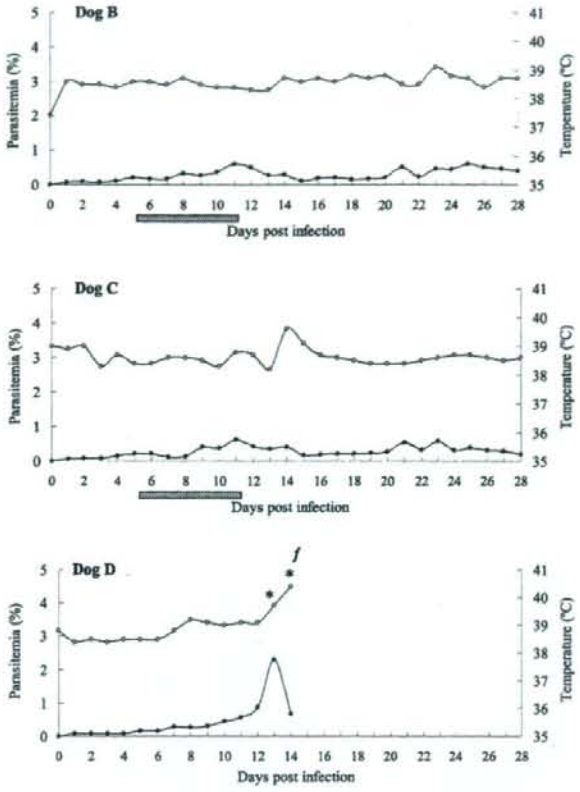

Fig. 2. Changes in parasitemia (-●-) and body temperature (-○-) in the dogs (B, C, and D) infected with *Babesia gibsoni*. Dogs B and C were administered bruceine A (6.4 mg/kg/day) orally from day 5 post-infection for 6 days. The dark grey bars indicate the period of bruceine A administration. Untreated dog D showed complete loss of appetite and movement (*) and reached humane endpoint (*f*).

stage of infection with *B. gibsoni* relieved some of the clinical signs of infected dogs. An untreated dog developed severe anemia, high fever, and complete loss of appetite and movement, whereas two bruceine A-treated dogs maintained healthy conditions. However, in these treated dogs decreases in PCV value and erythrocyte and platelet counts were observed and complete elimination of the parasites from the peripheral blood was not achievable. These results suggested that bruceine A inhibited the growth of parasites to a certain extent and prevented the manifestation of clinical signs associated with *B. gibsoni* infection in dogs.

It is reported that the degree of thrombocytopenia is more severe than that of anemia in *B. gibsoni*-infected dogs [9, 11, 17, 33]. This clinical finding was also observed in the present study. Dog C showed a temporal recovery from thrombocytopenia from day 15, however, this may not be related to the drug treatment since dog B did not show a similar recovery. Treatment with atovaquone alone, atovaquone in combination with azithromycin, and clindamycin alone against *B. gibsoni*-infected dogs has been reported to show a rapid decrease in parasitemia and significant improvement of clinical findings including anemia and thrombocyteopenia, in spite of unsuccess in total elimination of circulating parasites [11, 17, 33]. Unlike *in vitro* results, a rapid elimination of the parasites in the peripheral erythrocytes was not observed in the infected dogs by treatment with bruceine A in the present study. Since sequestration of *B. gibsoni*-infected erythrocytes within the tissues such as the lymph node and spleen has been suggested [11, 17], bruceine A might inhibit the growth of parasites in the

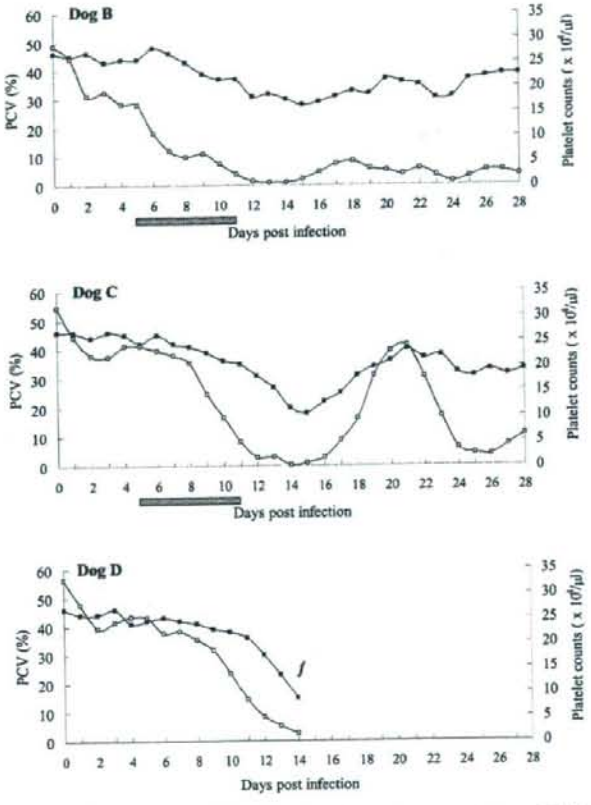

Fig. 3. Changes in packed cell volume (PCV) (-■-) and platelet counts (-□-) in the dogs (B, C, and D) infected with *Babesia gibsoni*. Dogs B and C were administered bruceine A (6.4 mg/kg/day) orally from day 5 post-infection for 6 days. The dark grey bars indicate the period of bruceine A administration. Humane endpoint is indicated by *f*.

tissues and prevent the manifestation of clinical signs. Another possible explanation is that metabolized-compounds derived from bruceine A exhibited reduced antiparasitic activities. In fact, a slight structural change and modification of quassinoid compounds have resulted in a variety of antibabesial activities [24]. Further studies including curative dosage, effective route, and period of administration of bruceine A as well as a rational design of combination therapy are required.

Bruceine A was found to have some toxic effects on treated animals [2, 23]. The risk of side-effects is likely to increase with higher dosages of the drug; therefore, reduction of the risk needs to be considered. In fact, liposomal encapsulation of another antibabesial drug, imidocarb, resulted in the reduction of its *in vivo* toxicity by 50-fold compared to the unencapsulated drug [27]. Hence, a similar approach may be useful for the development of more rational treatment regimens with bruceine A.

Brucea javanica, a plant species of the family Simaroubaceae, is widely distributed throughout South East Asian countries and its fruits have been used as a source of traditional medicine against malaria, dysentery, and cancer. The bitter principles of this plant are quassinoids, some of which have been investigated for their biological properties including antitumor [6, 12, 15], antiamoebic [31], antimalarial [7, 23], and antibabesial activities [24]. Kirby *et al.* [13] showed that seven quassinoids such as ailanthinone, bruceantin, bruceine B, glaucarubinone, holacanthone, chaparrin, and glaucarubol were proved to inhibit protein synthesis in malaria parasites. Inhibition of nucleic acid synthesis was also detected as a subsequent reaction. There are reports on the *in vitro* activity of bruceine A against protozoan parasites, including *Plasmodium falciparum*, *Entamoeba histolytica*, and *Giardia intestinalis* [23, 31, 32].

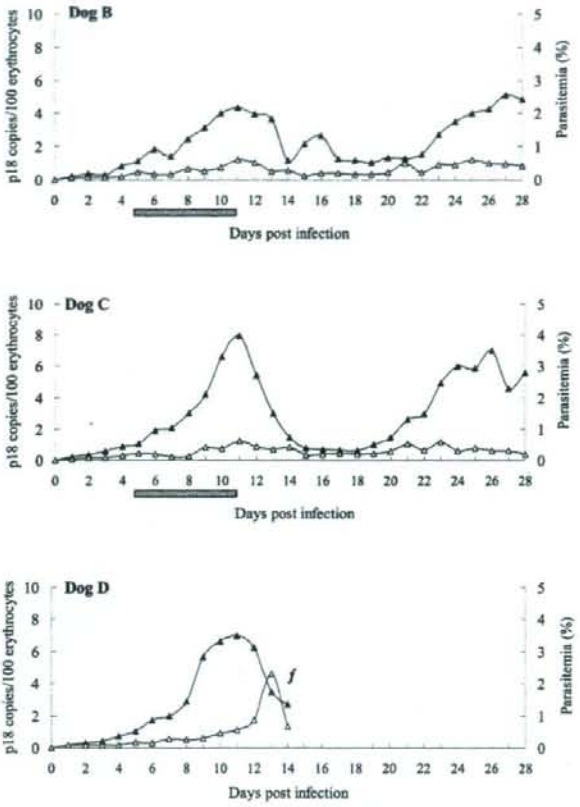

Fig. 4. Changes in copy number of *Babesia gibsoni* p18 gene (-▲-) and parasitemia (-△-) in the dogs (B, C, and D) infected with *Babesia gibsoni*. Dogs B and C were administered bruceine A (6.4 mg/kg/day) orally from day 5 post-infection for 6 days. The dark grey bars indicate the period of bruceine A administration. Humane endpoint is indicated by *f*.

Although the mode of action of bruceine A against *Babesia* species is unknown, it appears to be different from those of the currently available antibabesial drugs [25, 30]. Further studies are required to elucidate the mechanism by which bruceine A displays its potent antibabesial activity for the further development of novel combination chemotherapies with different antibabesial compounds.

Quassinoid compounds have anti-inflammatory activities *in vitro* and *in vivo* [8, 14, 28]. Hall *et al.* [8] reported that brusatol, another analogous quassinoid to bruceine A, reduced inflammation and arthritis in rodents. They found that the structures of 3-hydroxy-delta3-2-oxo moiety, a C-15 ester-bearing delta-lactone ring, and C-11 and C-12 free hydroxyl groups are important for the anti-inflammatory activity of brusatol. Bruceine A possesses a similar structure with potent active sites. Investigation of the anti-inflammatory properties of bruceine A may explain the mechanisms of the reduction in disease severity in dogs infected with *B. gibsoni*.

The standard method for quantification of babesial parasites is microscopic examination of a blood smear specimen. However, it is often difficult to quantify the low levels of parasites in the peripheral blood during acute phase of infection, asymptomatic infection and chemical treatment of infected animals. In the present study a real-time PCR method using the *B. gibsoni* p18 gene was applied to monitor the growth of *B. gibsoni in vitro* and a good correlation was found between p18 gene copy numbers and parasitemia levels in the bruceine A-treated cultures (unpublished). However, in this *in vivo* study, the copy number was not parallel to the level of parasitemia. In particular, the copy numbers were smaller than those expected from the data of

parasitemia on days 11–14 in dog D, and higher than those on days 23–28 in dogs B and C. Since multi-divided forms of *B. gibsoni* were not commonly detected in the peripheral blood of infected dogs, extra-erythrocytic merozoites existing in the blood or circulating macrophages may take the parasite DNA. In fact, *Plasmodium chabaudi* DNA could be detected in mouse peripheral blood by PCR at least until 24 hr after the injection of dead parasites [22]. This real-time PCR method will be useful not only for antibabesial chemotherapeutic studies but also for understanding the mechanisms of circulation and hiding/sequestration of *B. gibsoni* parasites in the host.

