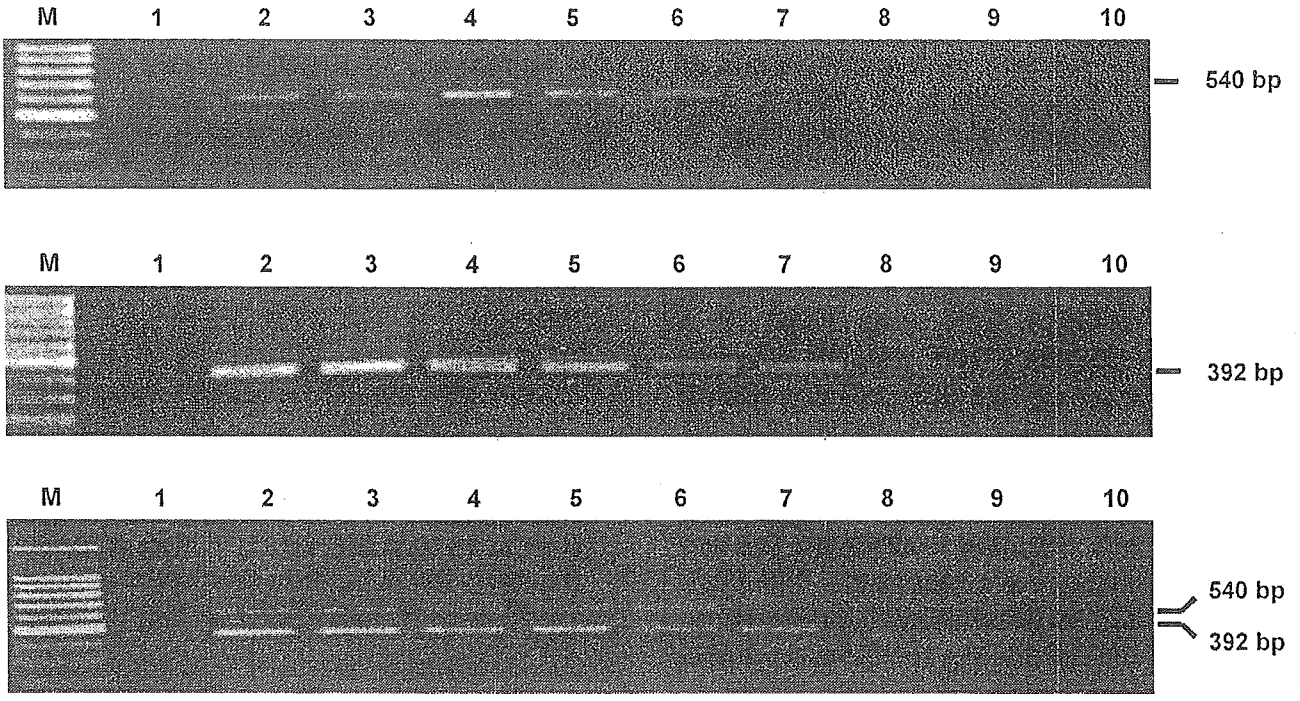

Fig. 2. Sensitivity of the single-round and multiplex PCR to 10-fold serial dilutions of *B. caballi* DNA (A), *B. equi* DNA (B), and the mixed DNA of both parasites (C). M: 100 bp ladder DNA marker; lane 1: equine whole blood DNA; lane 2: 10-fold dilution (18,000 parasites); lane 3: 10^2-fold dilution (1800 parasites); lane 4: 10^3-fold dilution (180 parasites); lane 5: 10^4-fold dilution (18 parasites); lane 6: 10^5-fold dilution (1.8 parasites); lane 7: 10^6-fold dilution (0.18 parasites); lane 8: 10^7-fold dilution (0.018 parasites); lane 9: 10^8-fold dilution (0.0018 parasites); lane 10: 10^9-fold dilution (0.00018 parasites). The band of 500 bp determined from the 100 bp ladder DNA marker is indicated on the left. The size of positive bands is indicated on the right.

