J. Protozool. Res. 27. 23-33 (2017)

Dynamics of erythrocyte indices in relation to anemia development in *Theileria orientalis*-infected cattle

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ABSTRACT
Theileria orientalis often causes clinical anemia in cattle. In the present study, the red blood cell (RBC) indices of cattle, as measured in *T. orientalis*-endemic regions of Hokkaido, Japan, were analyzed. In comparison with the non-infected animals, the mean values for RBC counts, hematocrit, and hemoglobin concentration (HGB) were lower in *T. orientalis*-infected Holstein and Hereford cattle but not in Japanese Shorthorn and Angus, suggesting that the hemolysis induced by *T. orientalis* is not pronounced in the later. Anemic animals (HGB < 8 g/dl) were detected only in Holstein and Hereford, and the anemia rate was higher for Holstein than that for Hereford. The mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values in the *T. orientalis*-infected but non-anemic animals were significantly higher than those for the non-infected Hereford, but not for the Holstein, suggesting that the host response to hemolysis influenced anemia development. To clarify this phenomenon, three Holstein calves experimentally infected with *T. orientalis* were monitored for the parasitemia and RBC indices. Among the infected animals, a calf with low parasitemia had elevated MCV and MCH values, but lacked anemia. In contrast, despite elevated MCV and MCH values, HGB in the calf with high parasitemia dropped to a low value. These findings indicate that the cattle breed, parasitemia level, and host response may influence anemia development in *T. orientalis*-infected cattle.

Keywords: Anemia; Cattle; Red blood cell indices; Host response; *Theileria orientalis*

INTRODUCTION

Theileria orientalis is a tick-transmitted protozoan parasite of cattle and several other ruminants, including buffaloes and yaks (Sivakumar *et al*., 2014). *T. orientalis* has a worldwide distribution, and the parasite infection caused by it often results in clinical disease outbreaks, leading to severe economic losses in the cattle industry (Perera *et al*., 2014; Sivakumar *et al*., 2014). The life cycle of *T. orientalis* involves tick vectors and vertebrate hosts (Shaw and Tilney, 1992; Bishop *et al*., 2004). The vectors that can transmit the parasite are usually 3-host ticks because, similarly to other *Theileria* species, *T. orientalis* lacks transovarian transmission in ticks (Sivakumar *et al*., 2014). *Haemaphysalis longicornis* is a well-known tick vector for *T. orientalis*, but the involvement of other tick species (e.g., *Ixodes persulcatus*, *H. megaspinosa*, *H. douglasi* and *Ixodes ovatus*) in the parasite lifecycle has also been suggested (Yokoyama *et al*., 2012).

The clinical signs observed in *T. orientalis*-infected cattle are thought to be associated with hemolysis in the host animals (Stockham *et al*., 2000). The hemoglobinuria commonly seen with extensive intravascular hemolysis is not usually observed in *T. orientalis*–infected cattle. Therefore, the *T. orientalis*-induced hemolysis is considered to be extravascular (Sugimoto and Fujisaki, 2002). Severe anemia from the hemolysis caused by *T. orientalis* infection has been reported in several countries with endemic theileriosis, including Japan, Australia, and New Zealand (Kobayashi *et al*., 1991; Kamau *et al*., 2011; McFadden *et al*., 2011; Eamens *et al*., 2013). In Japan, the rates of parasite infection often increase in cattle populations drastically after the grazing season begins, and peaks of infection rates are seen after about 45 days of grazing (Ota *et al*., 2009). Several cattle, which were apparently healthy before the start of the grazing period, experience very severe clinical anemia during the grazing season (Fukasawa *et al*., 2003; Ota *et al*., 2009).