In conclusion, bruceine A is a potent antibabesial compound. Further pharmacokinetic and pharmacodynamic studies could contribute for novel information on the efficacy of bruceine A for canine babesiosis and its rational administration schedule in dogs.

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REFERENCES

1. Abdullah, A.S., Sheikhomar, A.R., Baggot, J.D. and Zamri, M. 1984. Adverse effects of imidocarb dipropionate (Imizol) in a dog. *Vet. Res. Commun.* **8**: 55–59.
2. Anderson, M.M., O'Neill, M.J., Phillipson, J.D. and Warhurst, D.C. 1991. *In vitro* cytotoxicity of a series of quassinoids from *Brucea javanica* fruits against KB cells. *Planta Med.* **57**: 62–64.
3. Bedikian, A.Y., Valdivieso, M., Bodey, G.P., Murphy, W.K. and Freireich, E.J. 1979. Initial clinical studies with bruceantin. *Cancer Treat. Rep.* **63**: 1843–1847.
4. Boozer, A.L. and Macintire, D.K. 2003. Canine babesiosis. *Vet. Clin. North. Am. Small Anim. Pract.* **33**: 885–904.
5. Farwell, G.E., Legrand, E.K. and Cobb, C.C. 1982. Clinical observations on *Babesia gibsoni* and *Babesia canis* infections in dogs. *J. Am. Vet. Med. Assoc.* **180**: 507–511.
6. Fukamiya, N., Okano, M., Miyamoto, M., Tagahara, K. and Lee, K.H. 1992. Antitumor agents, 127. Bruceoside C, a new cytotoxic quassinoid glucoside, and related compounds from *Brucea javanica*. *J. Nat. Prod.* **55**: 468–475.
7. Guru, P.Y., Warhurst, D.C., Harris, A. and Phillipson, J.D. 1983. Antimalarial activity of bruceantin *in vitro*. *Ann. Trop. Med. Parasitol.* **77**: 433–435.
8. Hall, I.H., Lee, K.H., Imakura, Y., Okano, M. and Johnson, A. 1983. Anti-inflammatory agents III: Structure activity relationships of brusatol and related quassinoids. *J. Pharm. Sci.* **72**: 1282–1284.
9. Inokuma, H., Okuda, M., Yoshizaki, Y., Hiraoka, H., Miyama, T., Itamoto, K., Une, S., Nakaichi, M. and Taura, Y. 2005. Clinical observations of *Babesia gibsoni* infection with low parasitaemia confirmed by PCR in dogs. *Vet. Rec.* **156**: 116–118.
10. Jefferies, R., Ryan, U.M., Muhlnickel, C.J. and Irwin, P.J. 2003. Two species of canine Babesia in Australia: detection and characterization by PCR. *J. Parasitol.* **89**: 409–412.
11. Jefferies, R., Ryan, U.M., Jardine, J., Robertson, I.D. and Irwin, P.J. 2007. *Babesia gibsoni*: detection during experimental infections and after combined atovaquone and azithromycin therapy. *Exp. Parasitol.* **117**: 115–123.
12. Kim, I.H., Takashima, S., Hitotsuyanagi, Y., Hasuda, T. and Takeya, K. 2004. New quassinoids, javanicolides C and D and javanicosides B-F, from seeds of *Brucea javanica*. *J. Nat. Prod.* **67**: 863–868.
13. Kirby, G.C., O'Neill, M.J., Phillipson, J.D. and Warhurst, D.C. 1989. *In vitro* studies on the mode of action of quassinoids with activity against chloroquine-resistant *Plasmodium falciparum*. *Biochem. Pharmacol.* **38**: 4367–4374.
14. Kitagawa, I., Mahmud, T., Yokota, K., Nakagawa, S., Mayumi, T., Kobayashi, M. and Shibuya, H. 1996. Indonesian medicinal plants. 17. Characterization of quassinoids from the stems of *Quassia indica*. *Chem. Pharm. Bull. (Tokyo)* **44**: 2009–2014.
15. Lee, K.H., Hayashi, N., Okano, M., Nozaki, H. and Ju-Ichi, M. 1984. Antitumor agents, 65. Brusatol and cleomiscosin-A, antileukemic principles from *Brucea javanica*. *J. Nat. Prod.* **47**: 550–551.
16. Lobetti, R.G. 1998. Canine babesiosis. *Comp. Cont. Educ. Pract. Vet.* **20**: 418–430.
17. Matsuu, A., Koshida, Y., Kawahara, M., Inoue, K., Ikadai, H., Hikasa, Y., Okano, S. and Higuchi, S. 2004. Efficacy of atovaquone against *Babesia gibsoni in vivo* and *in vitro*. *Vet. Parasitol.* **124**: 9–18.
18. 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.
19. Meinkoth, J.H., Kocan, A.A., Loud, S.D. and Lorenz, M.D. 2002. Clinical and hematologic effects of experimental infection of dogs with recently identified *Babesia gibsoni*-like isolates from Oklahoma. *J. Am. Vet. Med. Assoc.* **220**: 185–189.
20. Milner, R.J., Reyers, F., Taylor, J.H. and van den Berg, J.S. 1997. The effect of diminazene aceturate on cholinesterase activity in dogs with canine babesiosis. *J. S. Afr. Vet. Assoc.* **68**: 111–113.
21. Miyama, T., Sakata, Y., Shimada, Y., Ogino, S., Watanabe, M., Itamoto, K., Okuda, M., Verdida, R.A., Xuan, X., Nagasawa, H. and Inokuma, H. 2005. Epidemiological survey of *Babesia gibsoni* infection in dogs in eastern Japan. *J. Vet. Med. Sci.* **67**: 467–471.
22. Mockenhaupt, F.P., Ulmen, U., von Gaertner, C., Bedu-Addo, G. and Bienzle, U. 2002. Diagnosis of placental malaria. *J. Clin. Microbiol.* **40**: 306–308.
23. O'Neill, M.J., Bray, D.H., Boardman, P., Chan, K.L., Phillipson, J.D., Warhurst, D.C. and Peters, W. 1987. Plants as sources of antimalarial drugs, Part 4: Activity of *Brucea javanica* fruits against chloroquine-resistant *Plasmodium falciparum in vitro* and against *Plasmodium berghei in vivo*. *J. Nat. Prod.* **50**: 41–48.
24. Subeki, Matsuura, H., Takahashi, K., Nabeta, K., Yamasaki, M., Maede, Y. and Katakura, K. 2007. Screening of Indonesian medicinal plant extracts for antibabesial activity and isolation of new quassinoids from *Brucea javanica*. *J. Nat. Prod.* **70**: 1654–1657.
25. Suzuki, K., Wakabayashi, H., Takahashi, M., Fukushima, K.,

Yabuki, A. and Endo, Y. 2007. A possible treatment strategy and clinical factors to estimate the treatment response in *Babesia gibsoni* infection. *J. Vet. Med. Sci.* **69**: 563–568.
26. Taboada, J. 1998. Babesiosis. pp. 473–481. *In*: Infectious Diseases of the Dog and Cat (Creene, C.E. ed.), Saunders, Philadelphia, PA.
27. Timofeev, B.A., Bolotin, I.M., Stepanova, L.P., Bogdanov, A.A.Jr., Georgiu, K., Malyshev, S.N., Petrovsky, V.V., Klibanov, A.L. and Torchilin, V.P. 1994. Liposomal diamidine (imidocarb): preparation and animal studies. *J. Microencapsul.* **11**: 627–632.
28. Utoguchi, N., Nakata, T., Cheng, H.H., Ikeda, K., Makimoto, H., Mu, Y., Nakagawa, S., Kobayashi, M., Kitagawa, I. and Mayumi, T. 1997. Bruceine B, a potent inhibitor of leukocyte-endothelial cell adhesion. *Inflammation* **21**: 223–233.
29. Vega, C.A., Buening, G.M., Green, T.J. and Carson, C.A. 1985. *In vitro* cultivation of *Babesia bigemina*. *Am. J. Vet. Res.* **46**: 416–420.
30. Vial, H.J. and Gorenflot, A. 2006. Chemotherapy against babesiosis. *Vet. Parasitol.* **138**: 147–160.
31. Wright, C.W., O'Neill, M.J., Phillipson, J.D. and Warhurst, D.C. 1988. Use of microdilution to assess *in vitro* antiamoebic activities of *Brucea javanica* fruits, *Simarouba amara* stem, and a number of quassinoids. *Antimicrob. Agents Chemother.* **32:** 1725–1729.
32. Wright, C.W., Anderson, M.M., Allen, D., Phillipson, J.D., Kirby, G.C., Warhurst, D.C. and Chang, H.R. 1993. Quassinoids exhibit greater selectivity against *Plasmodium falciparum* than against *Entamoeba histolytica, Giardia intestinalis* or *Toxoplasma gondii in vitro*. *J. Eukaryot. Microbiol.* **40**: 244–246.
33. Wulansari, R., Wijaya, A., Ano, H., Horii, Y., Nasu, T., Yamane, S. and Makimura, S. 2003. Clindamycin in the treatment of *Babesia gibsoni* infections in dogs. *J. Am. Anim. Hosp. Assoc.* **39**: 558–562.
34. Yamasaki, M., Asano, H., Otsuka, Y., Yamato, O., Tajima, M. and Maede, Y. 2000. Use of canine red blood cell with high concentrations of potassium, reduced glutathione, and free amino acid as host cells for in vitro cultivation of *Babesia gibsoni*. *Am. J. Vet. Res.* **61**: 1520–1524.

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Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples

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Abstract

We developed a TaqMan real-time polymerase chain reaction (PCR) assay for the quantitative detection of *Theileria equi* from the *in vitro*-cultured parasite and field blood samples collected from horses living in Ghana and Brazil. The detection limit for the assay was determined to be 1.5 parasites/μl per sample, and the quantitative capacity was demonstrated using the *in vitro*-cultured parasite. For field applications, the real-time PCR assay was compared to a previously established nested PCR assay used as the gold standard for the real-time PCR assay. Of 65 field blood samples, 46 samples were *T. equi*-positive in the nested PCR assay, while the real-time PCR assay also detected the parasite in all 46 of the nested PCR-positive samples but did not detect *T. equi* in the remaining 19 negative blood samples. This quantitative real-time PCR assay provides a valuable tool for fast laboratory diagnostic assessment of *T. equi* infection in horses.

Keywords: Real-time PCR; *Theileria equi*; Detection; Blood; Horse

1. Introduction

Equine piroplasmosis, caused by *Theileria equi* (recently re-classified from *Babesia equi*, Mehlhorn and Schein, 1998) and *Babesia caballi*, has emerged as an important protozoan infection from the veterinary and economic viewpoints (Schein, 1988). This disease is characterized by fever, anemia, icterus, and hepato- and splenomegaly and mainly occurs in tropical and subtropical areas of the world (Holbrook, 1969). Horses that recover from the initial infection often carry the parasites for the rest of their lives (De Waal, 1992). In such cases, it becomes very difficult to detect the parasites in microscopic examination, and the horses become potential disseminators of the parasites (Holbrook, 1969). Various ticks, including *Boophilus*, *Hyalomma*, *Dermacentor*, and *Rhipicephalus*, are known as transmission vectors for *T. equi* (Roby and Anthony, 1963; Stiller et al., 1980; Battsetseg et al., 2002a).

