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Parasitology

# A large-scale survey of *Theileria orientalis* infection in grazing dairy heifers in Kyushu, Japan

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**ABSTRACT.** The objective of the present study was to investigate the prevalence of *Theileria orientalis* infection and the influence of this disease on dairy grazing heifers in Kyushu by monitoring red blood cell (RBC) indexes, and to evaluate the efficacy of diminazene diaceturate treatment of *T. orientalis*-infected animals. A monthly epidemiological survey was conducted for Holstein heifers, which were reared from 10 to 16 months of age on a large commercial dairy farm and grazed on eight independent grasslands from April to November, 2009. During the survey, a total of 2,803 blood samples were collected from the 891 grazing heifers, in which the prevalence of *T. orientalis* infection was 52.4%. Compared with the heifers before infection, heifers with high parasitemia (more than 100 parasites per $10^4$ RBC) had significantly decreased RBC indexes, such as RBC count, hemoglobin concentration, and hematocrit ($P$<0.05), whereas heifers with low parasitemia (less than 100 parasites per $10^4$ RBC) had similar RBC indexes as those before infection. Treatment with diminazene diaceturate had lower efficacy in heifers with high parasitemia than those with low parasitemia (40.7% and 73.2% became negative, respectively, $P$<0.05). In summary, *T. orientalis* infection is a potentially serious problem in Kyushu, and it is important to routinely implement control programs for heifers that are grazed on grasslands in this region.

**KEY WORDS:** diary cattle, epidemiology, Kyushu, pasture, *Theileria orientalis*

*Theileria orientalis* is a tick-borne protozoan parasite that causes mild to severe anemia in infected cattle [18]. *T. orientalis* is also known to induce immune-mediated hemolytic anemia [11, 15], which enhances the disease severity. Although this parasite is distributed throughout Japan, only a few epidemiological studies have been conducted so far. A study carried out in Hokkaido, Japan, revealed that *T. orientalis* infections are a potentially serious problem in grazing cattle [12]. The livestock industry in Japan still suffers enormous economic losses from this disease, especially in grazing cattle [5, 12, 13], because no effective medicines or vaccines for controlling this disease are commercially available in Japan at present.

Kyushu island, in southern Japan, has a temperate climate with hot, humid summers and cold winters. The inland region is covered by a wide swathe of grassland, which is suitable for grazing or pasture. However, only beef cattle, such as Japanese Black and Brown cattle, are pastured in this region to reduce rearing costs, and few dairy cattle are grazed there because Holstein cattle are more susceptible to *T. orientalis* infection than beef cattle [15, 17]. To promote the effective use of grasslands in this region, it is important to quantify the prevalence of *T. orientalis* infections in dairy cattle. Yokoyama *et al*. [21] reported genotypic diversity of *T. orientalis* detected from cattle grazing in Kumamoto and Okinawa prefectures, but no other large-scale surveys have been conducted in this region.

The objective of the present study was to investigate the prevalence of *T. orientalis* infections and the influence of this disease on grazing dairy heifers in Kyushu by conducting a monthly epidemiological survey and monitoring red blood cell (RBC) indexes. The efficacy of diminazene diaceturate treatment in *T. orientalis*-infected heifers was also evaluated.

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## MATERIALS AND METHODS

### *Experimental design*

The present study focused on Holstein heifers grazed on eight independent grasslands (Fig. 1) in Kyushu (two grasslands located in Hita area, Oita prefecture and six grasslands located in Aso area, Kumamoto prefecture; Table 1) from 10 months to 16 months of age. These heifers were reared on a large commercial dairy farm and were produced on the farm or introduced from Australia. A monthly epidemiological survey was conducted from April to November, 2009. During this survey, a total of 2,803 blood samples were collected from the 891 grazing heifers to investigate the prevalence of *T. orientalis* infection and changes in *T. orientalis* infection throughout the grazing period. The mean (± SD) number of blood samples per heifer was 3.2 ± 2.0, ranging from 1 to 12.

