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Title	Flow cytometry to evaluate the level of Babesia gibsoni parasitemia in vivo and in vitro by using the fluorescent nucleic acid stain SYTO16
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Description	In the present study, we employed flow cytometry to evaluate the level of parasitemia of Babesia gibsoni infecting canine erythrocytes in vivo and in vitro by using fluorescent nucleic acid staining. Peripheral blood samples from a B. gibsoni-infected dog and cultured B. gibsoni parasitizing in canine erythrocytes were stained with a membrane-permeable fluorescent nucleic acid stain, SYTO16. In this study, we utilized normal canine erythrocytes (LK erythrocytes) and canine erythrocytes containing high concentrations of potassium, reduced glutathione, and some free amino acids (HK erythrocytes) as host cells for culture. Parasitized cells in vivo were discriminated completely from unparasitized cells and a correlation (r = 0.998) between the percentage of SYTO16-positive cells and parasitemia in vivo was observed. On the other hand, erythrocytes in vitro could not be divided clearly into parasitized and unparasitized cells. However, when LK erythrocytes were used as host cells, the percentage of SYTO16-positive cells was almost the same as, and was well correlated (r = 0.932) with, the level of parasitemia. When HK erythrocytes were used as host cells, the percentage of SYTO16-positive cells was almost half of, but was correlated (r = 0.982) with, the level of parasitemia. Therefore, we attempted to observe the changes in the percentage of parasitized cells after treatment with antiprotozoal drug or mitochondria inhibitors by using flow cytometry. The changes in the percentage of SYTO16-positive cells corresponded well with the changes of the level of parasitemia when the parasites in HK erythrocytes were cultured with each compound. The present results suggest that flow cytometric detection using SYTO16 is a rapid and reliable method for monitoring parasitemia both in vivo and in vitro.
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FULL PAPER

Flow cytometry to evaluate the level of *Babesia gibsoni* parasitemia *in vivo* and *in vitro* by using the fluorescent nucleic acid stain SYTO16

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Abstract

In the present study, we employed flow cytometry to evaluate the level of parasitemia of *Babesia gibsoni* infecting canine erythrocytes *in vivo* and *in vitro* by using fluorescent nucleic acid staining. Peripheral blood samples from a *B. gibsoni*-infected dog and cultured *B. gibsoni* parasitizing in canine erythrocytes were stained with a membrane-permeable fluorescent nucleic acid stain, SYTO16. In this study, we utilized normal canine erythrocytes (LK erythrocytes) and canine erythrocytes containing high concentrations of potassium, reduced glutathione, and some free amino acids (HK erythrocytes) as host cells for culture. Parasitized cells *in vivo* were discriminated completely from unparasitized cells and a correlation (r = 0.998) between the percentage of SYTO16-positive cells and parasitemia *in vivo* was observed. On the other hand, erythrocytes *in vitro* could not be divided clearly into parasitized and unparasitized cells. However, when LK erythrocytes were used as host cells, the percentage of SYTO16-positive cells was almost the same as, and was well correlated (r = 0.932) with, the level of parasitemia. When HK erythrocytes were used as host cells, the percentage of SYTO16-positive cells was almost half of, but was correlated (r = 0.982) with, the level of parasitemia. Therefore, we attempted to observe the changes in the percentage of parasitized cells after treatment with antiprotozoal drug or mitochondria inhibitors by using flow cytometry. The changes in the percentage of SYTO16-positive cells corresponded well with the changes of the level of parasitemia when the parasites in HK erythrocytes were cultured with each compound. The present results suggest that flow cytometric detection using SYTO16 is a rapid and reliable method for monitoring parasitemia both *in vivo* and *in vitro*.