Current diagnosis of equine piroplasmosis relies on microscopic examination, serological assays, and other molecular tools (Bose et al., 1995; Nicolaiewsky et al., 2001; Xuan et al., 2001a; Rampersad et al., 2003). Microscopic detection from blood smears has been used for the most standard diagnosis of equine piroplasmosis (Bose et al., 1995); however, it has many limitations. For example, the technique is relatively laborious when large numbers of blood smear samples need to be simultaneously quantified. Furthermore, it is difficult to

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detect the parasites from blood smears with low parasitemia (Rampersad et al., 2003). Several serological assays, such as the indirect fluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), and the immunochromatographic test (ICT), have been developed for the detection of *T. equi*-specific antibodies (Hirata et al., 2002, 2005; Huang et al., 2004; Asgarali et al., 2007). However, these assays are also restricted due to their antibody-detected limitation and/or potential cross-reactivity to other pathogens (Papadopoulos et al., 1996; Allred, 2003). On the other hand, the polymerase chain reaction (PCR) assay has also been described as a molecular tool for the genomic detection of *Babesia* (*Theileria*) parasites (Nicolaiewsky et al., 2001; Rampersad et al., 2003). The sensitivity of the PCR assay is higher than that of the classical microscopic examination (Rampersad et al., 2003; Alhassan et al., 2007b). Although the PCR assays have the advantage of rapid identification of target pathogens, they rely on end-point measurements that are less appropriate for accurate quantification of the target sequence. Quantification of the infection load has been used to assess the disease severity and treatment outcome in previous studies (Francino et al., 2006; Price et al., 2006). To date, there had been no comprehensive studies relating this application to *T. equi* infection. Thus, a more efficient assay is needed to provide a rapid and quantitative result for the diagnosis of *T. equi* infection in horses.

The recent advent of the real-time quantitative PCR technique has proven useful in various applications, including pathogenic detection (Francino et al., 2006), gene expression and regulation (Follo et al., 2006), and allelic discrimination (Best et al., 2005). The real-time PCR assay is an alternative method to the conventional PCR assay, in which the real-time PCR utilizes a fluorogenic detection system for the continuous measurement of amplified products throughout the reaction (Heid et al., 1996). In the present study, we describe the development of a TaqMan real-time PCR assay to detect *T. equi* in equine blood samples. The use of this methodology will facilitate the quantitative diagnosis of *T. equi* in clinical laboratories.

2. Materials and methods

2.1. In vitro-cultured parasite

The U.S. Department of Agriculture strain of *T. equi* was grown in purified equine red blood cells (RBC) by using a previously established continuous microaerophilous stationary-phase culture system (Bork et al., 2003). The infected RBC were subjected to DNA extraction as described below and then used for the determination of the detection limit and generation of the quantitative standard curve in the constructed real-time PCR assay.

2.2. Field blood samples and DNA extraction

Sixty-five field blood samples were collected from horses living in Mato Grosso do Sul, Brazil ($n = 35$) and the Amasaman district, Ghana ($n = 30$). Genomic DNAs were extracted from the blood samples by using a QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions and then stored at −20 °C until use.

2.3. Real-time PCR primers and TaqMan probe

For the real-time PCR assay, a forward primer (Be18SF), a reverse primer (Be18SR), and a TaqMan probe (Be18SP) were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA) to specifically amplify the 18S rRNA gene of *T. equi* (GenBank accession numbers, Z15105, AY150062, AY150063, AY150064, AY534882, DQ287951). In detail, two primers, Be18SF (5′-GCGGTGT-TTCGGTGATTCATA-3′) and Be18SR (5′-TGA-TAGGTCAGAAACTTGAATGATACATC-3′), amplified an 81-bp DNA fragment in the target gene. The fluorescent TaqMan probe (Be18SP: 5′-AAATTAGC-GAATCGCATGGCTT-3′) was labeled at the 5′-end with 6-carboxyfluorescein reporter dye and at the 3′-end with 6-carboxy-tetramethylrhodamine quencher dye (Saunders, 2004). These primers and probe were obtained from a commercial laboratory (Applied Biosystems).

2.4. Real-time PCR assay

For the real-time PCR assay, a PCR mixture consisting of 1 μl of DNA template, 12 μl of TaqMan 2× universal PCR master mix (Applied Biosystems), 450 nM of each primer, and 250 nM of the TaqMan probe was prepared in 20 μl volume. The real-time PCR was performed in a 96-well optical plate (Applied Biosystems) under the following conditions: 2 min at 50 °C to achieve optimal AmpErase uracil-*N*-glycosylase activity, 10 min at 95 °C to achieve optimal AmpliTaq Gold DNA polymerase activity, and then 45 cycles of 20 s at 95 °C and 1 min at 55 °C. During the PCR amplification, an ABI Prism sequence detector (Applied Biosystems) monitored the real-time PCR amplification by quantitatively analyzing the fluorescence emissions (Saunders, 2004). The collected

fluorescence data was analyzed using the PE 7900 Sequence Detection System Software (Version 2.1; Applied Biosystems). Cross-contamination and false-negative and false-positive results were prevented by using plugged tips, performing the PCR in a separate room from that used for DNA extraction, and including a negative DNA control in each run. A positive control was also run for each master mix batch.

2.5. Evaluation of the real-time PCR assay

In order to determine the detection limit of the real-time PCR assay, *T. equi*-infected RBC with 1% parasitemia (1.5×10^5 parasites/μl) were subjected to 10-fold serial dilutions with non-infected RBC. After all dilutions of the infected RBC were conducted in duplicate, DNA was then extracted from each sample as described above. DNAs extracted from other equine protozoan parasites, including *Trypanosoma evansi* and *Babesia caballi*, were also prepared and subjected to the *T. equi* real-time PCR assay to evaluate the specificity. Negative-control DNA was obtained from the blood of non-infected healthy horse that had been bred at Obihiro University of Agriculture and Veterinary Medicine in Japan, a country in which no *T. equi* infection has been detected. On the other hand, field blood samples collected from horses living in Brazil and Ghana were screened using the *T. equi* real-time PCR assay in order to evaluate its ability as a diagnostic tool. The results of the real-time PCR assay were compared to those of a previously established nested PCR assay (Battsetseg et al., 2001). Clinical sensitivity was calculated as the "(number of true positives, real-time- and nested PCR-double positives)/(number of true positives + number of false negative, real-time PCR-negatives and nested PCR-positives)," while clinical specificity was calculated as the "(number of true negatives, real-time- and nested PCR-double negatives)/(number of true negatives + number of false positives, real-time PCR-positives and nested PCR-negatives)," as described previously (Perandin et al., 2004).

3. Results

Application of the TaqMan real-time PCR assay for the detection of *T. equi* resulted in successful amplification of the target gene. By means of this amplification, fluorescent labels enable the continuous monitoring of amplicon (PCR product) formation throughout the reactions. The detection limit of the *T. equi* real-time PCR assay was determined using the *in vitro*-cultured *T. equi*-infected RBC that had been prepared by 10-fold serial dilutions with non-infected RBC. Positive signals were observed only from DNAs extracted from the infected blood samples of 1.5×10^5–1.5×10^0 parasites/μl (Fig. 1A). Thus, the detection limit for this assay was determined to be 1.5 parasites/μl per sample. In contrast, no fluorescence signal was detected in the *T. equi* real-time PCR assay with DNAs of other protozoan parasites (*T. evansi* and *B. caballi*) and non-infected equine blood samples (data not shown). Furthermore, a linear standard curve was successfully generated in each real-time PCR run with a series of the 10-fold serially diluted infected blood samples of 1.5×10^5–1.5×10^0 parasites/μl ($R^2 = 0.9985$). In the *T. equi* real-time PCR assay, there was a significant linear relationship between the log numbers of *T. equi* and the mean cycle threshold ($P < 0.001$) (C_t, indicative of the target gene amount at which the fluorescence exceeds a preset threshold value obtained by enabling a standard calibration curve to be produced) (Fig. 1B).

To evaluate the field usefulness of the *T. equi* real-time PCR assay, we tested 65 field blood samples collected from horses living in Ghana ($n = 30$) and Brazil ($n = 35$). The results of the real-time PCR assay were compared to those of a previously established nested PCR assay that had been reported to exhibited complete specificity and high sensitivity (Battsetseg et al., 2002b). Of 65 field blood samples, 16 samples from Ghana (53.3%) and 30 samples from Brazil (85.7%) were *T. equi*-positive in the nested PCR assay, while the real-time PCR assay also detected the parasite in all 46 of the nested PCR-positive samples (100% sensitivity) but did not detect *T. equi* in the remaining 19 negative blood samples (100% specificity) (Table 1). The quantities of the positive field blood samples in the real-time PCR assay were calculated on the basis of the standard curve as described above (Fig. 1B). The quantity of parasite among the positive samples was estimated to be from 1.5×10^3 to 15×10^0 parasites/μl in the 16 samples collected from Ghana and from 1.5×10^4 to 1.5×10^0 parasites/μl in the 30 samples collected from Brazil (Table 2).

4. Discussion

In this report, we first describe the application of the TaqMan real-time PCR assay for the quantitative detection of *T. equi*. An important consideration for the successful development of a DNA amplification assay is possibly to identify an appropriate gene target. In the present study, the primers and TaqMan probe were designed for the *T. equi* 18S rRNA gene. The 18S rRNA gene is evolutionarily stable, with limited intra-species sequence variation (Criado et al., 2006). In our

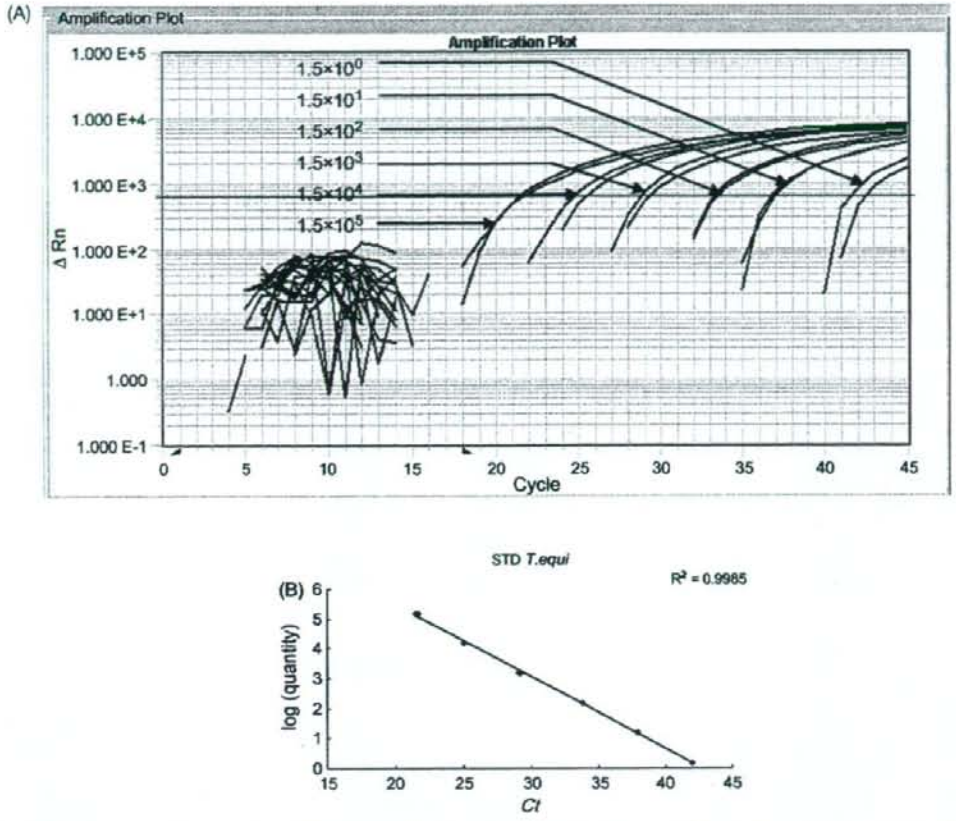

Fig. 1. Quantitative detection of *T. equi* in the TaqMan real-time PCR assay. (A) Amplification (as duplicate) of *T. equi* sample dilutions containing 1.5×10^{5}–1.5×10^{-4} parasites/μl of the infected blood. Amplification plots are observed only from 1.5×10^{5} to 1.5×10^{0} parasites/μl of the samples. (B) Standard curve for the quantification of the *T. equi* 18S rRNA gene. *T. equi* standard curve showing C_t values plotted versus the log of the initial 10-fold dilution series of parasite DNA equivalent to 1.5×10^{5}–1.5×10^{0} parasites/μl of the infected blood.