Table 2
Summary of PCR results with field blood samples using four different combinations of primers

Combinations of primers	Product size (bp)	Detection rate[a]
Bec-UF1 and -UR for detection of both *B. caballi* and *B. equi* without differentiation	867, 913	14/39 (35.9%)
Bec-UF2, Cab-R and Equi-R for differential detection of *B. caballi* and *B. equi*	*B. caballi*: 540; *B. equi*: 392	*B. caballi*: 7/39 (17.9%)[b]; *B. equi*: 10/39 (25.6%)[b]
BC-48-F and -R for detection of *B. caballi*	570	7/39 (17.9%)
EMA-1-F and -R for detection of *B. equi*	750	10/39 (25.6%)

[a] Number of positive samples/number of total blood samples (%).
[b] Three samples (7.7%) showed simultaneous infections of both parasites.

taneous infections of both parasites. Furthermore, blood samples with positive reactions for *B. caballi* and *B. equi* in the multiplex PCR were confirmed by the PCR using BC48 and EMA-1 species-specific primers, which showed positive bands of 570 and 750 bp, respectively (Table 2). Six negative control samples remained negative for the infections in all experiments. Specificity of the PCR methods was confirmed by sequencing the amplified DNA fragments (data not shown).

4. Discussion

In recent years, there have been several reports on the detection of equine *Babesia* parasites by PCR (Rampersad et al., 2003; Nicolaiewsky et al., 2001; Bashiruddin et al., 1999). However, these methods involve complex procedures, such as nested PCR or hybridization, which are time-consuming and not cost-effective for routine diagnosis. For the diagnosis of equine babesiosis,

especially in animals with acute infection, a rapid method that does not rely on a second round of amplification or hybridization to achieve high sensitivity and accurate species identification is desirable.

Our strategy for the diagnosis of two equine *Babesia* species was to use a single-round and multiplex PCR method with three kinds of primers. Recently, the multiplex PCR assay based on a combination of different primer pairs in the same reaction was introduced for differentiating several parasite species or genera simultaneously (Jessing et al., 2003; Kho et al., 2003; Markoulatos et al., 2002; Elnifro et al., 2000; Henegariu et al., 1997; Figueroa et al., 1993). These described methods are fast and easy to perform in diagnostic laboratories. Therefore, in the present study, we developed a single-round and multiplex PCR method for the simultaneous differentiation of *B. caballi* and *B. equi* with high sensitivity and specificity. Our method provides the detection of 0.18 and 0.018 parasites of *B. caballi* and *B. equi*, respectively. Additionally, the specificity of the multiples PCR method was confirmed by DNA sequencing.

Consistent with earlier findings (Battsetseg et al., 2001; Avarzed et al., 1997a), our study confirms the infection of horses in Mongolia with both equine *Babesia* species. There was total concordance of the results between the multiplex PCR and the PCR using the BC48 and EMA-1 primers, respectively. However, considering the land expanse of Mongolia, additional surveys would be helpful to assess the extent of distribution and endemicity of the disease. In conclusion, the single-round and multiplex PCR method offers a simple tool for the differential diagnosis of *B. caballi* and *B. equi* infections in routine diagnostic laboratory settings as well as in epidemiological studies.

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Research brief

Babesia caballi and *Babesia equi*: Implications of host sialic acids in erythrocyte infection