The hemolysis often leads to reduced oxygen supply, a status known as hypoxia, at tissue level. Consequently, the host may adopt several counter strategies, such as increasing the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin (MCH) level, as well as initiating high cardiac output and low oxygen metabolism, in order to avoid a reduced oxygen supply to the tissues (Gross *et al*., 1996). Therefore, in addition to low RBC counts, HCT and HGB, hemolytic anemia is often characterized by elevated MCV and MCH. Infections with virulent *Babesia* and *Theileria* parasites, such as *Babesia bovis*, *B. bigemina*, and *T. annulata,* which induce significant hemolysis in bovine hosts, have short prepatent periods, leaving little time for the body to adopt any compensatory mechanisms (Zhang, 1990; Bock *et al*., 2004). However, as disease development is relatively slow in *T. orientalis*-infected cattle (Ota *et al*., 2009; Stockham *et al*., 2000), we hypothesized that anemia development in such cattle might be influenced by the host responses to hemolysis. In the present study, to gain better understanding of the influence of host responses to parasite-induced anemia development, we analyzed the red blood cell (RBC) indices of cattle that were measured in a previous study on *T. orientalis* infection in cattle reared in the areas of Hokkaido, Japan, where this parasite is endemic (Ota *et al*., 2009). In addition, experimental infections in calves were conducted to confirm the results we obtained from the naturally infected cattle.

MATERIALS AND METHODS

RBC indices of cattle naturally infected with *T. orientalis*

RBC indices, which were measured in a previous study (Ota *et al*., 2009) for Holstein, Hereford, Japanese Shorthorn, and Angus cattle bred in Hokkaido, Japan, were analyzed in the present study. Briefly, 105 Holstein from Shin-Hidaka and Taiki districts, 48 Hereford from Shin-Hidaka and Shintoku districts, 22 Japanese Shorthorn from the Shin-Hidaka district and 17 Angus from Shintoku and Otofuke districts were blood-sampled approximately 1.5 months from the beginning of the grazing season in July, 2007. The RBC indices for the blood samples were measured using an automated hematology analyzer (Celltac, Nihon Koden, Tokyo, Japan). In the present analysis, an animal was considered to be anemic if the hemoglobin concentration (HGB) was less than 8 g/dl (Jain, 1993). When the blood DNA samples were screened by a *T. orientalis*-specific PCR assay based on the major piroplasm surface protein gene (MPSP-PCR assay), 62, 25, 20, and four animals were diagnosed as positive for the infection among the Holstein, Hereford, Japanese Shorthorn, and Angus cattle populations, respectively (Ota *et al*., 2009). Based on the parasite infection and anemia status, the RBC indices, including RBC counts, HGB, hematocrit (HCT), MCV, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), were analyzed for the following three different animal categories: 1) non-infected, 2) infected, but non-anemic, and 3) infected and anemic.

Experimental animals

Three Holstein calves reared in the Field Center of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine (Obihiro, Japan) in isolated pens under tick-free conditions, were selected for the experiment. When the calves were six months old, they were moved to an animal rearing facility in the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine. All three calves were tested to ensure they were negative for *T. orientalis* and other bovine blood pathogens (*Babesia* and *Anaplasma*) by microscopy, PCR, and an indirect fluorescent antibody test (Iseki *et al*., 2010; Sivakumar *et al*., 2012), as well as being clinically healthy. Each animal was fed following a standard feeding practice for calves, and had unlimited access to mineral blocks. The animal experiments were conducted in accordance with the Standards Related to the Care and Management of Experimental Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine (approval number: 21-48).

Experimental infections with parasites

A blood sample was collected in an EDTA-containing tube from a cow naturally infected with *T. orientalis* in the Taiki district of Japan. The infected blood sample was washed three times in phosphate buffered saline, and the buffy coat containing peripheral blood mononuclear cells was carefully removed during each washing step. When the experimental animals were eight months old, they were intravenously injected with the purified RBCs containing 2×10^{10} of the parasitized RBCs from the naturally infected cow.

RBC indices and parasitemia in experimentally infected animals

Blood sampling was carried out during the experimental period, starting from 2 months before the infection to 3 months post infection. During this period, whole blood samples were collected twice a week from all three calves in EDTA-containing tubes. The RBC indices for the samples were analyzed (i.e., RBC counts, HGB, HCT, MCV, MCH, and MCHC, as described above). Additionally, Giemsa-stained thin blood smears were prepared and examined under a light microscope to determine the parasitemia for *T. orientalis,* as described previously (Ota *et al*., 2009).