results, the specificity of the primers and probe targeting for the *T. equi* 18S rRNA gene was proven by successful amplification of the DNA product from the *in vitro*-cultured parasite, but not from other protozoan parasites, *B. caballi* and *T. evansi*, which are also known to infect horses (Brun et al., 1998; Alhassan et al., 2007b).

DNA amplification for the diagnostic detection of equine piroplasmosis has been known as a powerful tool

Table 1
Comparison of the real-time PCR assay with the nested PCR assay for the detection of *T. equi* from field blood samples collected from horses living in Ghana and Brazil

Real-time PCR			Number (%) with nested PCR results		Sensitivity	Specificity
Result		No. (%)	+	−		
+	Ghana	16 (53.3)	16 (53.3)	0		
	Brazil	30 (85.7)	30 (85.7)	0		
−	Ghana	14 (46.7)	0	14 (46.7)	100%	100%
	Brazil	5(14.3)	0	5 (14.3)		
Total	Ghana	30 (100)	16 (53.3)	14 (46.7)		
	Brazil	35 (100)	30 (85.7)	5 (14.3)		

Table 2
Quantities of *T. equi* in field equine blood collected from Ghana and Brazil

Quantity[a]	Number of infected cattle	
	Ghana	Brazil
1.5×10^4–1.5×10^3	0	1
1.5×10^3–1.5×10^2	1	11
1.5×10^2–1.5×10^1	10	12
1.5×10^1–1.5×10^0	5	6
Total	30	35

[a] The quantities are expressed as the number of *T. equi* parasites per microliter of infected blood.

both in the early phase of infection and in carrier animals (Rampersad et al., 2003). Although both nested and real-time PCR assays are useful for the analysis of clinical specimens and may achieve similar levels of specificity and sensitivity, the major advantages of the real-time PCR assay are not only its long linear range over at least 1.5 parasites/μl of infected blood but also its ability to quantify the infection load of clinical specimens. Moreover, the adaptability of this technique to a high-throughput 96-well format significantly reduces the overall time spent per sample in a clinical laboratory. Recently, various real-time PCR protocols have been developed for the specific detection of protozoan parasites (Saunders, 2004). Among them, multiplex real-time PCR assays were interestingly introduced, in which the assays can simultaneously detect more than 2 different species with different fluorescence probes (Perandin et al., 2004; Haque et al., 2007). In the future, multiplex real-time PCR assays for the simultaneous detection of *T. equi* and *B. caballi* would be desired because these two species often show an overlapping geographical distribution (Battsetseg et al., 2001; Alhassan et al., 2007b; Asgarali et al., 2007).

With the real-time PCR assay, we obtained novel epidemiological results suggestive of the presence of *T. equi* in field blood samples. This assay showed 100% clinical sensitivity and specificity in field blood samples when compared to results of a previously established nested PCR assay. The results indicate that the real-time PCR assay developed in this study can therefore be practically implemented as a diagnostic tool to detect *T. equi* in field blood samples. Previous studies have also shown that *T. equi* infection was widespread in horses living in Brazil and Ghana (Xuan et al., 2001b; Battsetseg et al., 2002b; Alhassan et al., 2007a). The authors reported that *T. equi* infection in Brazil showed high infection rates of 96 and 81% using the nested PCR and serodiagnostic methods, respectively (Xuan et al., 2001b; Battsetseg et al., 2002b), and that the *T. equi* infection in Ghana showed a rate of 60% using the PCR method (Alhassan et al., 2007a). Considering the high prevalence of *T. equi* infection in Brazil and Ghana, further studies are required to define the population dynamics of the infection. The quantitative analysis developed in the present study may be applied to compare different drug regimens and determine the prognostic value of treatment as well as to diagnose *T. equi* infection.

In summary, the present study is the first to develop a TaqMan real-time PCR assay for the diagnostic detection of *T. equi*. We demonstrated that the assay is a rapid and accurate method for quantifying *T. equi*. Additionally, the *T. equi* real-time PCR assay can test a large number of blood samples, and the results may be obtained in a short time. Therefore, the real-time PCR assay may be suitable for the routine screening of *T. equi* infection in clinical laboratories. We anticipate that future research will maximize the effectiveness of the present study for clarifying the epidemiology of equine piroplasmosis.

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References

Alhassan, A., Iseki, H., Kim, C., Yokoyama, N., Igarashi, I., 2007a. Comparison of polymerase chain reaction methods for the detection of *Theileria equi* infection using whole blood compared with pre-extracted DNA samples as PCR templates. Trop. Anim. Health Prod. 39, 369–374.

Alhassan, A., Thekisoe, O.M., Yokoyama, N., Inoue, N., Motloang, M.Y., Mbati, P.A., Yin, H., Katayama, Y., Anzai, T., Sugimoto, C., Igarashi, I., 2007b. Development of loop-mediated isothermal amplification (LAMP) method for diagnosis of equine piroplasmosis. Vet. Parasitol. 143, 155–160.

Allred, D.R., 2003. Babesiosis: persistence in the face of adversity. Trends Parasitol. 19, 51–55.

Asgarali, Z., Coombs, D.K., Mohammed, F., Campbell, M.D., Caesar, E., 2007. A serological study of *Babesia caballi* and *Theileria equi* in thoroughbreds in Trinidad. Vet. Parasitol. 144, 167–171.

Battsetseg, B., Lucero, S., Xuan, X., Claveria, F., Byambaa, B., Battur, B., Boldbaatar, D., Batsukh, Z., Khaliunaa, T., Battsetseg, G., Igarashi, I., Nagasawa, H., Fujisaki, K., 2002a. Detection of

equine *Babesia* spp. gene fragments in *Dermacentor nuttalli* Olenev 1929 infesting Mongolian horses and their amplification in egg and larval progenies. J. Vet. Med. Sci. 64, 727–730.

Battsetseg, B., Lucero, S., Xuan, X., Claveria, F.G., Inoue, N., Alhassan, A., Kanno, T., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2002b. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. Vet. Parasitol. 107, 351–357.

Battsetseg, B., Xuan, X., Ikadai, H., Bautista, J.L., Byambaa, B., Boldbaatar, D., Battur, B., Battsetseg, G., Batsukh, Z., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2001. Detection of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. Int. J. Parasitol. 31, 384–386.

Best, E.L., Fox, A.J., Frost, J.A., Bolton, F.J., 2005. Real-time single-nucleotide polymorphism profiling using Taqman technology for rapid recognition of *Campylobacter jejuni* clonal complexes. J. Med. Microbiol. 54, 919–925.

Bork, S., Yokoyama, N., Matsuo, T., Claveria, F.G., Fujisaki, K., Igarashi, I., 2003. Growth inhibitory effect of triclosan on equine and bovine *Babesia* parasites. Am. J. Trop. Med. Hyg. 68, 334–340.

Bose, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T., De vos, A.J., 1995. Current state and future trends in the diagnosis of babesiosis. Vet. Parasitol. 57, 61–74.

Brun, R., Hecker, H., Lun, Z.R., 1998. *Trypanosoma evansi* and *T. equiperdum*: distribution, biology, treatment, and phylogenetic relationship (a review). Vet. Parasitol. 79, 95–107.

Criado, A., Martinez, J., Buling, A., Barba, J.C., Merino, S., Jefferies, R., Irwin, P.J., 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. Vet. Parasitol. 142, 238–247.

De Waal, D.T., 1992. Equine piroplasmosis: a review. Br. Vet. J. 148, 6–14.

Follo, M.Y., Bosi, C., Finelli, C., Fiume, R., Faenza, I., Ramazzotti, G., Gaboardi, G.C., Manzoli, L., Cocco, L., 2006. Real-time PCR as a tool for quantitative analysis of PI-PLCbeta1 gene expression in myelodysplastic syndrome. Int. J. Mol. Med. 18, 267–271.

Francino, O., Altet, L., Sanchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Sanchez, A., Roura, X., 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. Vet. Parasitol. 137, 214–221.

Haque, R., Roy, S., Siddique, A., Mondal, U., Rahman, S.M., Mondal, D., Houpt, E., Petri Jr., W.A., 2007. Multiplex real-time PCR assay for detection of *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium* spp. Am. J. Trop. Med. Hyg. 76, 713–717.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real-time PCR quantitative PCR. Genome Res. 6, 986–994.

Hirata, H., Ikadai, H., Yokoyama, N., Xuan, X., Fujisaki, K., Suzuki, N., Mikami, T., Igarashi, I., 2002. Cloning of a truncated *Babesia equi* gene encoding an 82-kilodalton protein and its potential use in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 40, 1470–1474.

Hirata, H., Yokoyama, N., Xuan, X., Fujisaki, K., Suzuki, N., Igarashi, I., 2005. Cloning of a novel *Babesia equi* gene encoding a 158-kilodalton protein useful for serological diagnosis. Clin. Diagn. Lab. Immunol. 12, 334–338.

Holbrook, A.A., 1969. Biology of equine piroplasmosis. Am. J. Vet. Med. Assoc. 155, 453–454.

Huang, X., Xuan, X., Xu, L., Zhang, S., Yokoyama, N., Suzuki, N., Igarashi, I., 2004. Development of an immunochromatographic test with recombinant EMA-2 for the rapid detection of antibodies against *Babesia equi* in horses. J. Clin. Microbiol. 42, 359–361.

Mehlhorn, H., Schein, E., 1998. Redescription of Babesia equi Laveran. 1901 as Theileria equi. Parasitol. Res. 84, 467–475.