Key Words: *Babesia gibsoni,* flow cytometry, SYTO16

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Introduction

Babesia gibsoni is a known blood protozoan in dogs and causative pathogen of hemolytic anemia in infected dogs. The infection of *B. gibsoni* is generally demonstrated by the observation of Giemsa-stained specimens. This method is also utilized to monitor the change of the level of parasitemia by many researchers. However, this method need much time and labor, and is less objective. Flow cytometric analysis of intraerythrocytic protozoa using a membrane-penetrative nucleic acid-binding fluorochrome has been performed in several cases of protozoan infection, including malaria[4,5] and babesiosis[3]. Hoechst 33258 is a common fluorochrome for flow cytometric detection of intraerythrocytic protozoa. However, UV excitation (351 nm) is essential for detection of the Hoechst fluorescence. Hydroethidine (HE) can be excited by a single argon laser at 488 nm. HE has been employed to determine the number of intraerythrocytic protozoa *Babesia* sp.[1,12] and *Plasmodium sp.*[11]. However, this fluorochrome cannot clearly distinguish *Theirelia sergenti,* renamed *T. orientalis*-infected erythrocytes from uninfected erythrocytes[13]. SYTO16 can also be excited by a single argon laser at 488 nm, and can penetrate intact live cells and bind strongly to nucleic acid. Therefore, it is a convenient nucleic acid-staining fluorochrome for flow cytometry. Parasitized cells in the peripheral blood from *Theirelia orientalis*-infected cattle were discriminated completely from unparasitized cells by the SYTO16 staining method[13]. However, no studies have focused on the use of SYTO16 in *B. gibsoni*-infected dogs or in the culture of *B. gibsoni.* The present study was designed to determine whether flow cytometry using SYTO16 could detect *B. gibsoni*-infected erythrocytes. In addition, we also investigated whether this method could be utilized to assess the antibabesial potentials of various drugs and compounds.

Materials and Methods

Preparation of erythrocytes from a Babesia gibsoni-infected dog.

For preparation of *B. gibsoni*-infected erythrocytes, blood was collected from an experimentally *B. gibsoni*-infected dog with a parasitemia rate of 2.9%. Blood was also collected from a healthy dog for preparing uninfected erythrocytes. Heparinized blood samples were centrifuged and the buffy coat was removed. Samples were washed three times with phosphate-buffered saline (PBS, pH 7.4). Washed infected and uninfected erythrocytes were resuspended in PBS to yield a packed cell volume (PCV) of 0.5%. To dilute the parasitized erythrocytes in each sample, the infected erythrocyte suspension was serially diluted 2-fold to a parasitemia level of 0.045% with uninfected erythrocyte suspension. SYTO16 (Molecular Probes, Eugene, OR) was added to each sample at a final concentration of 1 μM. The mixture was incubated for 20 min at 37℃ in the dark. Cells were washed twice in PBS and resuspended in 1 mL of PBS for flow cytometric analysis. The suspension of uninfected erythrocytes was also stained with SYTO16 as a control by the same procedure. A thin smear of each sample was made and the percent of parasitemia was calculated by counting the number of parasitized cells per 2,000 erythrocytes.

Preparation of Babesia gibsoni-infected erythrocytes from culture

The cultured *B. gibsoni* used in the present study have been maintained in cultures as described in a previous report[14]. We utilized either normal canine erythrocytes (LK erythrocytes) or canine erythrocytes containing high concentrations of potassium, reduced glutathione, and some free amino acids (HK erythrocyte) as host cells[15]. Erythrocytes were obtained from a dog that had inherited HK erythrocytes and a normal dog that had LK erythrocytes. Infected and uninfected erythrocytes were harvested from culture and resuspended in PBS to yield a PCV of 0.5%. At that time, infected LK erythrocytes had a level of para-

sitemia of 2.8%, and infected HK erythrocytes had one of 4.9%. To dilute the parasitized erythrocytes in each sample, the infected erythrocyte suspension was diluted at various ratios with uninfected erythrocytes, which were cultured without the parasites by the same procedure. The staining with SYTO16 and the measurement of the level of parasitemia of each sample were performed by the procedure described above.

Flow cytometry

Flow cytometry was performed according to the method of Yagi et al.[13]. Flow cytometric data acquisition and analysis were performed on an EPICS XL (Beckman-Coulter, Miami, FL), equipped with a single argon laser operating at 488 nm, using SYSTEM software. The green fluorescence of SYTO16 was detected at FL1 (525 nm). A total of 5 x 10^4 events (cells) were analyzed per sample with the detection of forward (FSC) and side scatter (SSC) and expressed as the percentage of fluorescence-positive cells.