Statistical analysis

The standard deviation values for each of the RBC indices of the field blood samples were determined, using Microsoft Excel. The mean values of the RBC indices of the field blood samples were analyzed by an unpaired *t* test using an online version of GraphPad software (http://graphpad.com/quickcalcs/ttest1.cfm?Format=SD). For Holstein cattle, the mean values RBC indices between non-infected, infected non-anemic, and infected anemic animals were also analyzed by one way ANOVA followed by Tukey post-hoc test using an online tool (http://astatsa.com/OneWay_Anova_with_TukeyHSD/_get_data/). *P* values of < 0.05 were considered to be statistically significant.

RESULTS

Anemia development in different cattle breeds naturally infected with *T. orientalis*

Anemia was detected in the *T. orientalis*-infected Holstein (n=9) and Hereford (n=1) cattle, but none of the Japanese Shorthorn and Angus infected cattle were anemic. As expected, none of the animals from the four non-infected cattle breeds were anemic in the present study.

The *T. orientalis*-infected anemic Holstein cattle had low mean RBC counts ($359 \times 10^4/\mu l$), low HCT (20.7%) and HGB (6.7 g/dl) values, and elevated mean values for MCV (61.3 fl) and MCH (19.8 pg), in comparison with those of the non-infected cattle ($757 \times 10^4/\mu l$, 33%, and 10.8 g/dl, respectively) ($P < 0.0001$) (Fig. 1). In addition, the mean values of the RBC counts, HCT, and HGB were also significantly lower in the *T. orientalis*-infected but non-anemic Holstein animals ($687 \times 10^4/\mu l$, 30.7%, and 10.1 g/dl, respectively) than those of the non-infected cattle (*P* values 0.0021, 0.0019, and 0.0029, respectively), while the values for MCV and MCH in the infected but non-anemic Holstein cattle (45.4 fl and 14.9 pg, respectively) were no different from those of the latter category (43.8 fl and 14.4 pg, respectively) (Fig. 1). The ANOVA and Tukey post-hoc test analyses also indicated that the mean RBC counts, HCT, and HGB were significantly different ($P < 0.01$) between non-infected and infected non-anemic, non-infected and infected anemic, and infected non-anemic and infected anemic Holstein animals. On the other hand, mean MCV and MCH values were significantly different ($P < 0.01$) between non-infected and infected anemic, and infected non-anemic and infected anemic but not between non-infected and infected anemic Holstein cattle.

Only a single Hereford animal was anemic, based on its HGB level (6.1 g/dl) and low RBC count ($325 \times 10^4/\mu l$) and HCT (18.5%), while the MCV (56.9 fl) and MCH (18.8 pg) values were comparable to the means of these parameters in the non-infected category (Fig. 1). In contrast, the *T. orientalis*-infected but non-anemic Hereford animals had a lower mean RBC count ($544 \times 10^4/\mu l$), lower mean HCT (32.3%), and HGB (11.3 g/dl) values, but higher mean MCV (60.6 fl) and MCH (21.2 pg) values, in comparison to those of the non-infected animals ($757 \times 10^4/\mu l$, 39.3%, 13.4 g/dl, 52.3 fl, and 18.1 pg, respectively) (*P* values < 0.0001, 0.0002, < 0.0001, 0.0006, and 0.0002, respectively) (Fig. 1).

None of the Japanese Shorthorn animals were anemic, although 20 of 22 were infected with *T. orientalis*. Furthermore, none of the RBC indices analyzed in the present study were found to differ between the infected and non-infected categories (Fig. 1). Similarly, while four of 17 Angus cattle were infected with *T. orientalis*, all the RBC indices, except HCT, did not differ between the infected and non-infected animals. Although the mean HCT value for the *T. orientalis*-infected Angus animals (33.2%) was lower than that of the non-infected animals (35.3%), the *P* value was only 0.0137. For all of the cattle breeds analyzed in the present study, the mean MCHC values did not differ significantly between the non-infected and infected groups (Fig. 1).