Nicolaiewsky, T.B., Richte, M.F., Lunge, V.R., Cunha, C.W., Delagostin, O., Ikuta, N., Fonseca, A.S., da Silva, S.S., Ozaki, L.S., 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. Vet. Parasitol. 101, 9–21.

Papadopoulos, B., Perie, N.M., Uilenberg, G., 1996. Piroplasms of domestic animals in the Macedonia region of Greece. 1. Serological cross-reactions. Vet. Parasitol. 63, 41–56.

Perandin, F., Manca, N., Calderaro, A., Piccolo, G., Galati, L., Ricci, L., Medici, M.C., Arcangeletti, M.C., Snounou, G., Dettori, G., Chezzi, C., 2004. Development of a real-time PCR assay for detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. J. Clin. Microbiol. 42, 1214–1219.

Price, R.N., Uhlemann, A.C., van Vugt, M., Brockman, A., Hutagalung, R., Nair, S., Nash, D., Singhasivanon, P., Anderson, T.J., Krishna, S., White, N.J., Nosten, F., 2006. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. Clin. Infect. Dis. 42, 1570–1577.

Rampersad, J., Cesar, E., Campbell, M.D., Samlal, M., Ammons, D.A., 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. Vet. Parasitol. 114, 81–87.

Roby, T.O., Anthony, D.W., 1963. The transmission of equine piroplasmosis by the tropical horse tick (*Dermacentor nitens* Neumann). Am. J. Vet. Res. 142, 768–769.

Saunders, N.A., 2004. Real-Time PCR: An Essential Guide. Horizon Bioscience Press, London.

Schein, E., 1988. Equine babesiosis. In: Rictic, M. (Ed.), Babesiosis of Domestic Animals and Man. CRC Press, Boca Raton, FL, pp. 197–209.

Stiller, D., Frerichs, W.M., Leatch, G., Kuttler, K.L., 1980. Transmission of equine babesiosis and bovine anaplasmosis by *Dermacentor albipictus* (Packard) (Acari: Ixodidae). J. N. Y. Entomol. Soc. 88, 75–76.

Xuan, X., Larsen, A., Ikadai, H., Tanaka, T., Igarashi, I., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 2001a. Expression of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 39, 705–709.

Xuan, X., Nagai, A., Battsetseg, B., Fukumoto, S., Makala, L.H., Inoue, N., Igarashi, I., Mikami, T., Fujisaki, K., 2001b. Diagnosis of equine piroplasmosis in Brazil by serodiagnostic methods with recombinant antigens. J. Vet. Med. Sci. 63, 1159–1160.

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Babesia: The protective effects of killed *Propionibacterium acnes* on the infections of two rodent *Babesia* parasites in mice

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Abstract

In the present study, we investigated the protective effects of killed *Propionibacterium acnes* on the infections of two rodent *Babesia* parasites in mice. Pre-treatment with "EqStim" (a commercially available immunostimulant containing killed *P. acnes*) showed significant resistance to both infections. To elucidate the immunological status in the mice, the concentrations of multiple cytokines were measured in serum collected from infected mice. After *B. microti* infection, the levels of interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12p70, and tumor necrosis factor (TNF)-α in the treated group were significantly lower than in the control group. In contrast, after *B. rodhaini* infection, only IL-12p70 and TNF-α were detectable at significantly higher levels in the treated group than in the control group. The present findings indicated the protective effects of killed *P. acnes* on rodent babesiosis even with different immune responses between the *B. microti* and *B. rodhaini* infections. Killed *P. acnes* might be a powerful tool for the control of serious livestock babesiosis.

Index Descriptors and Abbreviations: *P. acnes*, *Propionibacterium acnes*; *B. microti*, *Babesia microti*; *B. rodhaini*, *Babesia rodhaini*; TNF-α, tumor necrosis factor; IFN-γ, interferon; IL, interleukin; Cytokine; Mice

1. Introduction

Members of the genus *Babesia* belong to a diverse group of intraerythrocytic, tick-transmitted protozoa that infect a broad variety of vertebrate hosts (Krause, 2002). *Babesia* parasites destroy host erythrocytes and induce fever, anemia, and icterus in the infected animals (Irwin, 2002). The vaccine control of severe livestock babesiosis, such as bovine and equine babesiosis, has faced increasing failures (Bock et al., 1995). For example, because their wide use may put the parasites under further selection pressure, it may result in the possible appearance of emerging strains capable of evading the host immune responses (Dalrymple, 1992). Two kinds of rodent *Babesia* parasites, *Babesia microti* and *Babesia rodhaini*, have been used as experimental models to study the immunological responses for livestock babesiosis (Chen et al., 2000; Igarashi et al., 1999; Yokoyama et al., 2003). Mice infected with *B. microti* exhibit a transiently high parasitemia, but they subsequently recover from acute infection (Pastusiak et al., 2003). On the other hand, *B. rodhaini* causes a lethal infection in mice (Kamiya et al., 2005; Shimada et al., 1991).

An extract of killed *Propionibacterium acnes* has been reported to stimulate non-specific immunity to viral (Glasgow et al., 1977; Kirchner et al., 1977), bacterial (Adlam et al., 1972; Miyata et al., 1980), and protozoan (Clark et al., 1977; Nussenzweig, 1967) diseases. The protective effect of *P. acnes* pre-treatment on an intraerythrocytic protozoan infection was first reported by Nussenzweig (1967), who found that the pre-treated mice showed complete resistance against the challenge of *Plasmodium berghei* sporozoites. Additionally, the treated mice also exhibited a significant resistance to the subsequent challenge infection of *B. microti* on day 120 after the treatment (Clark et al., 1977). In *Babesia bigemina*, *P. acnes*-treated calves

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showed consistently lower parasitemia and less marked disease (Corrier and Wagner, 1984). At present, the immunomodulation of killed *P. acnes* is being used for the prophylaxis of respiratory diseases and the chemotherapy of chronic pulmonary inflammation in horses (Davis et al., 2003). However, the correct mechanism underlying this effect is poorly understood.

The present study was conducted to confirm that killed *P. acnes* has a preventive effect on babesiosis using the mouse models. First, we studied the effect of heat-killed *P. acnes* on *B. microti* infection based on parasitemia development. Next, the effect of "EqStim," which is a commercially available immunostimulant consisting of killed *P. acnes*, was studied on *B. microti* and *B. rodhaini* infections. Finally, we discussed the protective immunity of killed *P. acnes* against rodent babesiosis using the data of multiple cytokine responses and its possible utility for livestock babesiosis.

2. Materials and methods

2.1. Parasites and mice

The Munich strain of *Babesia microti* and the Australian strain of *B. rodhaini* were maintained by blood passage in mice (Nishisaka et al., 2001). Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Six-week-old mice were prepared at the beginning of the mouse experiments described below.

2.2. Propionibacterium acnes and EqStim

Propionibacterium acnes was purchased from ATCC (American Type Culture Collection, No. 6919). The bacterium was cultured under an aerobic condition for 2 days at 37 °C in a GAM medium (NISSUI, Tokyo, Japan) (Ninomiya et al., 1976). The bacterial pellet was collected, washed with phosphate-buffered saline (PBS), and resuspended in PBS. The bacterial suspension was treated at 80 °C for 30 min and then stored at −80 °C until use (Kuwahara et al., 2005). A commercially available immunostimulant EqStim, consisting of killed *P. acnes*, was purchased from the NEOGEN Corporation (Lexington, KY, USA).

2.3. Mouse experiments

Approximately 6 weeks old mice were injected intraperitoneally (i.p.) with 0.4 mg of the heat-killed *P. acnes* or EqStim diluted with PBS and then challenged with 1×10^7 *B. microti* or *B. rodhaini*-infected erythrocytes at the indicated interval. In an initial experiment, a total of 40 mice were divided into 8 groups. The first to fourth groups were inoculated (i.p.) with the heat-killed *P. acnes* and challenged with *B. microti* at weekly intervals (second group; 2 weeks, third group; 3 weeks, fourth group; 4 weeks) except the first group. Fifth to seventh groups were challenged with *B. microti* as positive control at the indicated interval (fifth group; 2 weeks, sixth group; 3 weeks, seventh group; 4 weeks), and eighth group was inoculated PBS instead of the heat-killed *P. acnes*. On the other hands, a total of 48 mice were also divided into 6 groups in the second experiment. The first, second, and third group were inoculated (i.p.) with the EqStim and then challenged with *B. microti* or *B. rodhaini* at 3 weeks interval except the first group. Fourth and fifth group were challenged as positive control, and sixth group was inoculated with PBS instead of the EqStim as negative control. After the challenge infection, Giemsa-stained thin blood smears were prepared from the tail veins every 2 days (*B. microti*) or daily (*B. rodhaini*), and the parasitemia and survival rate were monitored for a total of 35 days or until the death of the mice. The serum fraction was also prepared from the collected blood. All mouse experiments were conducted in accordance with the Standard Relating to the Cage and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

2.4. Cytokine analyses

Th1 and Th2 cytokines, including interleukin (IL)-2, IL-4, IL-5, IL-10, IFN-γ, and inflammatory cytokines, including IL-6, IL-12p70, and tumor necrosis factor (TNF)-α, were simultaneously quantified from the collected sera using mice Th1/ Th2 cytokine and inflammatory cytometric bead array (CBA) kits (BD Biosciences Pharmingen, San Diego, CA, USA). These kits provided a mixture of 8 microbead populations with distinct fluorescent intensities (FL-3); the populations were pre-coated with capture antibodies specific for each cytokine. Fifty microliters of each serum diluted 10 times with PBS was added into the pre-mixed microbeads in a 12 mm × 75 mm Falcon tube (BD Biosciences Pharmingen). After the addition of 50 μl of phycoerythrin-conjugated antibodies against the cytokines, the mixture was incubated for 3 h in the dark at room temperature. This mixture was washed by centrifuging at 500*g* for 5 min, and the pellet was resuspended in 300 μl of a washing buffer (BD). The FACSCalibur flow cytometer (BD Biosciences Pharmingen) was calibrated with setup beads, and 3000 events were acquired for each sample. The concentration of individual cytokines was indicated by their fluorescent intensities (FL-2) using the standard reference curve of the CBA software (BD Biosciences Pharmingen).

2.5. Statistical analysis

The survival rates of the *B. rodhaini*-infected mice were plotted according to a Kaplan–Meier method, and the statistical significance in the survival rate was analyzed among the groups by the generated Wilcoxon test. Differences in parasitemia were analyzed using an independent Student's *t*-test. In the cytokine analyses, the independent Student's

t-test was used to assess differences between the groups. Differences were accepted as significant at a level of $P < 0.05$.

3. Results

3.1. Protective effect of heat-killed P. acnes on B. microti infection in mice

As the initial experiment, mice (5 for each group) were injected i.p. with heat-killed *P. acnes* and then challenged with non-lethal *B. microti* at 2 or 4 weeks after the treatment, as shown in Fig. 1. In control mice that were only infected with the parasite, the parasitemia reached 40–60% on day 7–10 after the infection and then declined. Significant protection was achieved in the pre-treated group in 4 weeks after the treatment, as determined by the development of parasitemia on days 6 ($P = 0.00037$), 8 ($P = 0.00004$), and 10 ($P = 0.00005$) after the infection, when the parasites were hardly observed in the mice (Fig. 1B). In contrast, the mice in the 2-week interval experiment did not show any protection against the challenge infection (Fig. 1A).