### *Determination of parasitemia*

Approximately 4 ml of blood was collected from the jugular or tail vein of cattle. Blood sampling was made by using a 4 ml vacuum container with ethylenediaminetetraacetic acid (EDTA-2K, Japan Becton, Dickinson and Co., Tokyo, Japan). Thin blood smears were made and stained with Giemsa solution using Diff-Quick stain (Sysmex, Kobe, Japan). Parasitemia was determined by counting the *T. orientalis*-infected RBC in the blood smears under the microscope. Animals were defined as *T. orientalis*-infection-negative if there were no parasites in $10^4$ RBC. *T. orientalis*-infection-positive was classified based on the upper and lower 33 percentile of proportion of the *T. orientalis*-infected RBC: low parasitemia (<1%, less than 100 parasites per $10^4$ RBC), medium parasitemia (1%), and high parasitemia (>1%). The detection limit of infected RBC was $1/10^4 < \times < 1/10^5$.

We calculated prevalence of *T. orientalis* on heifer-basis and blood-sample-basis in accordance with the following formula:

$$\text{Heifer-basis prevalence } (\%) = \frac{\text{The number of heifers with } T.\,orientalis\text{-infection-positive}}{\text{The number of blood heifers}},$$

$$\text{Blood-sample-basis prevalence } (\%) = \frac{\text{The number of samples with } T.\,orientalis\text{-infection-positive}}{\text{The number of blood samples}}.$$

At the calculation of heifer-basis prevalence, a heifer was counted if she had at least one *T. orientalis*-infection-positive record during the studied period. On the other hand, at the calculation of blood-sample-basis prevalence, heifers were counted multiple times if they had multiple *T. orientalis*-infection-positive records.

### *RBC index*

The collected samples mentioned above were used to assess the RBC index. In the present study, the RBC indexes used were as follows: 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).


**Fig. 1.** Geographical map of the Kyushu, Japan. Holstein heifers were grazed on eight independent grasslands, two grasslands located in Hita area, Oita prefecture and six grasslands located in Aso area, Kumamoto prefecture.

**Table 1.** Prevalence of *Theileria orientalis* infection in eight independent grasslands, two grasslands located in Hita area, Oita prefecture and six grasslands located in Aso area, Kumamoto prefecture

| Grasslands | N | Prevalence of *T. orientalis* infection (%) |
|---|---|---|
| Hita area | | |
| Amagase | 689 | 42.2 |
| Kamitsue | 39 | 94.9 |
| Aso area | | |
| Moutani | 59 | 83.1 |
| Shinmiya | 21 | 57.1 |
| Yamada | 87 | 58.6 |
| Makino | 40 | 45.0 |
| Katamata | 74 | 28.4 |
| Sasakura | 84 | 52.4 |

### *Treatment of T. orientalis-infected heifers*

During the study period, *T. orientalis*-infected heifers were treated with an intramuscular injection of diminazene diaceturate (8 mg/kg; Ganazeg®, Novartis Animal Health, Tokyo, Japan). Basically, diminazene diaceturate was injected to heifers showed severe anemia. Additionally, prophylactic administration was performed to some heifers based on their RBC indexes. Flumethrin (Bayticol®, Bayer, Osaka, Japan) and ivermectin (Ivomec® Pour-On, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) were also administered to all heifers at the beginning of grazing, and subsequently administered once per month on each grazing area to exterminate ixodid ticks.

### *Statistical analysis*

Model 1 was constructed to compare the prevalence of *T. orientalis* infection and proportion of *T. orientalis* parasitemia between heifers produced on the farm and those introduced from Australia. The observation unit was one heifer. The dependent variables were *T. orientalis* infection status (whether a heifer was with or without *T. orientalis* infection [1 or 0] during the study period), which were compared using a logistic regression model [9], and the proportion of *T. orientalis* parasitemia, compared using a generalized linear model [9]. The independent variable was whether the heifers were produced on the farm or introduced from Australia.

Model 2 was built to compare RBC indexes between the heifers before and after *T. orientalis* infection using a mixed-effects linear model [9]. The observation unit was the blood sample, and 343 heifers that had records for both before and after *T. orientalis* infection were used. The dependent variables were the above-mentioned RBC indexes, and the independent variable was before and after *T. orientalis* infection. Month and heifers were included as random effects.