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Abstract

The present study investigated the involvement of host sialic acids in the erythrocyte infection by two equine *Babesia* parasites, *Babesia equi* and *Babesia caballi*. We observed that the in vitro growth of both parasites is influenced by the removal of sialic acids from the surface of equine erythrocytes (RBC). When the parasites were cultured with neuraminidase (Nm, EC 3.2.1.18)-treated RBC, in which α2–3-linked sialic acid residues were removed from four membrane proteins of the RBC, *B. caballi* showed a significant inhibition of the erythrocyte invasion, while the intracellular development of *B. equi* seemed to be significantly affected. The possible involvement of host sialic acid in the erythrocyte invasion by *B. caballi* was also supported by a significant reduction in the parasite growth accompanied by an increased number of extracellular merozoites after the addition of exogenous 3′-sialyllactose (Neu5Acα(2–3)Galβ(1–4)Glc) into the culture. These results suggest that the α2–3-linked sialic acid residues on host RBC play important roles in the erythrocyte infections by *B. caballi* and *B. equi*.
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Index descriptors and Abbreviations: *Babesia equi*; *Babesia caballi*; Neuraminidase; α2–3-linked sialic acid; Erythrocyte infection; MAL II, *Maackia amurensis* lectin II; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PBS, phosphate-buffered saline; RBC, erythrocytes; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNL, *Sambucus nigra* lectin

Equine babesiosis, mainly caused by two intra-erythrocytic parasites, *Babesia equi* and *Babesia caballi*, results in significant economic losses to the horse industry in tropical and subtropical areas worldwide (de Waal, 1992). The disease is characterized by fever, anemia, jaundice, and edema, and, in some cases, the death of affected horses (Friedhoff, 1982; Friedhoff and Soule, 1996). To develop effectively preventive strategies for equine babesiosis, the molecular interaction(s) between the merozoites and host erythrocytes (RBC) should be examined. Recently, host sialic acids were reported to play an important role in the erythrocyte invasion by bovine *Babesia* parasites (Gaffar et al., 2003; Kania et al., 1995; Zintl et al., 2002) on the basis of the findings that the pretreatment of bovine RBC with neuraminidase (Nm) affects their in vitro growth or erythrocyte invasion. However, the invasion mechanism of equine *Babesia* parasites into RBC has not been investigated yet. Therefore, the role of sialic acid of equine RBC in *B. equi* and *B. caballi* invasion was examined in the present study.

Equine RBC was treated with different concentrations (8, 40, and 200 mU/ml) of *Vibro cholerae* Nm (EC 3.2.1.18: Sigma) or phosphate-buffered saline (PBS) for 3 h at 37 °C with a slow rotation. To check the desialylation of equine RBC, erythrocyte ghosts were prepared from Nm- or PBS-treated RBC by hypotonic lysis (Dodge et al., 1963) and subjected to sodium dodecyl sulfate–polyacrylamide

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gel electrophoresis (SDS–PAGE) (Laemmli, 1970). Nm- and PBS-treated RBC were also examined by lectin blot. Ghost samples loaded on the same gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The PVDF membrane was incubated with biotinylated *Maackia amurensis* lectin II (MAL II) or *Sambucus nigra* lectin (SNL) (Vector Laboratories, Burlingame, CA, USA) followed by reaction with horseradish peroxidase-coupled streptavidin (Molecular Probes, Eugene, OR, USA) and subsequent exposure to 0.003% H_2O_2-containing diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan). MAL II and SNL selectively recognize α2–3-linked and α2–6-linked sialic acids, respectively (Knibbs et al., 1991; Shibuya et al., 1987, 1989; Wang and Cummings, 1988). The removal of α2–3- or α2–6-linked sialic acids on the surface of Nm-treated RBC was also studied by flow cytometric analyses using biotinylated lectins followed by a streptavidin–Alexa Fluor 488 conjugate (Molecular Probes) at the optimal setting previously described for bovine RBC (Yagi et al., 2000).

The Nm-treated RBC were used for *B. equi* and *B. caballi* cultures to see how the desialylation of host RBC affects the parasite growth. United States Department of Agriculture strains of *B. equi* and *B. caballi* were maintained in normal equine RBC using a continuous micro-aerophilous stationary phase culture system in our laboratory (Avarzed et al., 1997; Vega et al., 1985; Zweygarth et al., 1995). This experiment was initiated after *B. equi*- and *B. caballi*-infected cultures, adjusted to 5% parasitemia with intact RBC, were mixed with 200 mU/ml Nm- or PBS-treated RBC at a final parasitemia of 1% in 96-well tissue culture plates (Nalge Nunc International). This procedure allowed the culture system to contain 80% of Nm-treated RBC and 20% of normal cells. Parasitemia was determined by counting more than 1000 RBC on Giemsa-stained thin smears.