Effects of quinine and anti-mitochondrial compounds on the percentage of parasitized cells

Effects of the anti-protozoan parasite drug quinine and two anti-mitochondrial compounds, potassium cyanide (KCN) and *N, N'*-dicyclohexylcarbodiimide (DCCD), on the percentage of parasitized cells in 72 hr assays were determined by flow cytometry as described above. The parasites in HK erythrocytes were incubated in medium containing various concentrations of quinine, KCN and DCCD. After 72 hr incubation, infected HK erythrocytes were harvested and stained with SYTO16 as described above. A thin smear of each sample was made for estimating the level of parasitemia under light microscopy.

Statistical analysis

Statistical analysis was performed using a Student's *t*-test. The level of parasitemia in the presence of KCN or DCCD were compared to that without compounds. The difference between data was considered to be significant if the P value was < 0.01.

Results

Figure 1 shows fluorescence histograms obtained from SYTO16-stained *B. gibsoni*-infected erythrocytes. SYTO16-stained intraerythrocytic parasites were detected at FL1 by their green fluorescence. Parasitized cells *in vivo* were discriminated completely from unparasitized cells (Figs. 1A and 1B). There was an obvious peak of parasitized cells in *in vivo* samples (Fig. 1B). However, there was no obvious peak of parasitized cells in *in vitro* samples (Fig. 1C). Instead, the strength of fluorescence intensity of the parasitized cells varied, and there were cells having much stronger fluorescence intensity in *in vitro* samples (Fig. 1C). In *in vivo* samples, the percentage of SYTO16-positive cells was well correlated (r = 0.998) with, and was slightly higher than, the level of parasitemia (Fig. 2A). In LK erythrocytes cultured with *B. gibsoni*, the percentage of SYTO16-positive cells was also well correlated (r = 0.932) with, and was almost the same as, the level of parasitemia (Fig. 2B). In HK erythrocytes cultured with *B. gibsoni,* the percentage of SYTO16-positive cells was well correlated (r = 0.982) with, but was almost half of, the level of parasitemia (Fig. 2C).

Moreover, we attempted to detect the changes of the percentage of the parasitized cells by flow cytometry. When the parasites in HK erythrocytes were cultured in medium contained various concentrations of quinine, the percentage of SYTO16-positive cells was gradually decreased with an escalating dose of quinine, and was well corresponding with the change of, but was only about half of, the level of parasitemia (Figs. 3A and 3B). The percentage of SYTO16-positive cells was well correlated (r = 0.919) with the level of parasitemia (Fig. 3B). When the parasites in HK erythrocytes were exposed to various concentrations of DCCD, the percentage of SYTO16-positive cells was significantly ($P < 0.01$) decreased between 0.001 mM and 0.1 mM DCCD (Fig. 4A), and was well