Model 3 was constructed to investigate the efficacy of diminazene diaceturate treatment in *T. orientalis*-infected heifers using a mixed-effects logistic regression model [9]. The observation unit was blood sample, and 260 heifers infected with *T. orientalis* and subsequently administrated diminazene diaceturate were used. The dependent variable was *T. orientalis* infection status after diminazene diaceturate treatment (whether a heifer was with or without *T. orientalis* infection [1 or 0] after treatment), and the independent variable was *T. orientalis* infection status before treatment (low, middle, and high parasitemia). Blooding month and heifers were included as random effects.

All statistical analyses were performed using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). In each model, $P$-values <0.05 were considered significant.

## RESULTS

The present study involved 2,803 blood samples from 891 heifers. In the 891 heifers, the prevalence of *T. orientalis* infection was 52.4% (466/891). The prevalences of the parasite in the eight grasslands are shown in Table 1. There was a large variation in the prevalences among the grasslands, ranging from 28.4% to 94.9%. Heifer-based and blood-sample-based relative frequencies of the proportion of *T. orientalis* parasitemia are shown in Fig. 2. The most frequent parasitemia proportion was found to be 1%, which was apparent in 27.9% of heifers and 33.5% of blood samples. In addition, the second most frequent proportion was found to be 0.1% parasitemia, which was seen in 22.5% of heifers and 28.0% of blood samples.

The prevalences of *T. orientalis* infection in heifers produced on the farm and those introduced from Australia were 58.8% (80/136) and 51.1% (386/755), respectively, and there was no significant difference between the groups. In addition, there was no significant difference in the *T. orientalis* parasitemia proportion between heifers produced on the farm and those introduced from Australia.

Comparisons of RBC indexes between the heifers before and after *T. orientalis* infection are shown in Table 2. Compared with the values for the heifers before infection, heifers with high parasitemia had significantly different RBC indexes ($P<0.05$), i.e., decreased RBC count, HGB, HCT, MCHC, and PLT and elevated WBC, MCV, and MCH. Out of 124 heifers with high parasitemia, 24 became severe having RBC count less than $2 \times 10^6/\mu l$ and HCT less than 15%. Compared with the values for heifers before infection, heifers with medium parasitemia had some significantly different RBC indexes ($P<0.05$): decreased RBC count, HGB, HCT, and PLT and increased MCH. However, heifers with low parasitemia had similar RBC indexes to those before infection.

The efficacy of diminazene diaceturate treatment for *T. orientalis*-infected heifers is shown in Table 3. Of the 260 heifers that were infected with *T. orientalis* and subsequently administrated diminazene diaceturate, 140 (53.8%) were negative for *T. orientalis* infection after treatment. In addition, the efficacy of diminazene diaceturate treatment differed between heifers with different parasitemia statuses before treatment ($P<0.05$). Diminazene diaceturate treatment was less efficacious for heifers with high levels of parasitemia than those with low levels (40.7% and 73.2% became negative after treatment, respectively, $P<0.05$). Of 113 heifers with high parasitemia before administration, 29.2% (33/113) still had high parasitemia after treatment (Table 3).

## DISCUSSION

This is the first large-scale epidemiological investigation of *T. orientalis* infections conducted in Kyushu. The present study revealed that approximately half (52.4%) of grazing dairy heifers were infected with *T. orientalis* during their grazing period, which is similar to the prevalence in Hokkaido, at 10.0% to 64.8% [12], and New Zealand, at 58% [7]. In the study region, our previous research showed that Ikeda and Chitose types were mainly presented by sequencing analysis (unpublished data), which is