3.2. Protective effects of EqStim on B. microti and B. rodhaini infections in mice

The second experiments were performed to test the protective effects of EqStim on *B. microti* and *B. rodhaini* infections by altering the interval between the pre-treatment and challenge infection to 3 weeks. In the *B. microti* experiment (8 mice per group), a significant difference in parasitemia development was reproduced on days 4 ($P = 0.00582$), 6 ($P = 0.00566$), 8 ($P = 0.00411$), and 10 ($P = 0.01962$) after the infection (Fig. 2A). In the *B. rodhaini* experiment (10 mice per group), all of the control mice showed high parasitemia and then died within 3–5 days, whereas 2 mice were protected against the lethal *B. rodhaini* infection (Fig. 2B). In the 2 surviving mice, the maximum parasitemia did not exceed 45%, and the parasites were undetected by thin blood smears after the 11th day. The survival rate indicated a significant difference between the pre-treated and control groups ($P = 0.00065$).

3.3. Different immune responses between B. microti and B. rodhaini infections in the treated mice

To evaluate the immune responses of EqStim inoculation, 8 kinds of cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF-α, and IFN-γ) were measured in the serum that had been collected from mice inoculated with EqStim immediately before the challenge infection. Significant differences were observed only in the concentrations of IL-6 ($P = 0.01936$), IL-10 ($P = 0.00048$), and TNF-α ($P = 0.00131$) between the EqStim-treated and control groups (average ± standard deviations; 72.16 ± 10.46 and 17.21 ± 12.26, 162.06 ± 29.90 and 37.36 ± 32.47, 321.09 ± 153.21 and 55.42 ± 68.17, respectively), in which significant increases of these cytokines were observed in the treated mice (Fig. 3A). After infection of *B. microti* and *B. rodhaini*, serum samples were also collected on days 8 and 4 after the infections, respectively, to measure the cyto-

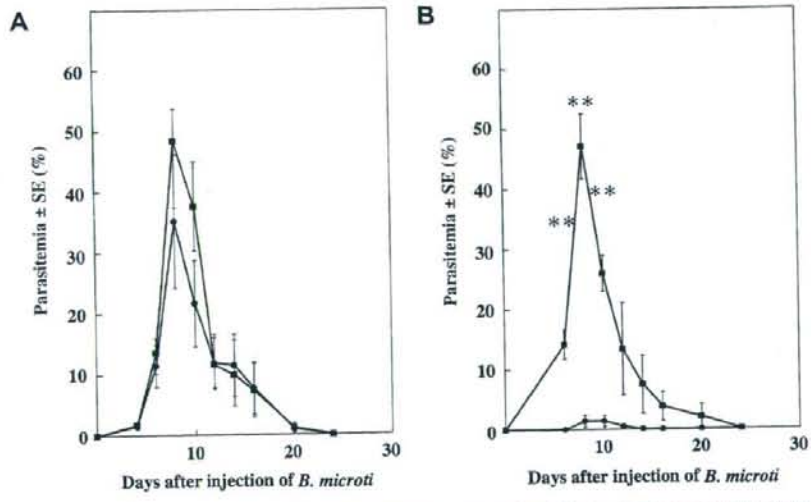

Fig. 1. Protective effect of heat-killed *P. acnes* on *B. microti* infection in mice. Parasitemia of *B. microti* was monitored in the infected mice at different intervals of 2 (A) or 4 (B) weeks between pre-treatment with 0.4 mg heat-killed *P. acnes* and subsequent infection with 1×10^7 of *B. microti*-infected erythrocytes. The values of the P. *acnes*-treated and non-treated control groups are shown as ● and ■, respectively, in both panels. Each value represents mean ± standard error (SE). The asterisks indicate significant differences between the pre-treated and control groups ($^*P < 0.05$, $^{**}P < 0.01$).

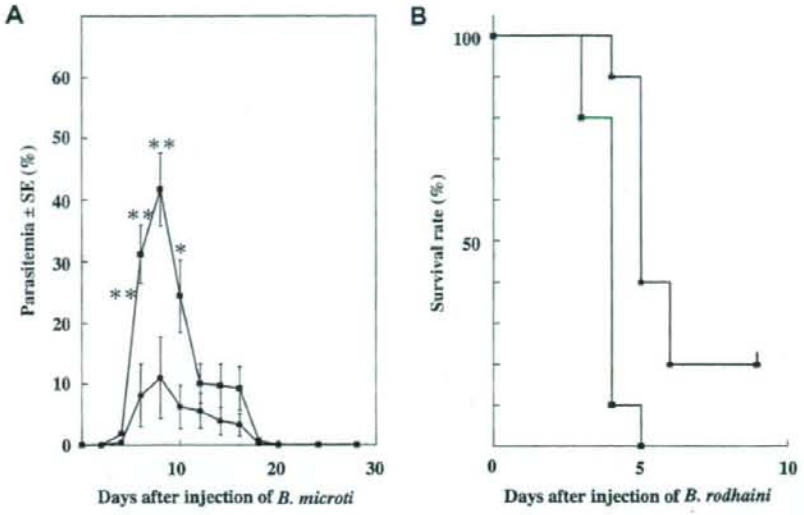

Fig. 2. Protective effects of EqStim on *B. microti* and *B. rodhaini* infections in mice. Parasitemia in the *B. microti*-infected mice (A) and survival rates in the *B. rodhaini*-infected mice (B) are shown after infections at 3-week intervals between pre-treatment with 0.4 mg EqStim and subsequent infection with 1×10^7 of the infected erythrocytes. The values of the EqStim-treated and non-treated control groups are shown as ● and ■, respectively. (A) Each value of parasitemia represents mean ± SE. The asterisks indicate significant differences between the pre-treated and control groups ($^*P < 0.05$, $^{**}P < 0.01$). (B) A significant difference between the pre-treated and control groups was determined in the survival rates ($P < 0.05$).

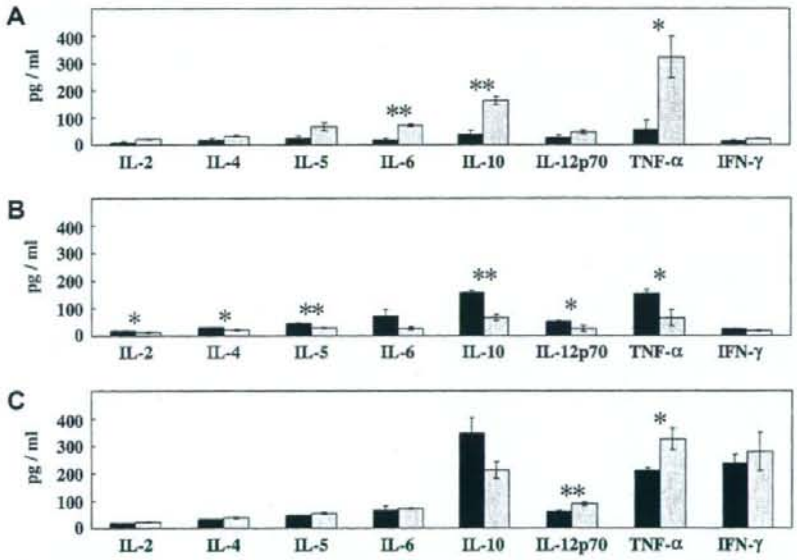

Fig. 3. Concentrations of multiple cytokines in serum collected from EqStim pre-treated and control mice. The serum samples were collected from pre-treated (gray square) or control (black square) mice at 3 weeks after inoculation (A) and, subsequently, on days 8 (*B. microti*; B) and 4 (*B. rodhaini*; C) after the challenge infections. The concentrations of 8 mouse cytokines were determined by CBA kits using flow cytometry. Data represent means ± SE of 4 mice per group and are representative of two separate experiments. The asterisks indicate significant differences between the pre-treated and control groups ($^*P < 0.05$, $^{**}P < 0.01$).

kine concentrations. In the *B. microti* infection, 6 cytokine levels, IL-2 ($P = 0.02068$), IL-4 ($P = 0.03674$), IL-5 ($P = 0.00118$), IL-10 ($P = 0.00052$), IL-12p70 ($P = 0.01075$), and TNF-α ($P = 0.03654$) were significantly lowered in the pre-treated group with EqStim than in the control group (average ± standard deviations in treated

and control group; 14.08 ± 2.94 and 19.15 ± 1.40, 22.83 ± 5.64 and 31.15 ± 2.61, 29.87 ± 3.47 and 45.16 ± 4.50, 66.35 ± 19.95 and 156.31 ± 17.81, 38.06 ± 7.66 and 62.13 ± 10.75, 64.9 ± 58.62 and 152.45 ± 28.87, respectively) (Fig. 3B). On the other hand, in the case of the *B. rodhaini* infection, only the levels of IL-12p70 ($P = 0.00931$) and TNF-α ($P = 0.03130$) were significantly higher in the pre-treated group than in the control group (average ± standard deviations in treated and control group; 89.17 ± 13.01 and 60.51 ± 7.87, 324.06 ± 79.00 and 209.25 ± 22.34, respectively) (Fig. 3C), while there was no significant difference among the groups regarding the other 6 cytokine levels.

4. Discussion

In the present study, we demonstrated that pre-treatment with killed *P. acnes* protects susceptible BALB/c mice from severe infections with *B. microti* and *B. rodhaini*. This protective effect was achieved at an interval of at least 3–4 weeks. This finding is consistent with a previous report in which mice pre-treated with killed *P. acnes* showed a significant resistance to the subsequent infection with *B. microti* (Clark et al., 1977). Surprisingly, they achieved protection at an interval of 120 days. On the other hand, one inoculation of EqStim showed 20% protection against challenge infection with 1×10^7 *B. rodhaini* which cause lethal infection with a small number of parasites. Therefore, there is a possibility that EqStim will enhance more strong protection against challenge infection by changing of inoculation times or inoculation intervals of this drug.

To assess the *in vivo* cytokine productions by EqStim treatment, we determined the cytokine concentrations in the serum collected from treated mice before the challenge infection. Significant increases in IL-6, IL-10, and TNF-α were detected in the serum samples of treated mice. TNF-α has an inflammation-promoting activity (Zheng and Atherton, 2005) and is often upregulated in the early stage of various diseases (Biron, 2001). The increase of the TNF-α level might be due to a non-specific reaction against the injected *P. acnes*. The DNA sequence of *P. acnes* contains repetitive unmethylated cytosine guanine (CpG) sequences, which may be responsible for its immunostimulatory activity (Buck and Kelly, 1980; Megid and Kaneno, 2000; Pisetsky, 1996; Sakao et al., 1999; Verthelyi et al., 2001). Signaling of the CD40 ligand due to the binding of CpG sequences results in the synergistic production of IL-6, IL-10, and IL-12 for the activation of innate immunity (Wagner et al., 2004). In the EqStim-treated group, increased production of IL-12p70 was not statistically detected, but the value was higher than that of the control group. These cytokine responses support the hypothesis that *P. acnes* induces the activation of innate immunity due to the CpG motif.