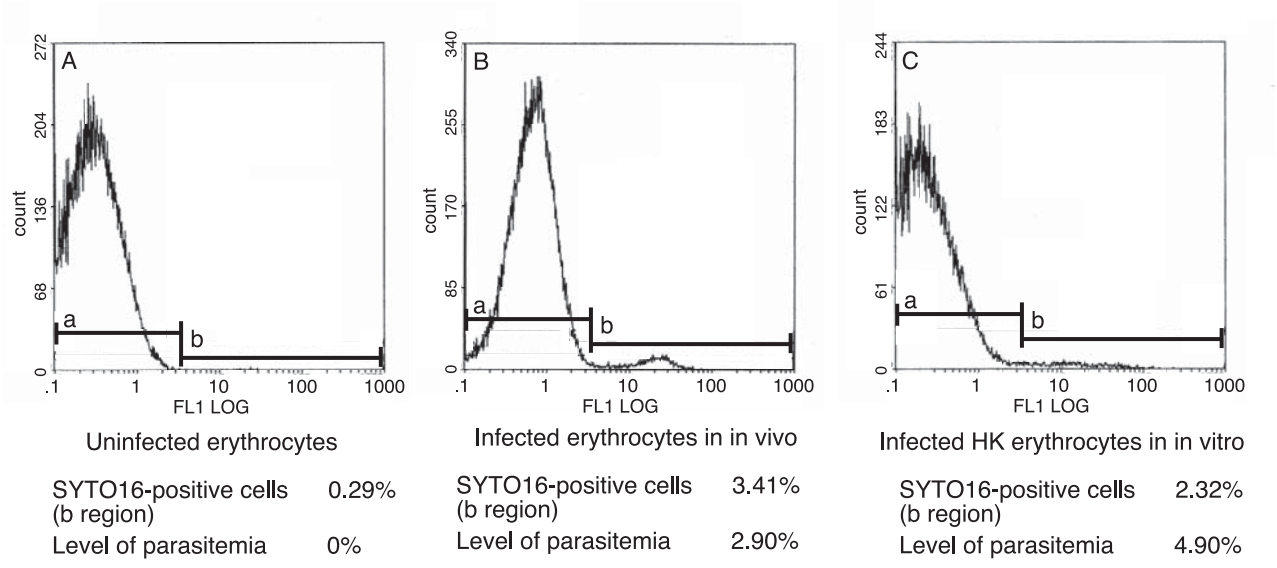

Fig. 1. Fluorescence histograms of SYTO16-stained *Babesia gibsoni*-infected erythrocytes. A. Histogram of uninfected erythrocytes from a healthy dog. B. Histogram of *B. gibsoni*-infected erythrocytes from an infected dog. C. Histogram of *B. gibsoni*-infected HK erythrocytes from culture.

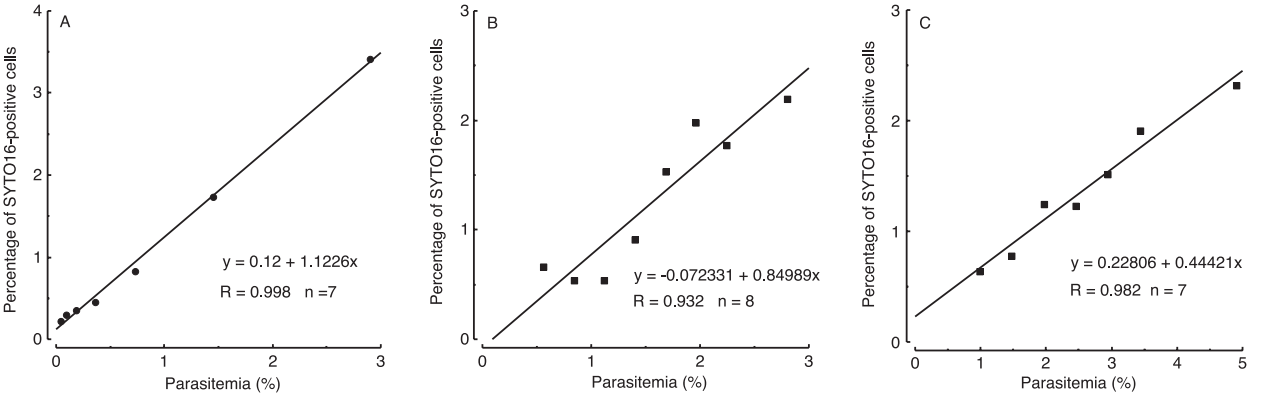

Fig. 2. Relationship between the percentage of SYTO16-positive cells observed by flow cytometry and the levels of parasitemia in *in vivo* and *in vitro* samples. A. *Babesia gibsoni*-infected blood samples from an infected dog were serially diluted with uninfected blood. B. *B. gibsoni*-infected LK erythrocytes from the culture were diluted at various ratios with uninfected LK erythrocytes from the culture. C. *B. gibsoni*-infected HK erythrocytes from the culture were diluted at various ratios with uninfected HK erythrocytes from the culture.

correlated (r = 0.989) with the level of parasitemia (Fig. 4B). When the parasites in HK erythrocytes were exposed to various concentrations of KCN, the percentage of SYTO16-positive cells was not changed between 1 x 10^{-7} and 10 mM KCN (Fig. 5 A), and was also well correlated (r = 0.951) with the level of parasitemia (Fig. 5B).

Discussion

In the present study, SYTO16-stained intraerythrocytic *B. gibsoni* from an infected dog were detected by flow cytometry, and parasitized cells could be discriminated from unparasitized cells. Though SYTO16-stained intraerythrocytic parasites from cultures were also detected by flow cytometry, parasitized cells from cultures were incompletely discriminated from unparasitized cells.