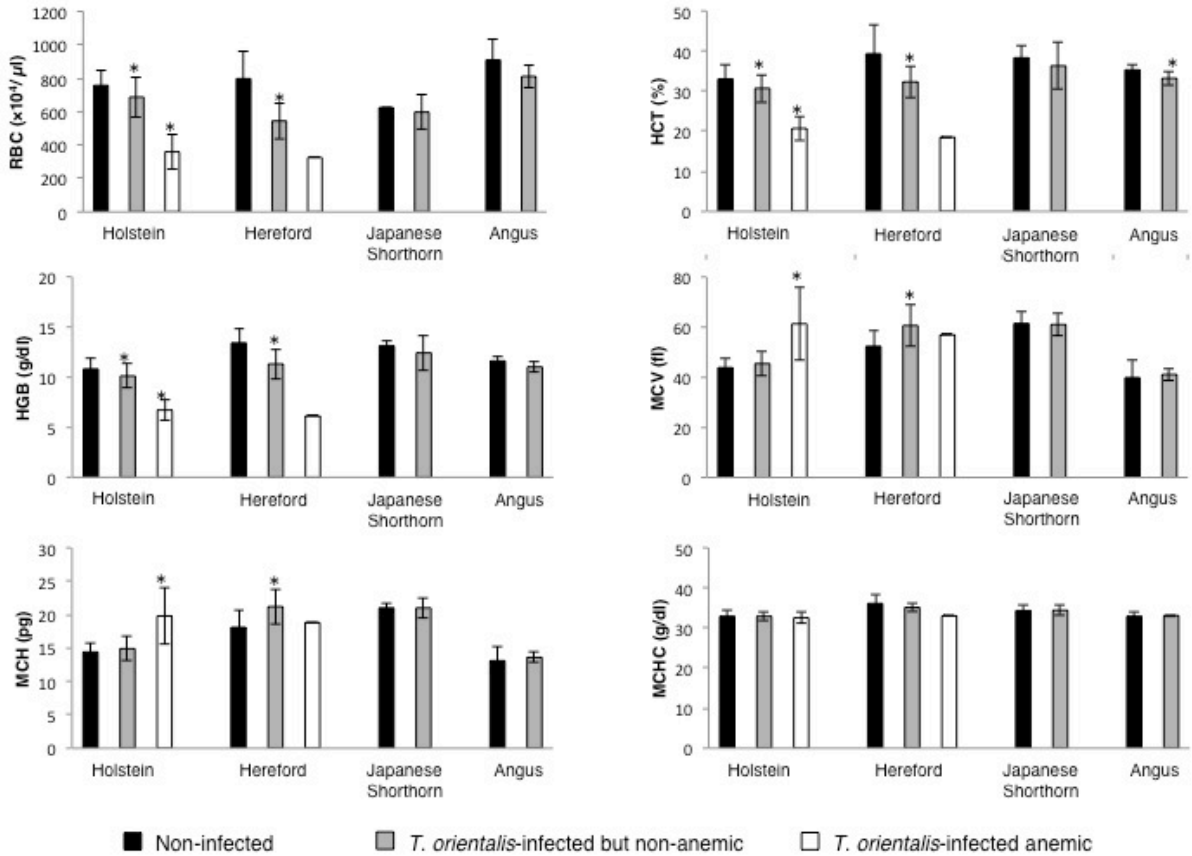

Fig. 1. Analyses of RBC indices for cattle naturally infected with *T. orientalis*. The mean RBC counts and HCT, HGB, MCV, MCH and MCHC values were determined for non-infected, *T. orientalis*-infected but non-anemic, *T. orientalis*-infected and anemic Holstein (n=43, 53, and 9, respectively), Hereford (n=23, 24, and 1, respectively), Japanese Shorthorn (n=2, 20, and 0, respectively), and Angus cattle (n=13, 4, and 0, respectively). Asterisks indicate the parameters that differed significantly from the non-infected categories ($P<0.05$).