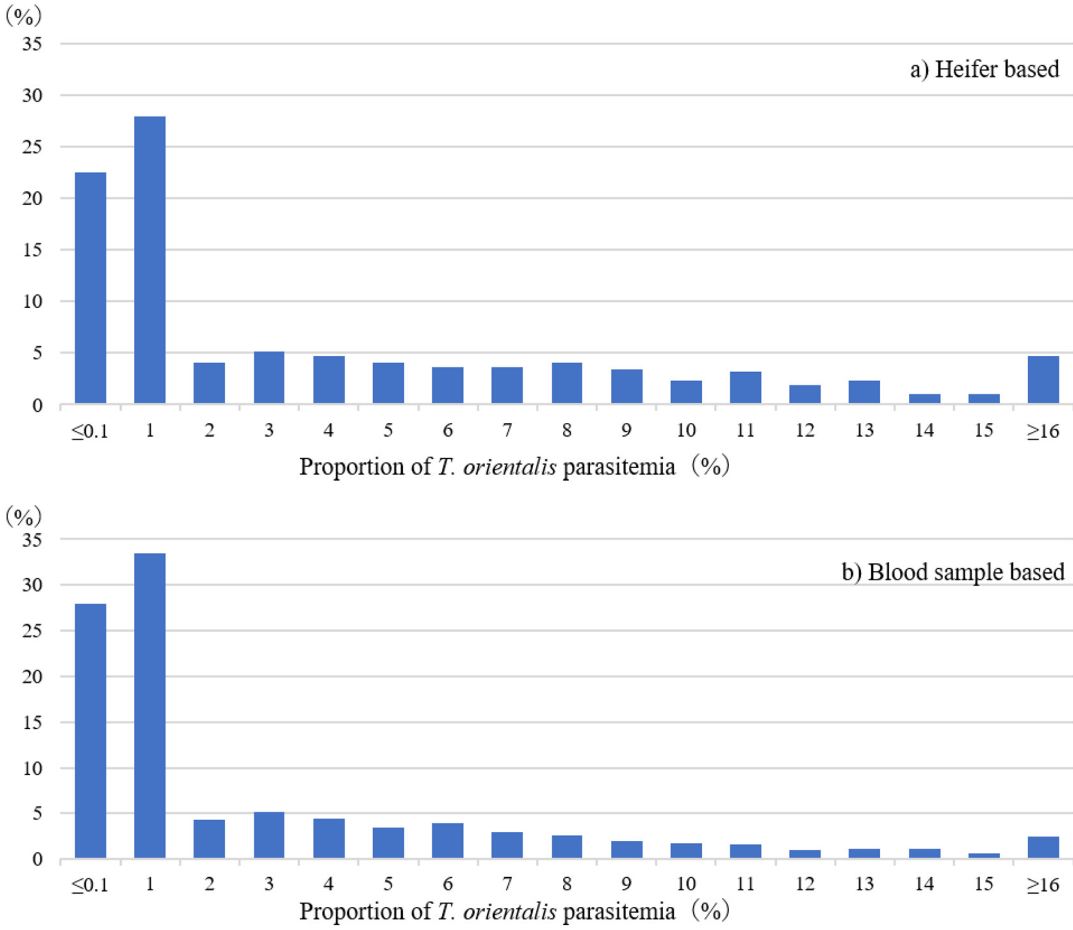


**Fig. 2.** Relative frequency of the proportion of *Theileria orientalis* parasitemia in 466 heifers (a) and 1,025 blood samples (b). In heifer basis, a heifer was included if she had at least one record of *T. orientalis*-infection-positive during the studied period. If she had multiple records of *T. orientalis*-infection-positive, maximum value of the proportion of *T. orientalis* parasitemia was used in this figure. In blood-sample basis, a blood sample was included if it was *T. orientalis*-infection-positive. Each value of the proportion of *T. orientalis* parasitemia was used in this figure.