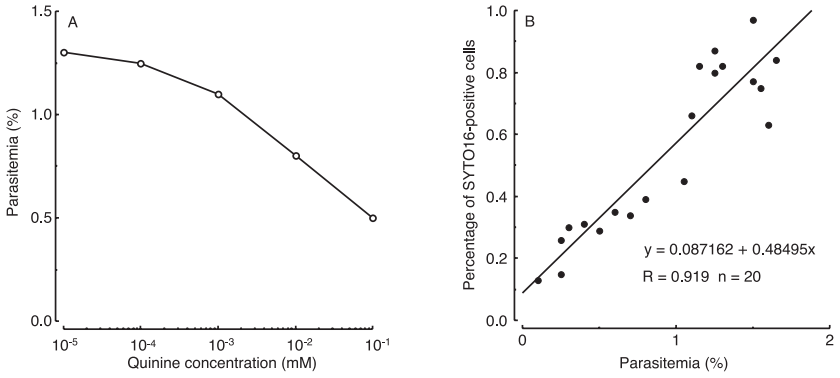

Fig. 3. The effects of various concentrations of quinine on parasite viability observed by flow cytometry using SYTO16 and light microscopy. A. The effect of quinine is expressed as the change of the level of parasitemia. B. Correlation between the percentage of SYTO16-positive cells and the level of parasitemia when the parasites were cultured in medium containing various concentrations of quinine.

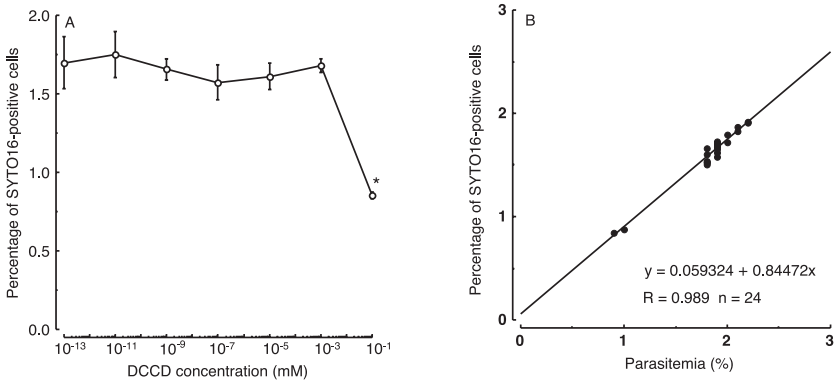

Fig. 4. The effects of various concentrations of *N, N'*-dicyclohexylcarbodiimide (DCCD) on parasite viability observed by flow cytometry using SYTO16 and light microscopy. A. The effect of DCCD is expressed by the percentage of SYTO16-positive cells. *Significantly ($P < 0.01$) different from the percentage of SYTO16-positive cells without DCCD. B. Correlation between the percentage of SYTO16-positive cells and the level of parasitemia when the parasites were cultured in medium containing various concentrations of DCCD.

The majority of parasites are small oval in shape and occur singly or in pairs in the erythrocytes *in vivo*[10]. However, in culture, the large oval shaped parasites appeared inside and outside the erythrocytes, and several clusters of parasites and the erythrocytes including 4, 8, 16, or 32 parasites were frequently observed[10]. Erythrocytes including a lot of parasites would have strong fluorescence intensities, because those would include a large amount of nucleic acids. Therefore, there was no obvious peak of parasitized cells in *in vitro* samples. It is noteworthy that the percentage of SYTO16-positive cells in cultured HK erythrocytes was much lower than the level of parasitemia. Osmotic fragility of HK erythrocytes naturally showed marked increase as compared to that of LK erythrocytes[6]. Moreover, superoxide anions are produced in *B. gibsoni*-infected erythrocytes *in*