Anemia in calves experimentally infected with *T. orientalis*

Three Holstein calves (animal IDs: 5690, 5692, and 5694,) were injected with *T. orientalis*-parasitized RBCs. When these animals were monitored for parasitemia development and their RBC indices were measured, their profiles differed among the individual animals. Parasites were not detectable in the experimental calves until 5 days post-infection (dpi), while two of them (5690 and 5694) were parasite-positive by microscopy at 8 dpi (data not shown). However, after 11 dpi, the parasites were detectable in all three calves. The percentage parasitemia in calves 5690 and 5692 gradually increased, peaking at 1.63% and 2.17% at 43 and 36 dpi, respectively, and then decreased gradually (Fig. 2 and Table 1). In contrast, the parasitemia in calf 5694 increased, peaking at 5.59% at 36 dpi. Some post-peak fluctuations were observed in this calf, such as the parasitemia remaining high in comparison to the parasitemias of the other two calves, but eventually started to decrease from 68 dpi.

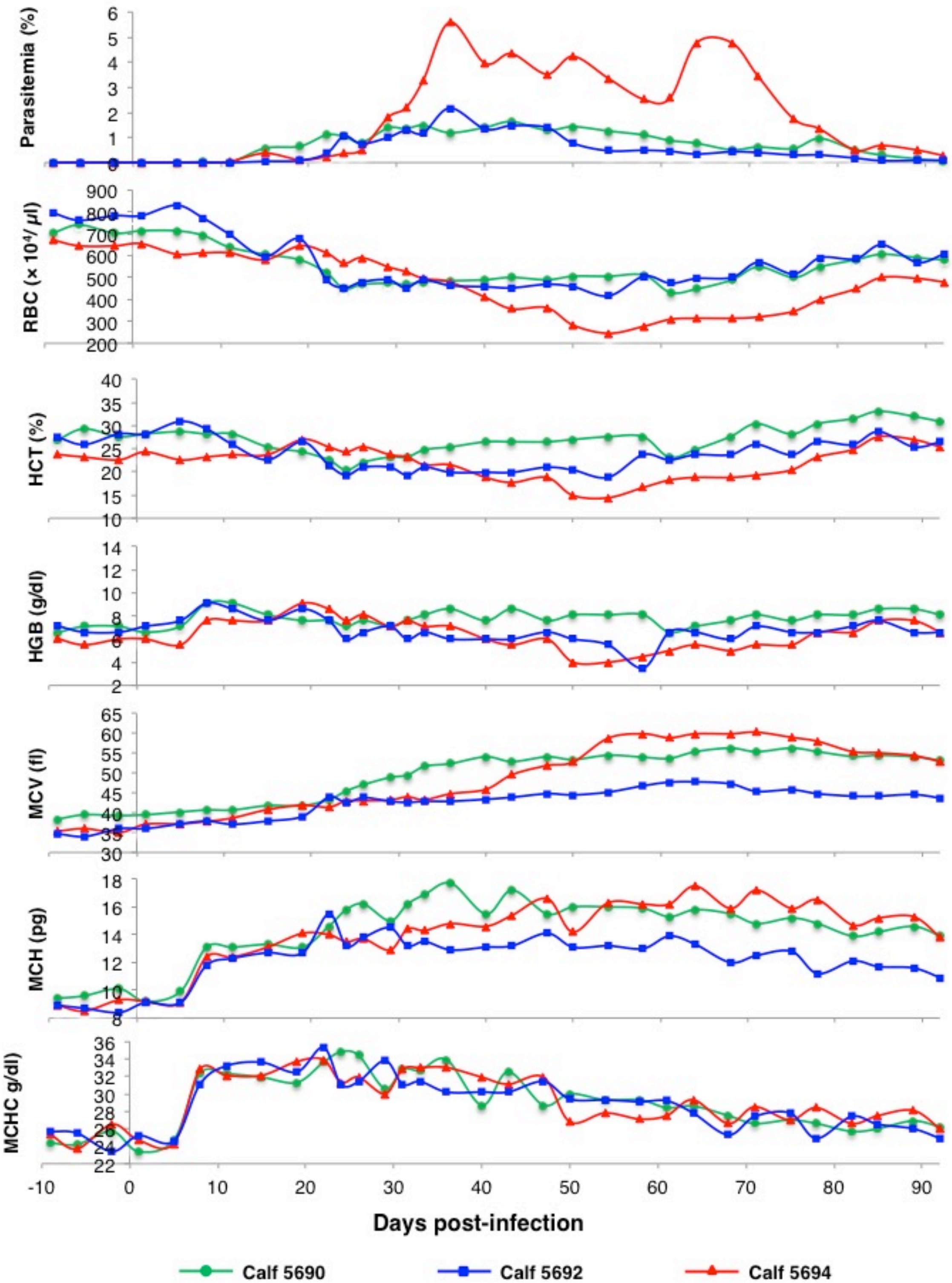

Fig. 2. Temporal dynamics of the RBC indices for the three Holstein calves experimentally infected with *T. orientalis*.