**Table 2.** Comparison of red blood cell (RBC) indexes before and after *Theileria orientalis* infection for 343 heifers

| RBC indexes[2] | Before infection | Parasitemia[1] after infection | | |
|---|---|---|---|---|
| | | Low | Medium | High |
| WBC ($10^2$/µl) | 97.2 ± 1.6$^{b}$ | 92.5 ± 2.4$^{ab}$ | 97.9 ± 2.3$^{ab}$ | 102.8 ± 2.7$^{a}$ |
| RBC count ($10^4$/µl) | 636.7 ± 7.3$^{a}$ | 634.6 ± 9.8$^{a}$ | 586.2 ± 10.9$^{b}$ | 449.8 ± 12.0$^{c}$ |
| HGB (g/dl) | 10.4 ± 0.1$^{a}$ | 10.2 ± 0.2$^{a}$ | 9.6 ± 0.2$^{b}$ | 8.0 ± 0.2$^{c}$ |
| HCT (%) | 32.2 ± 0.2$^{a}$ | 32.8 ± 0.4$^{a}$ | 31.1 ± 0.4$^{b}$ | 26.4 ± 0.5$^{c}$ |
| MCV (fl) | 52.0 ± 0.5$^{b}$ | 52.3 ± 0.7$^{b}$ | 54.1 ± 0.7$^{b}$ | 61.4 ± 1.1$^{a}$ |
| MCH (pg) | 16.6 ± 0.1$^{c}$ | 16.2 ± 0.2$^{bc}$ | 16.8 ± 0.3$^{b}$ | 18.4 ± 0.3$^{a}$ |
| MCHC (g/dl) | 32.2 ± 0.1$^{a}$ | 31.1 ± 0.4$^{a}$ | 30.9 ± 0.5$^{ab}$ | 30.3 ± 0.3$^{b}$ |
| PLT ($\times 10^4$/µl) | 32.5 ± 0.9$^{a}$ | 28.9 ± 1.4$^{ab}$ | 28.0 ± 1.2$^{b}$ | 22.4 ± 1.0$^{c}$ |

Mean ± SEM. [1]Parasitemia was classified based on the proportion of *T. orientalis* parasitemia: low (<1%; N=98), medium (1%; N=121), and high (>1%; N=124). [2]RBC indexes used 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) were also assessed. Values without the same letters (a, b, c) within a row differed significantly ($P<0.05$).

agreed with a previous report conducted in Kumamoto and Okinawa prefectures [21]. Additionally, we found a large variation in the prevalence of the parasite among grasslands in the studied regions, and some grasslands had a *T. orientalis* infection prevalence of 80% to 90%. These findings indicate the potential for high *T. orientalis* infection rates in Kyushu, and routine programs to

**Table 3.** Efficacy of diminazene diaceturate treatment of 260 *Theileria orientalis*-infected heifers

| | N | Parasitemia[1] after treatment | | | |
|---|---|---|---|---|---|
| | | Negative (%) | Low (%) | Medium (%) | High (%) |
| All heifers | 260 | 53.8 | 10.8 | 15.4 | 20.0 |
| Parasitemia[1] before administration | | | | | |
| Low | 56 | 73.2 | 10.7 | 10.7 | 5.4 |
| Medium | 91 | 58.2 | 9.9 | 14.3 | 17.6 |
| High | 113 | 40.7 | 11.5 | 18.6 | 29.2 |

[1]Parasitemia was classified based on the proportion of *T. orientalis* parasitemia: negative (no detection), low (<1%; N=98), medium (1%; N=121) and high (>1%; N=124).

control *T. orientalis* are important to protect heifers grazed in this area.

The present study compared the heifers' RBC indexes before and after *T. orientalis* infection and found the *T. orientalis* parasitemia status was associated with different RBC indexes. In particular, heifers with medium and high parasitemias had different RBC indexes compared with before infection, but heifers with low parasitemia had similar RBC indexes as before infection, indicating that it is important to evaluate not just whether a heifer is infected with *T. orientalis* but also the proportion of *T. orientalis* parasitemia. Our result suggests that a "1% *T. orientalis* parasitemia" could be used as an indicator of infection strength.