Interestingly, after the subsequent challenge infections, different immune responses were observed between *B. microti* and *B. rodhaini* infections. On day 8 after the *B. microti* infection, which showed the highest parasitemia, IL-2, IL-4, IL-5, IL-10, IL-12p70, and TNF-α were significantly controlled at lower levels in the treated group than in the control group. Consequently, the cytokines were scarcely detected at lower levels, suggesting that pre-treatment with *P. acnes* led to the suppression of activity but the excess responses were possibly based on Th1 and Th2 immunity during the infectious development. The suppression might be helpful for the effective eradication of *B. microti* in the mouse body. After the animals clear the infections, the levels of cytokines; IL-2, IL-4, IL-10, and IL-12p70 were high on day 28 and 35 compared with those on day 8, significant difference was not found among these cytokine levels between treated and untreated groups in recovery stage. These findings suggest that suppression of increasing the parasites in early stage is important rather than in recovery stage on resistance for *B. microti* infection.

On the other hand, on day 4 after *B. rodhaini* infection, IL-12p70 and TNF-α were detected at significantly higher levels in the treated group than in the control group. Since expression of IL-12 and TNF-α indirectly induces the activation of macrophage and natural killer cells, these cells might play a critical role in the control of *B. rodhaini*. Understanding the different immune responses between two kinds of rodent babesial infections would be useful for developing successful immunological responses to livestock babesiosis.

The present findings indicated the protective effects of killed *P. acnes* on rodent babesiosis even though different immune responses for the *B. microti* and *B. rodhaini* infections were obtained. Killed *P. acnes* might be a powerful tool for the control of serious livestock babesiosis in the future.

Acknowledgments

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References

Adlam, C., Broughton, E.S., Scott, M.T., 1972. Enhanced resistance of *Corynebacterium parvum*-treated mice to infection by bacteria. J. Med. Microbiol. 5, Pvii.

Biron, C.A., 2001. Interferons and other cytokines. In: Knipe, D.M., Howley Fields, P.M., (Eds.), Virology, pp. 321–351.

Bock, R.E., de Vos, A.J., Lew, A., Kingston, T.G., Fraser, I.R., 1995. Studies on failure of T strain live *Babesia bovis* vaccine. Aust. Vet. J. 72, 296–300.

Buck, G.E., Kelly, M.T., 1980. Investigation of the component of *Propionibacterium acnes* (*Corynebacterium parvum*) responsible for macrophage activation. Infect. Immun. 27, 620–627.

Chen, D., Copeman, D.B., Burnell, J., Hutchinson, G.W., 2000. Helper T cell and antibody responses to infection of CBA mice with *Babesia microti*. Parasite Immunol. 22, 81–88.

Clark, I.A., Cox, F.E., Allison, A.C., 1977. Protection of mice against Babesia spp. and Plasmodium spp. with killed *Corynebacterium parvum*. Parasitology 74, 9–18.

Corrier, D.E., Wagner, G.G., 1984. The protective effect of pretreatment with killed *Corynebacterium parvum* against acute babesiosis in calves. Vet. Parasitol. 15, 165–168.

Dalrymple, B.P., 1992. Diversity and selection in *Babesia bovis* and their impact on vaccine use. Parasitol. Today 8, 21–23.

Davis, E.G., Rush, B.R., Blecha, F., 2003. Increases in cytokine and antimicrobial peptide gene expression in horses by immunomodulation with *Propionibacterium acnes*. Vet. Ther. 4, 5–11.

Glasgow, L.A., Fischbach, J., Bryant, S.M., Kern, E.R., 1977. Immunomodulation of host resistance to experimental viral infections in mice: effects of *Corynebacterium acnes*, *Corynebacterium parvum*, and Bacille calmette-guerin. J. Infect. Dis. 135, 763–770.

Igarashi, I., Suzuki, R., Waki, S., Tagawa, Y., Seng, S., Tum, S., Omata, Y., Saito, A., Nagasawa, H., Iwakura, Y., Suzuki, N., Mikami, T., Toyoda, Y., 1999. Roles of CD4(+) T cells and gamma interferon in protective immunity against *Babesia microti* infection in mice. Infect. Immun. 67, 4143–4148.

Irwin, P.J., 2002. Companion animal parasitology: a clinical perspective. Int. J. Parasitol. 32, 581–593.

Kamiya, S., Takabatake, N., Fujii, K., Okamura, M., Iseki, H., Yokoyama, N., Igarashi, I., 2005. Protective immunity of blood plasma fraction containing *Babesia rodhaini* exoantigens in mice. J. Protozool. Res. 15, 29–40.

Kirchner, H., Hirt, H.M., Munk, K., 1977. Protection against herpes simplex virus infection in mice by *Corynebacterium parvum*. Infect. Immun. 16, 9–11.

Krause, P.J., 2002. Babesiosis. Med. Clin. North Am. 86, 361–373.

Kuwahara, K., Kitazawa, T., Kitagaki, H., Tsukamoto, T., Kikuchi, M., 2005. Nadifloxacin, an antiacne quinolone antimicrobial, inhibits the production of proinflammatory cytokines by human peripheral blood mononuclear cells and normal human keratinocytes. J. Dermatol. Sci. 38, 47–55.

Megid, J., Kaneno, R., 2000. Natural killer activity in mice infected with rabies virus and submitted to *P. acnes* (*Propionibacterium acnes*) as immunomodulator. Comp. Immunol. Microbiol. Infect. Dis. 23, 91–97.

Miyata, H., Nomoto, K., Takeya, K., 1980. Characteristics of resistance to Listeria monocytogenes enhanced by *Corynebacterium parvum* in mice. Immunology 40, 33–39.

Ninomiya, K., Kobata, S., Imamura, H., Mochizuki, I., Watanabe, K., 1976. Antibacterial and bactericidal activity of tinidazole against anaerobic bacteria comparing with metronidazole (author's transl.). Jpn. J. Antibiot. 29, 325–331.

Nishisaka, M., Yokoyama, N., Xuan, X., Inoue, N., Nagasawa, H., Fujisaki, K., Mikami, T., Igarashi, I., 2001. Characterization of the gene encoding a protective antigen from *Babesia microti* identified it as eta subunit of chaperonin containing T-complex protein 1. Int. J. Parasitol. 31, 1673–1679.

Nussenzweig, R.S., 1967. Increased nonspecific resistance to malaria produced by administration of killed *Corynebacterium parvum*. Exp. Parasitol. 21, 224–231.

Pastusiak, K., Konopka, E., Doligalska, M., Sinski, E., 2003. *Babesia microti*: parasitemia and antibody responses to primary and challenge infections in BALB/c mice. Folia Parasitol. (Praha) 50, 237–239.

Pisetsky, D.S., 1996. Immune activation by bacterial DNA: a new genetic code. Immunity 5, 303–310.

Sakao, Y., Takeda, K., Tsutsui, H., Kaisho, T., Nomura, F., Okamura, H., Nakanishi, K., Akira, S., 1999. IL-18-deficient mice are resistant to endotoxin-induced liver injury but highly susceptible to endotoxin shock. Int. Immunol. 11, 471–480.

Shimada, T., Igarashi, I., Maki, Y., Claveria, F.G., Saito, A., Suzuki, N., 1991. Cellular subsets involved in protective immunity to *Babesia rodhaini* infected in BALB/c mice. J. Protozool. Res. 1, 35–44.

Verthelyi, D., Ishii, K.J., Gursel, M., Takeshita, F., Klinman, D.M., 2001. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. J. Immunol. 166, 2372–2377.

Wagner, M., Poeck, H., Jahrsdoerfer, B., Rothenfusser, S., Prell, D., Bohle, B., Tuma, E., Giese, T., Ellwart, J.W., Endres, S., Hartmann, G., 2004. IL-12p70-dependent Th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA. J. Immunol. 172, 954–963.

Yokoyama, N., Bork, S., Nishisaka, M., Hirata, H., Matsuo, T., Inoue, N., Xuan, X., Suzuki, H., Sugimoto, C., Igarashi, I., 2003. Roles of the Maltese cross form in the development of parasitemia and protection against *Babesia microti* infection in mice. Infect. Immun. 71, 411–417.

Zheng, M., Atherton, S.S., 2005. Cytokine profiles and inflammatory cells during HSV-1-induced acute retinal necrosis. Invest. Ophthalmol. Vis. Sci. 46, 1356–1363.

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Babesia gibsoni: Serodiagnosis of infection in dogs by an enzyme-linked immunosorbent assay with recombinant BgTRAP

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Abstract

The thrombospondin-related adhesive protein of *Babesia gibsoni* (BgTRAP) is known as an immunodominant antigen and is, therefore, considered as a candidate for the development of a diagnostic reagent for canine babesiosis. The recombinant BgTRAP (rBgTRAP) expressed in *Escherichia coli* was tested in an enzyme-linked immunosorbent assay (ELISA) for detecting antibodies to *B. gibsoni* in dogs. The ELISA with rBgTRAP clearly differentiated between *B. gibsoni*-infected dog sera and specific pathogen-free (SPF) dog sera. The sera collected from dogs experimentally infected with closely related parasites, *B. canis canis*, *B. canis vogeli*, *B. canis rossi*, and *Neospora caninum*, showed no cross-reactivity by the ELISA with rBgTRAP. A total of 107 blood samples collected from dogs that had been diagnosed as having babesiosis at veterinary hospitals in Japan were examined for the diagnosis of *B. gibsoni* infection by the ELISA and PCR. Ninety-six (89.7%) and 89 (83.2%) of the tested samples were positive by the ELISA and PCR, respectively, while 11 (10.3%) and 4 (3.7%) were ELISA+/PCR− and ELISA−/PCR+, respectively. In addition, the sensitivity of the ELISA with rBgTRAP was much higher than that of previously established ELISAs with rBgP50, rBgSA1, and rBgP32. These results indicate that the rBgTRAP is the most promising diagnostic antigen for the detection of an antibody to *B. gibsoni* in dogs and that the combined ELISA/PCR approach could provide the most reliable diagnosis for clinical sites.

Index Descriptors and Abbreviations: *Babesia gibsoni*; Dogs; Diagnosis; ELISA, enzyme-linked immunosorbent assay; TRAP, thrombospondin-related adhesive protein

1. Introduction

Babesia gibsoni is a tick-borne apicomplexan parasite that causes piroplasmosis in dogs. The disease, which is characterized by remittent fever, progressive anemia, hemoglobinuria, and marked splenomegaly as well as hepatomegaly, sometimes causes death (Wozniak et al., 1997). The infection is endemic in many regions of Asia, Africa, Europe, and America. In addition, molecular phylogenetic analysis based on genetic sequencing has revealed that there are at least three distinct subtypes or subspecies of small piroplasms: a classic Asian *B. gibsoni* mainly present in Japan, a small *Babesia* related to *Theileria annae* identified in Spain and a small-unnamed organism identified in California (Boozer and Macintire, 2003; Birkenheuer et al., 2003). In general, *B. gibsoni*-infection is characterized by recurrent infections even after treatment with antibabesia drugs (Miyama et al., 2006), necessitating the development of effective diagnostic methods and vaccines to prevent this disease.