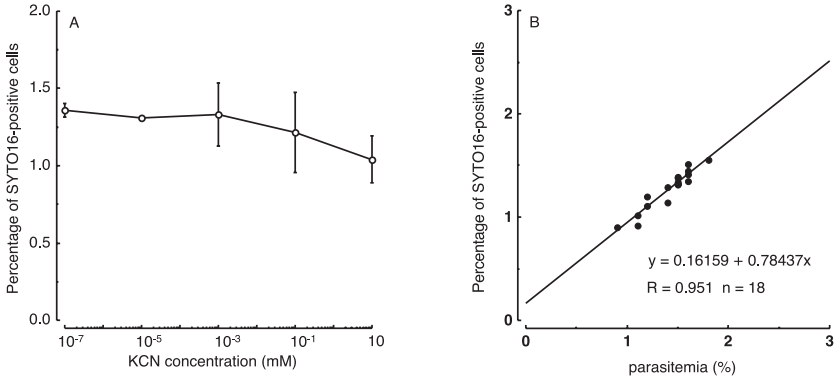

Fig. 5. The effects of various concentrations of potassium cyanide (KCN) on parasite viability observed by flow cytometry using SYTO16 and light microscopy. A. The effect of KCN is expressed by the percentage of SYTO16-positive cells. B. Correlation between the percentage of SYTO16-positive cells and the level of parasitemia when the parasites were cultured in medium containing various concentrations of KCN.

vitro, which suggests that the parasite causes measurable oxidation of the host erythrocytes, resulting in increase of fragility of erythrocytes[8]. From these previous reports, it is supposed that infected HK erythrocytes in culture might be much fragile, and might hemolyze during washing or measurement. However, the percentage of SYTO 16-positive cells was well correlated with the level of parasitemia both *in vivo* and *in vitro.* These results suggested that flow cytometry using SYTO16 could be applied to evaluate the percentage of the parasitized cells both in peripheral blood from infected dogs and in culture of *B. gibsoni.* However, the sensitivity of this method is not determined in the present study. It is reported that the sensitivity of flow cytometry for Hoechst 33258-stained *Plasmodium*-infected erythrocytes[5] and SYTO16-stained *Theileria orientalis*-infected erythrocytes[13] is not sufficient to detect very low numbers of infected erythrocytes. Recently, it was reported that real-time polymerase chain reaction (PCR) assay could also be used to detect *B. gibsoni* quantitatively[7]. Though real-time PCR assay has high specificity and high sensitivity, this method needs cumbersome procedures. Flow cytometry using SYTO16 might be a simple method for evaluating the percentage of parasitized cells compared to real-time PCR assay, though the sensitivity of flow cytometry is lower. Since SYTO16 reacts with any intraerythrocytic microorganism, including *Theileria sp.* and *Anaplasma sp.*[13], this method cannot be used for specific diagnosis of babesiosis. However, the method employed here is a rapid and reliable technique for the detection and analysis of blood infection of some microorganism.

Moreover, to verify whether flow cytometry using SYTO16 could correctly detect the change of the percentage of infected erythrocytes by antibabesial compounds, we compared the percentage of SYTO16-positive cells to the level of parasitemia after the incubation of the parasites with quinine, KCN, and DCCD. The decrease of the parasites caused by quinine was correctly detectable by flow cytometry just as by the observation of Giemsa-stained specimens, when the parasites were cultured with HK erythrocytes. A high correlation between the percentage of SYTO16-positive cells and parasitemia was observed at that time. KCN did not affect the percentage of infected erythrocytes, nor could the high concentration of DCCD decrease that. A high correlaion between the percentage of SYTO16-positive cells and parasitemia was also observed. These results indicated that flow cytometry using SYTO16 could also be employed to evaluate the changes of the percentage of the parasitized cells in cultures and to assess the

anti-babesial potentials of various drugs and compounds. Additionally, objectivity is important in assessment of the anti-babesial potentials of various compounds, and the observation of specimens need much time and exacting labor. Flow cytometry seems to be more objective and less exacting than the observation of Giemsa-stained specimens. Gozar et al.[2] and Shikano et al.[9] assessed the influence of anti-mitochondrial compounds on parasite viability by using [^{3}H]hypoxanthine. However, an appropriate facility is necessary for using [^{3}H] hypoxanthine because it is a radioisotope. SYTO 16 can be easily utilized compared to [^{3}H]hypoxanthine. Thus, it is suggested that flow cytometry using SYTO16 is an easily-handled and convenient method for monitoring the level of parasitemia.

