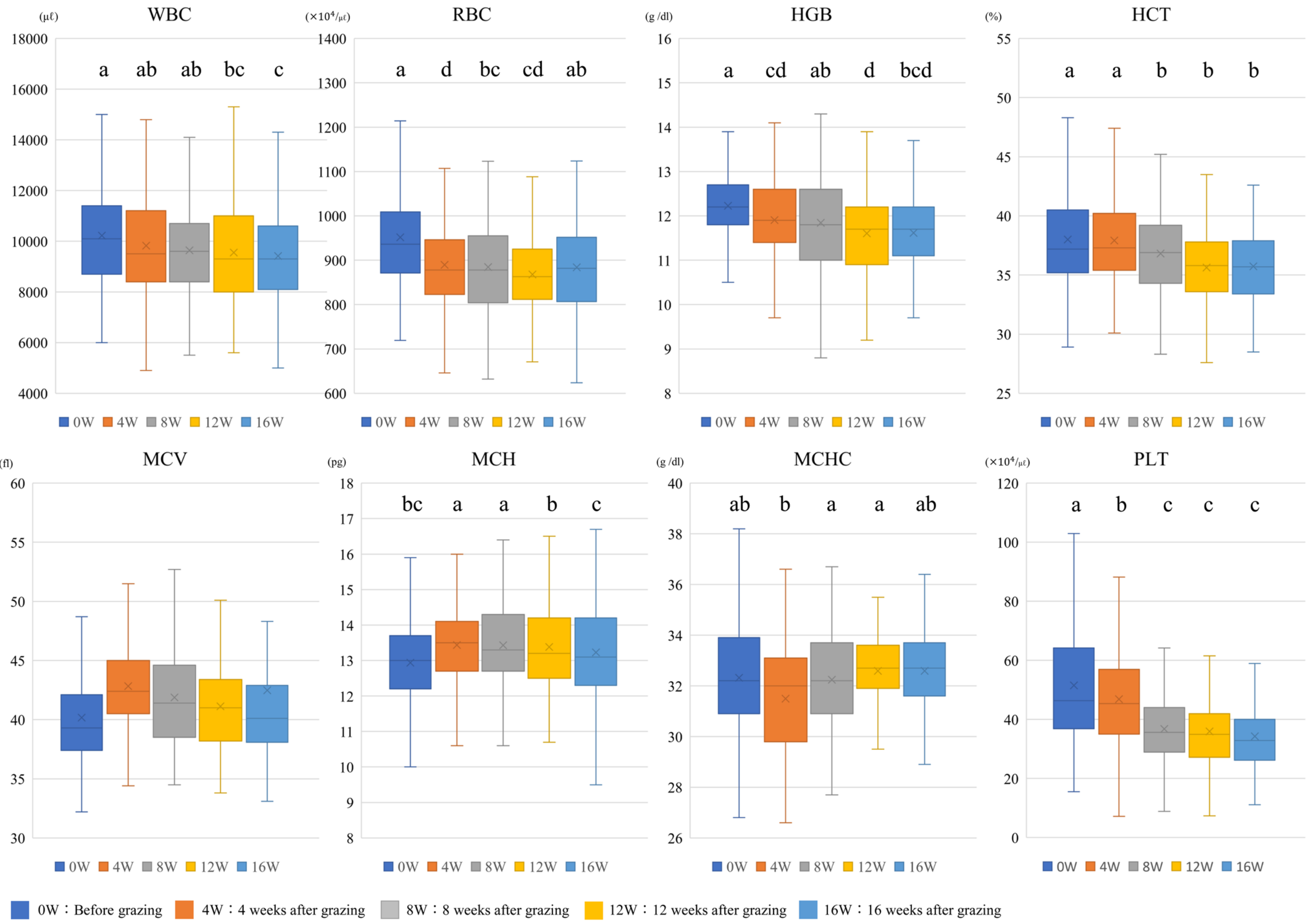

Fig. 3. Serial changes in red blood cell (RBC) parameters in 210 heifers grazed during the re-pasturing period. The RBC parameters were RBC count, hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). White blood cell (WBC) and platelet (PLT) counts were also assessed. Values without the same letters (a, b, c, d) within a row differed significantly (P<0.05).