The HGB values for all three calves increased abruptly from the pre-infection values of 6 – 7.1 g/dl to 7.6 – 9 g/dl, which is consistent with the elevation in MCH values (from 8.4 – 10.1 pg to 11.8 – 13.1 pg at 8 dpi) (Fig. 2). The RBC counts in calves 5690 and 5692 reached low values, which were comparable between both animals, and then remained almost unchanged until 68 dpi. Because of the elevated MCV and MCH values after 19 dpi (maximum MCV, 56.3 fl; maximum MCH, 17.7 pg), the HGB values for calf 5690 never dropped to values lower than those measured during the pre-infection period (Fig. 2 and Table 1). However, after the initial elevation at 8 dpi, notable changes were not observed in the MCV and MCH values in calf 5692, and the HGB dropped to low values (minimum HGB was 3.5 g/dl) after 47 dpi in this calf. On the other hand, the RBC counts continued to decrease in calf 5694, until the minimum count of $245 \times 10^4/\mu l$ was recorded at 54 dpi. Despite the elevated MCV and MCH values (maximum MCV, 60.3 fl; maximum MCH, 17.5 pg), calf 5694 experienced lower HGB levels (minimum HGB was 4 g/dl) during 47 – 75 dpi than those during the pre-infection stage.

Table 1. Peak parasitemia, minimum RBC counts, HCT, and HGB values, and maximum MCV and MCH values in calves experimentally infected with *T. orientalis*

Parameters	Calves		
	5690 (dpi[a])	5692 (dpi)	5694 (dpi)
Peak parasitemia (%)	1.63 (43)	2.17 (36)	5.59 (36)
Min. RBC value ($\times$ $10^4/\mu l$)	432 (61)	416 (54)	245 (54)
Min. HCT (%)	20.4 (24)	18.8 (54)	14.4 (54)
Min. HGB (g/dl)	6.6 (61)	3.5 (58)	4 (50-54)
Max. MCV (fl)	56.3 (68)	47.8 (64)	60.3 (71)
Max. MCH (pg)	17.7 (36)	15.5 (22)	17.5 (64)

[a] Day post-infection
Min. denotes minimum
RBC, red blood cell; HCT , hematocrit; HGB, hemoglobin concentration, MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

DISCUSSION

T. orientalis has become a hot topic in the field of veterinary parasitology because of the recent disease outbreaks caused by it in Australia and New Zealand (Kamau *et al*., 2011; McFadden *et al*., 2011; Eamens *et al*., 2013; Perera *et al*., 2014). Several *T. orientalis*-related pathological mechanisms such as oxidative damage (Shiono *et al*., 2003), protease-mediated damage (Hagiwara *et al*., 1995), and iron-overload (Watanabe *et al*., 1998) have been studied for the extravascular hemolysis that results in severe anemia in *T. orientalis*-infected cattle. Although hematological parameters were sometimes analyzed in *T. orientalis*-infected cattle in previous investigations, the anemia development based on the cattle breed, MCV and MCH dynamics, which can be considered as the host response to hemolysis, and parasitemia level was not studied. Therefore, we investigated here the changes occurring in RBC indices in relation to anemia development in *T. orientalis*-infected cattle.