In conclusion, flow cytometric detection using SYTO16 is a rapid, simple, and reliable method of monitoring parasitemia both in peripheral blood from infected dogs and in culture of *B. gibsoni.*

Acknowledgments

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References

1) Fukata, T., Ohnishi, T., Okuda, S., Sasaki, K., Baba, E. and Arakawa, A. 1996. Detection of canine erythrocytes infected with *Babesia gibsoni* by flow cytometry. *J. Parasitol.,* **82**: 641-642.
2) Gozar, M. M. G., O'Sullivan, W. J. and Bagnara, A. S. 1992. Mitochondrial function in *Babesia bovis. Int. J. Parasitol.,* **22**: 165-171.
3) Howard, R. J. and Rodwell, B. J. 1979. *Babesia rodhaini, Babesia bovis,* and *Babesia bigemina:* analysis and sorting of red cells from infected mouse or calf blood by flow fluorimetry using 33258 Hoechst. *Exp. Parasitol.,* **48**: 421-431.
4) Janse, C. J., van Vianen, P. H., Tanke, H. J., Mons, B., Ponnudurai, T. and Overdulve, J. P. 1987. *Plasmodium* species: flow cytometry and microfluorometry assessments of DNA content and synthesis. *Exp. Parasitol.,* **64**: 88-94.
5) Janse, C. J. and van Vianen, P. H. 1994. Flow cytometry in malaria detection. *Methods Cell Biol.,* **42**: 295-318.
6) Maede, Y., Inaba, M. and Taniguchi, N. 1983. Increase of Na-K-ATPase activity, glutamate, and aspartate uptake in dog erythrocytes associated with hereditary high accumulation of GSH, glutamine, and aspartate. *Blood,* **61**: 493-499.
7) Matsuu, A., Ono, S., Ikadai, H., Uchide, T., Imamura, S., Onuma, M., Okano, S. and Higuchi, S. 2005. Development of a SYBR green real-time polymerase chain reaction assay for quantitative detection of *Babesia gibsoni* (Asian genotype) DNA. *J. Vet. Diagn. Invest.,* **17**: 569-573.
8) Otsuka, Y., Yamasaki, M., Yamato, O. and Maede, Y. 2001. Increased generation of superoxide in erythrocytes infected with *Babesia gibsoni. J. Vet. Med. Sci.,* **63**: 1077-1081.
9) Shikano, S., Katoh, R., Adachi, K., Bonkobara, M., Inaba, M. and Ono, K. 1998. Mitochondrial function in *Babesia microti* and *Babesia rodhaini. Int. J. Parasitol.,* **28**: 567-570.
10) Sunaga, F., Namikawa, K. and Kanno, Y. 2002. Continuous *in vitro* culture of erythrocytic stages of *Babesia gibsoni* and virulence of the cultivated parasites. *J. Vet. Med.Sci.,* **64**: 571-575.
11) van der Heyde, H. C., Elloso, M. M., vande Waa, J., Schell, K. and Weidanz, W. P. 1995. Use of hydroethidine and flow cytometry to assess the effects of leukocytes on the malarial parasite. *Clin. Diag. Lab. Immunol.,* **2**: 417-425.
12) Wyatt, C. R., Goff, W. and Davis, W. C. 1991. A

flow cytometric method for assessing viability of intraerythrocytic hemoparasites. *J. Immunol. Methods,* **140**: 23-30.

13) Yagi, Y., Shiono, H., Kurabayashi, N., Yoshihara, K. and Chikayama, Y. 2000. Flow cytometry to evaluate *Theileria sergenti* parasitemia using the fluorescent nucleic acid stain, SYTO16. *Cytometry,* **41**: 223-225.

14) Yamasaki, M., Hossain, M. A., Jeong, J. R., Chang, H. S., Satoh, H., Yamato, O. and Maede, Y. 2003. *Babesia gibsoni*-specific isoenzymes related to energy metabolism of the parasite in infected erythrocytes. *J. Parasitol.,* **89**: 1142-1146.

15) Yamasaki, M., Takada, A., Yamato, O. and Maede, Y. 2005. Inhibition of Na,K-ATPase activity reduces *Babesia gibsoni* infection of canine erythrocytes with inherited high K, low Na concentrations. *J. Parasitol.,* **91**: 1287-1292.