The diagnostic method of *B. gibsoni* infection is mainly represented by the detection of small intraerythrocytic *Babesia* organisms in a microscopic examination of Giemsa-

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stained thin blood smear films. The microscopic examination method is simple, easy, and appropriate for the diagnosis of *B. gibsoni* infection in the acute stage. However, this method is difficult for the detection of *B. gibsoni* organisms in unapparent or chronic infections because of low levels of parasitemia (Verdida et al., 2004). Recently, the polymerase chain reaction (PCR) has been used to diagnose *B. gibsoni* infection with high specificity and sensitivity (Fukumoto et al., 2001; Bose et al., 1995; Birkenheuer et al., 2003). Since the introduction of PCR, *B. gibsoni* infection with low parasitemia has been easily diagnosed (Inokuma et al., 2005). Therefore, *B. gibsoni*-specific PCR is a useful method for the diagnosis of *B. gibsoni* infection in the acute stage. However, the test requires skilled personnel and specialized laboratory, moreover, analysis of large numbers of samples is usually tedious. Alternatively, the enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT) with infected erythrocytes as an antigen have been used for the serological diagnosis of *B. gibsoni* infection (Bose et al., 1995). These tests are useful for the identification of chronically infected dogs with significantly low parasitemia. However, it is possible that the use of whole parasites or parasite-derived antigens may result in a false-positive due to cross-reaction with other closely related parasites, such as *B. canis*. Hence, it is necessary to develop a reliable, sensitive, and specific diagnostic test using *B. gibsoni*-specific antigens.

Thrombospondin-related adhesive proteins (TRAPs) are a conserved family identified in several apicomplexan parasites. A number of studies have suggested that the TRAP protein plays a crucial role in host cell invasion by parasites (Baum et al., 2006; Gaffar et al., 2004; Naitza et al.; 1998). Recently, Zhou et al. (2006) identified the TRAP of *B. gibsoni* (BgTRAP) as an immunodominant antigen. In addition, the recombinant antigens namely, rBgP50, rBgSA1, and rBgP32 have been identified from *B. gibsoni* merozoite in our laboratory (Verdida et al., 2004; Jia et al., 2006; Aboge et al., 2007). Although the antigens have shown promising results while using sera from *B. gibsoni*-naturally infected dogs, their sensitivities have not achieved perfect result. Therefore, we believe that the sensitivities of the tests can be improved by developing new candidate recombinant antigens.

In this regard, we expressed the recombinant BgTRAP (rBgTRAP) in *Escherichia coli* and then evaluated its diagnostic potential in an ELISA using 107 blood samples collected from dogs that were clinically diagnosed as having babesiosis at veterinary hospitals in Japan. In addition, we compared the results of the ELISA with those of a seminested PCR.

2. Materials and methods

2.1. Parasite

Babesia gibsoni isolated from a hunting dog of Hyogo Prefecture, Japan, designed as the NRCPD strain, was maintained in splenectomized beagles as described earlier (Fukumoto et al., 2001). The *B. gibsoni*-infected dog erythrocytes were collected from the experimentally infected dog at peak parasitemia (14%) and stored at −80 °C.

2.2. Expression and purification of rBgTRAP

The open reading frame (ORF) of the BgTRAP gene in the pBluescriptSK(+) vector was subcloned into a pGEM-4T-3 *E. coli* expression vector (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting plasmid was checked for accurate insertion by sequencing. The BgTRAP gene was expressed as a glutathione *S*-transferase (GST)-fusion protein in the *E. coli* BL21 (DE3) strain according to the manufacturer's instructions (Amersham Pharmacia Biotech). The resulting *E. coli* cells were washed three times with phosphate-buffered saline (PBS), lysed in 1% Triton X-100–PBS, sonicated, and then centrifuged at 12,000*g* for 10 min at 4 °C. Supernatants containing the soluble rBgTRAP were purified with glutathione–Sepharose 4B beads according to the manufacturer's instructions (Amersham Pharmacia Biotech). The expression of the recombinant protein was confirmed by performing SDS–PAGE using 12% as described previously (Zhou et al., 2006).

2.3. Expression and purification of rBgP50, rBgSA1, and rBgP32

A series of recombinant antigens of *B. gibsoni*, rBgP50, rBgSA1, and rBgP32, was expressed in *E. coli* and purified according to previously described methods (Verdida et al., 2004; Jia et al., 2006; Aboge et al., 2007).

2.4. ELISA

The ELISA with rBgTRAP was essentially carried out according to a previously described method (Fukumoto et al., 2004). rBgTRAP (50 ng/well) and GST (50 ng/well) in an antigen coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6) were coated on 96-well microplates (Nunc, Denmark) for overnight at 4 °C and then the plate was blocked with a 3% (w/v) skimmed milk solution for 1 h at 37 °C. After washing, the plate was incubated with serum samples at a dilution 1:200. The second antibody was detected by treatment with horseradish peroxidase-conjugated (BETHYL, Laboratories, INC) anti-dog IgG (1:5000) and ABTS [2,2′-azinobis (3-ethylbenzthiazoline sulfonic acid)] (Sigma). The color was allowed to develop at room temperature. The optical density (OD) was measured using the MTP-500 microplate reader (Corona Electric, Tokyo, Japan) at 415 nm. The ELISA titer was expressed as the reciprocal of the maximum dilution that showed an ELISA value equal to or greater than 0.18, which is the difference in absorbance between that for the antigen (GST–BgTRAP) well and that for the control antigen (GST) well. The OD value (0.18) was calculated by

obtaining the mean OD value plus 3-fold standard deviations of 25 specific pathogen-free (SPF) dog sera.

The ELISAs with rBgP50, rBgSA1, and rBgP32 were performed as described previously (Verdida et al., 2004; Jia et al., 2006; Aboge et al., 2007).

2.5. PCR

DNA was extracted from canine whole-blood samples using the QIAamp DNA Blood Mini-Kit according to the manufacturer's instructions. A seminested PCR was performed to detect the parasite DNA using a method described by Birkenheuer et al. (2003). Amplification was performed using 50-μl reactions. Each 50-μl reaction contained a 1× concentration of PCR buffer (Roche), 2.5 U of Taq polymerase, 2 μl of DNA template, 10 pmol of each primer, 2 mM concentration of each deoxynucleoside triphosphate. An outer primer pair (455–479F; GTCTTGTAATTGGA ATGATGGTGAC and 793–772R; ATGCCCCCAACCG TTCCTATTA) was designed to amplify approximately ~340 bp fragment from *B. gibsoni* (Asian genotype) and specific internal primer (BgibAsia-F; ACTCGGCTACTTGC CTTGTC) was designed, and paired with the outer reverse primer in the seminested secondary reaction to amplify *B. gibsoni*-specific 185-bp amplicons. The PCR conditions were as follows; initial denaturation at 95 °C for 5 min, followed by 50 amplification cycles (95 °C for 45 s, 58 °C for 45 s, and 72 °C for 45 s), and a final extension step at 72 °C for 5 min. And the reaction was performed using the specific internal primer and the outer reverse primer mentioned above and the amplicon from the initial reaction was used as the DNA template. The reaction conditions were the same as above and amplification was performed for 30 cycles. The PCR products were electrophoresed in 1% agarose gel, stained with ethidium bromide and visualized under UV-transillumination.

2.6. Sera and blood samples

In this study, we used 182 sera and 136 blood samples in total. In detail, the canine serum samples used for the evaluation of the ELISA with rBgTRAP were as follows: 10 sera from dogs experimentally infected with *B. gibsoni*; 25 sera from specific pathogen-free (SPF) dogs; three sera from dogs experimentally infected with *B. canis canis*; two sera from dogs experimentally infected with *B. canis vogeli*; three sera from dogs experimentally infected with *B. canis rossi*; three sera from dogs experimentally infected with *Neospora caninum*. In addition, 107 sera and blood samples from dogs with anemic symptoms from 13 veterinary hospitals of seven prefectures in Japan (Hyogo and Wakayama prefectures in Honshu; Tokushima and Kagawa prefectures in Shikoku; Miyazaki, Ooita, and Kagoshima prefectures in Kyushu); and 29 sera and blood samples from dogs in non-endemic area of Japan were used for evaluation of ELISA with rBgTRAP and the detection of *B. gibsoni* organisms in peripheral blood by PCR, respectively.

3. Results

3.1. Evaluation of the ELISA with rBgTRAP

To evaluate whether the rBgTRAP expressed in *E. coli* can be used as a suitable antigen for the diagnosis of the *B. gibsoni* infection, the purified rBgTRAP was tested in an ELISA using sera from dogs experimentally infected with *B. gibsoni* and other closely related protozoan parasites. As shown in Fig. 1, 10 sera from dogs infected with *B. gibsoni* were positive (lane 1, optical density > 0.18), whereas 25 sera from SPF dogs (lane 2), three sera from dogs infected with *B. canis canis* (lane 3), two sera with *B canis vogeli* (lane 4), three sera with *B. canis rossi* (lane 5), three sera with *N. caninum* (lane 6), or 29 sera from dogs in non-endemic areas of Japan (lane 7) were negative (optical density < 0.18). As shown in Fig. 2, a dog experimentally infected with *B. gibsoni* developed high titer of antibody response to BgTRAP from eight days post-infection. The high antibody titer was maintained until 541 days post-infection even when the dog became chronically infected with low level of parasitemia.

3.2. Diagnosis of B. gibsoni infection in dogs by the ELISA with rBgTRAP and PCR

A total of 107 blood samples collected from dogs that were diagnosed as having babesiosis at veterinary hospitals in Japan were tested for the detection of antibodies to *B. gibsoni* by the ELISA with rBgTRAP and for the detection of *B. gibsoni* DNA by PCR. As shown in Table 1, 96 (89.7%) and 89 (83.2%) of the tested samples were positive by the ELISA and PCR, respectively. On the other hand, 85 (79.5%) samples were positive by both the ELISA and

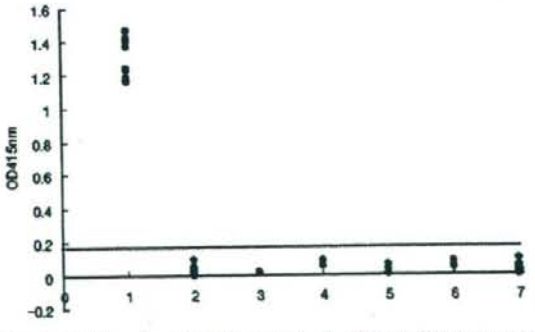

Fig. 1. ELISA with rBgTRAP. Lane 1, *B. gibsoni*-infected dog sera ($n = 10$); lane 2, SPF dog sera ($n = 25$); lane 3, *B. canis canis*-infected dog sera ($n = 3$); lane 4, *B. canis vogeli*-infected dog sera ($n = 2$); lane 5, *B. canis rossi*-infected dog sera ($n = 3$); lane 6, *N. caninum*-infected dog sera ($n = 3$); lane 7, sera from dogs in *B. gibsoni* non-endemic area in Japan ($n = 29$).