HGB is thought to be the most suitable parameter for determining the anemia status of an animal, as HGB levels reflect changes in RBC counts, as well as in the MCV and MCH (Graitcer *et al*.,, 1981; Gross *et al*., 1996). Consequently, in the present study, an animal was considered to be anemic when its HGB value is lower than the lower limit (8 g/dl) of this parameter (Jain, 1993). Based on this criterion, anemic animals were found only in the Holstein and Hereford cattle breeds. The percentage anemia in the *T. orientalis*-infected Holstein (14.5%) was, however, higher than that in the parasite-infected Hereford cattle (4%). The elevated MCV and MCH were observed in the *T. orientalis*-infected but non-anemic Hereford animals as compared to those of non-infected animals. With the elevated MCV and MCH, the infected Hereford animals are likely to maintain the HGB within the normal range. In contrast, the MCV and MCH values in Holstein cattle were comparable between non-infected and infected non-anemic cattle. Thus the *T. orientalis*-infected Holstein animals may progress to anemia status, as the HGB continue to fall. This could explain why the anemia percentage is lower among Hereford cattle breed as compared with the Holstein cattle. It is also worth noting that the single anemic animal detected in the Hereford cattle had MCV and MCH values comparable to those of the non-infected cattle. These findings suggest that host-specific responses to hemolysis might have influenced anemia development in the *T. orientalis*-infected Holstein and Hereford cattle, and that the host response is more pronounced in Hereford than in Holstein cattle.

The mean values of the RBC indices, including the mean RBC counts, were not significantly different between the *T. orientalis*-positive and -negative Japanese Shorthorn and Angus cattle. These findings indicate that hemolysis is not pronounced in Japanese Shorthorn and Angus cattle, in comparison with Holstein and Hereford cattle. However, only two Japanese Shorthorn animals were parasite-negative and only four Angus animals were parasite-positive, making a fair comparison of the RBC indices between the parasite-positive and -negative animals difficult to obtain.

Three Holstein calves were experimentally infected with *T. orientalis* and monitored thereafter for the dynamics of parasitemia development and RBC indices in order to clarify the findings we obtained in the naturally infected cattle. Generally, the experimental infections of *T. orientalis* yielded results comparable to those from the naturally infected cattle from this study. The dynamics of parasitemia and the RBC indices varied among the three experimental animals. At 8 dpi, all three animals showed sudden increases in their MCH values. The MCV values for the experimental calves 2 days before the parasite infection were 35 – 39.3 fl, which is comparable to the mean MCV values of the non-infected grazing Holstein cattle (43.8 fl). However, the MCH values in the experimental calves (8.4 – 10.1 pg) were much lower than the mean MCH values of the grazing cattle (14.4 pg). Therefore, we believe that the MCH values were elevated within the normal range without altering the MCV as an early response to hemolysis.

Calves 5690 and 5692 had comparable parasitemias, and the peak parasitemia for both calves was lower than that for calf 5694. However, calf 5690 never experienced low HGB caused by elevated MCH values, a finding consistent with an increased MCV in this animal, while such a drastic elevation in MCH or MCV values was not observed in calf 5692, which eventually had low HGB values. These findings suggest that in animals with low parasitemias, the anemia development may be influenced by changes in MCH values. However, calf 5694 developed a much higher parasitemia than the other two animals, and despite its elevated MCH and MCV values, HGB in this animal dropped to low values, suggesting that animals with higher parasitemias might develop anemia notwithstanding the increased MCH values. Collectively, the findings from the animal experiment suggest that the changes in the MCV and MCH values and the level of parasitemia in *T. orientalis*-infected host animals might influence the development of clinical anemia.

In conclusion, the present study, which analyzed the RBC indices in naturally and experimentally infected cattle, found evidence that anemia development in *T. orientalis*-infected cattle might be influenced by cattle breed, host responses, and parasitemia levels.

ACKNOWLEDGEMENTS

We are grateful to Ms. Hiroko Yamamoto, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine (Obihiro, Japan), for her excellent technical assistance. This study was supported by Grants from the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry, from JSPS KAKENHI (Grant numbers 25292169 and 2604087), from the project of the NARO Biotechnology Research Advancement Institution (the special scheme project on regional developing strategy), and from the AMED/JICA, Science and Technology Research Partnership for Sustainable Development (SATREPS).

CONFLICT OF INTEREST

The authors declare no conflicts of interest in association with this study.

SUBMISSION DECLARATION AND VERIFICATION

This manuscript is approved by all authors, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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