T. orientalis. Heath *et al.* (1988) reported that one dose of flumethrin pour-on formulation was effective for 23 days in the case of *H. longicornis* [5], and flumethrin pour-on every 2–3 weeks, conducted in the present study, might have been effective to reduce tick numbers in the field and on cattle [12]. Additionally, the interval of administration of flumethrin can be extended more than 3 weeks during March-April or September-November when no adhering ticks were found. However, complete eradication of ticks in the field has been said to be difficult [4, 9], and only administration of flumethrin may be not enough in order to completely control *T. orientalis*. In addition, regular blood testing of grazing heifers is useful to identify animals with anemia, which can facilitate earlier diagnosis of *T. orientalis* infection. Furthermore, the most important measure is likely to be grazing only *T. orientalis*-uninfected heifers. This helps prevent the spread of *T. orientalis* infection because vertical transmission occurs between approximately 10% of *T. orientalis*-infected dams and their calves [7, 10]. In 2017, PCR analysis for *T. orientalis* infection before grazing was not conducted and three heifers were found to be *T. orientalis*-infected after grazing. In contrast, after 2018, we performed PCR analysis before grazing and only grazed *T. orientalis*-uninfected heifers. In 2019, only one heifer was infected with *T. orientalis*, and those infections was not detected last 2 years (2020–2021). These findings indicate that the measures we conducted were effective in establishing a *T. orientalis*-free grazing system for dairy heifers in Kyushu. Further research is needed to construct cost-effective and sustainable management system for establishing *T. orientalis*-free grazing. In addition, it is noteworthy that if *T. orientalis*-infected heifers are detected, they should be moved immediately to the inside barn to receive appropriate care and management that minimizes the negative impact of infection [2]. In the present study, when *T. orientalis*-infected heifers were detected during pasturing, they were moved immediately to the inside barn, observed regularly and conducted only standard management because they did not show any clinical symptom. The isolation of infected animals is essential for maintaining *T. orientalis*-free pasture.

Our results showed that the *T. orientalis*-positive heifer group had decreased RBC count, HGB, HCT, MCHC, and PLT and elevated MCV and MCH compared with the *T. orientalis*-negative group and the re-pasturing group. These data are consistent with previous studies [1, 3, 13]. However, even the *T. orientalis*-negative group had decreased RBC count, HGB, HCT, MCHC, and

PLT and elevated WBC, MCV, and MCH when compared with the re-pasturing group, indicating that the *T. orientalis*-spreading pasture had a negative impact on RBC parameters even in *T. orientalis*-uninfected heifers. These findings suggest that some of the *T. orientalis*-uninfected heifers on the *T. orientalis*-spreading grazing system may have been infected with *T. orientalis* that could not be detected by counting cells on blood smears.

This is the first study to investigate the serial changes in RBC parameters in heifers grazed on *T. orientalis*-free pasture. Our results showed that heifers had the highest WBC, HGB, HCT, and PLT before grazing and those parameters gradually decreased with time during the grazing period. In contrast, the RBC remained at a higher level during the grazing period. Although it is difficult to explain exactly how such high RBC counts were maintained in the pasturing heifers, the daily movements of heifers on pasture in a mountainous area may induce hematopoiesis.

Our results indicate that regular blood sampling and measurement of RBC parameters is an effective way to monitor animal health. In a grazing system, the health condition of grazing dairy heifers can be influenced by changes in grass nutrition or weather conditions, as well as by *T. orientalis* infection. The RBC parameter values during the *T. orientalis*-free grazing can be used as standards for blood testing and enable managers and/or veterinarians to quickly identify problem heifers, perform *T. orientalis* PCR testing, and conduct isolation management or other measures such as modulation of additional feeding, and changing the grazing program.

This study has several limitations that should be noted when interpreting the results. First, the present study did not have access to some information about nutritional conditions in each period (*T. orientalis*-spreading period and re-pasturing period). Nutritional status may influence *T. orientalis* infections. Second, this study was conducted on one farm and the results cannot be generalized to all grasslands in Kyushu. Third, the data in the *T. orientalis*-spreading period is relatively old because we conducted long-term deferred grazing. Last, this study did not measure the blood concentration of erythropoietin. Nevertheless, this is the first study to publish methods of establishing *T. orientalis*-free grazing in dairy heifers. Further studies analyzing more data are warranted to improve our understanding of this problem.

In conclusion, it was possible to establish a *T. orientalis*-free grazing system in Kyushu, Japan by conducting a combination of four measures. Heifers grazed on *T. orientalis*-free pasture had significantly different RBC parameters compared with those grazed during the *T. orientalis*-spreading period. Our results can contribute to the creation of guidelines for *T. orientalis*-free grazing management of dairy heifers.

CONFLICT OF INTEREST. The authors have no conflicts of interest to declare.

ACKNOWLEDGMENT. The authors thank the producers and farm staff for their cooperation with this project.

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