Our results showed that heifers with high levels of *T. orientalis* parasitemia showed decreased RBC counts, HGB, HCT, MCHC, and PLT and elevated WBC, MCV, and MCH compared with before infection, which agrees with previous studies [2, 15]. The *T. orientalis*-infected cattle had reduced RBC counts, which led to anemia and, subsequently, decreased HGB and HCT. Anemia is mostly caused by hemolysis or phagocytosis of the infected erythrocytes by macrophages in the spleen or liver [19, 20]. Consequently, the host may adopt several counter-strategies, such as increasing MCV and MCH levels and decreasing MCHC, as well as initiating high cardiac output and low oxygen metabolism to avoid a reduction in oxygen supply to the tissues [3]. Additionally, the activation of phagocytic macrophages in the spleen results in oxidative injuries that decrease the PLT [14, 16]. The reduction of PLT levels observed in heifers with high parasitemia indicates that, in addition to the RBC indexes, PLT can be used as an indicator of anemia in *T. orientalis*-infected cattle.

We also evaluated the efficacy of diminazene diaceturate treatment of *T. orientalis*-infected heifers. Diminazene diaceturate is anti-piroplasm drug commonly used in the treatment of bovine babesiosis caused by *Babesia bovis*. At present, diminazene diaceturate is only available in Japan for the treatment with *T. orientalis* infection in cattle, however, drug efficacy has not tested well. Although, 8-aminoquinoline drugs have been usually used for *Theleria* treatment and showed effectiveness so far, it has been unavailable at that time. Our results showed that half of the heifers infected with *T. orientalis* tested negative for *T. orientalis* infection after treatment, but the rest remained positive. In particular, the success of the treatment depended on the parasitemia status before treatment: treatment was less effective in heifers with high levels of parasitemia than in those with low levels. These findings elucidate that cattle with high *T. orientalis* infection levels are poorly responsive to diminazene diaceturate treatment. In this study, we chose rather mild dosage, a dose of 8 mg/kg, to avoid side-effect because of difficulty of adequate care of cattle during grazing on several large pastures. Those may be resulted insufficient efficacy of diminazene diaceturate for the treatment of *T. orientalis*-infected heifers. Furthermore, a study in New Zealand found no evidence supporting the use of toltrazuril to control *T. orientalis* Ikeda-type infections and prevent disease [8]. Further research should be implemented to develop a specific treatment for *T. orientalis* infection and to investigate detailed drug efficacy of diminazene diaceturate on *T. orientalis* infection in cattle.

The present study found no difference in the prevalence of *T. orientalis* infection or proportion of *T. orientalis* parasitemia between heifers produced on the farm and those introduced from Australia. However, this result should be interpreted cautiously, as we had no information on the *T. orientalis* infection status before grazing. Recently, there have been many outbreaks of *T. orientalis* reported in Australia and New Zealand [1, 4, 6, 10]. Therefore, additional investigations will be needed to detect this relationship.

The present study had several limitations that should be noted when interpreting the results. First, the present study did not have access to some information about nutritional conditions and grazing management issues, which might influence *T. orientalis* infections. Second, this was a study of one farm, and the results cannot be generalized to all grasslands in Kyushu. Third, the present study evaluated parasitemia by counting the *T. orientalis*-infected RBC in the blood smears under the microscope, and some samples with no parasites in $10^4$ RBC could be had *T. orientalis*-infected RBC. Lastly, the data in this study is relatively old, and detail data in recent years in the study region was not available. To our knowledge, no grazing of dairy cattle was recently performed in this area. Nevertheless, the study was worthwhile, as it was the first evaluation of *T. orientalis* infections in grazing dairy heifers in Kyushu, Japan. Further studies analyzing more data are warranted to improve our understanding of this problem.

In conclusion, approximately half of the grazing dairy heifers studied were infected with *T. orientalis* during their grazing period, suggesting *T. orientalis* infection is a serious problem in Kyushu. The RBC indexes after *T. orientalis* infection differed between heifers with different parasitemia statuses: heifers with high levels of parasitemia had significantly different RBC indexes compared with before infection, whereas heifers with low parasitemia proportions had similar RBC indexes than before infection.

Furthermore, *T. orientalis*-infected cattle were less sensitive to diminazene diaceturate treatment. Therefore, it is important to maintain routine *T. orientalis* control programs, as well as to conduct nutritional management and select suitable breeds, to protect heifers grazed in this region.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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