To examine the role of host sialic acids in erythrocyte invasion by *B. caballi*, growth inhibition assays were performed using *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) (both from Dextra Laboratories, Reading, UK), 3′-sialyllactose (NeuAcα(2–3)Galβ(1–4)Glc) and 6′-sialyllactose (NeuAcα(2–6)Galβ(1–4)Glc) (both from Glycotech, Gaithersburg, MD, USA), and lactose (Sigma). Neu5Ac is the commonest structure of sialic acids, while Neu5Gc is its 5-position-substituted variant from the acetyl to the hydroxyl base (Varki, 2001). 3′- and 6′-sialyllactoses contain Neu5Ac attached to lactose via α2–3- and α2–6-linkages, respectively. Parasitemia was measured at 24 h after 1.0 mM of these compounds were added into the serum-free cultures of *B. caballi* (Ikadai et al., 2001).

After treatment of RBC with 200 mU/ml of Nm, a hemolytic effect was not seen (data not shown), indicating that the treatment did not influence the integrity of the erythrocyte membrane. SDS–PAGE revealed little difference in the protein profiles between PBS- and Nm-treated RBC (Fig. 1A, left). The sialic acid content of Nm-treated RBC was also examined by lectin blot analysis. Although MAL II-positive bands of 63, 58, 29–31, and 22 kDa were observed in the PBS-treated RBC, these bands disappeared in the Nm-treated RBC (Fig. 1A, middle). However, when equine RBC were treated

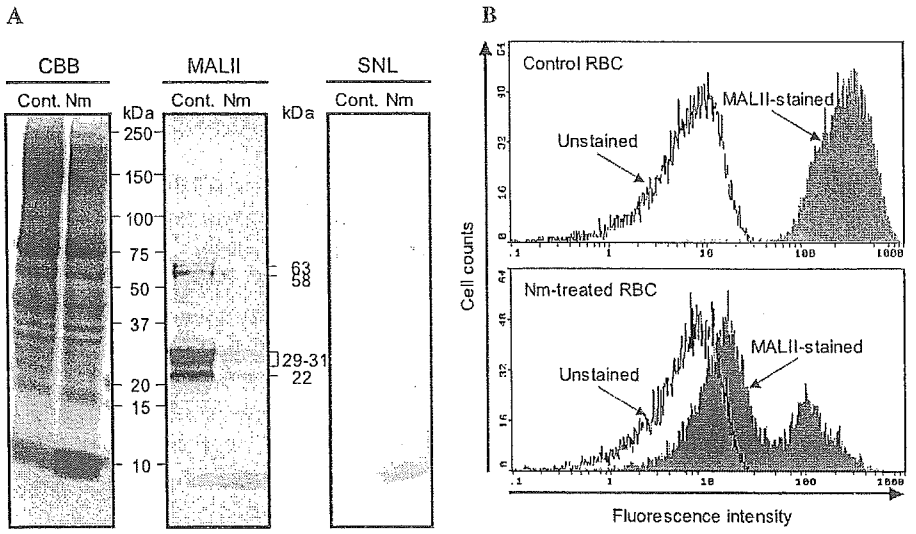

Fig. 1. Effects of Nm treatment on equine RBC. (A) SDS–PAGE and lectin blot analyses. SDS–PAGE followed by CBB staining of a PBS (control)- or 200 mU/ml Nm-treated erythrocyte ghost is shown (left). Molecular-weight-marker standards (kDa) are indicated on the right. On a PVDF membrane with the erythrocyte ghosts transferred from the identical gel, lectin blot analyses with MAL II (middle) and SNL (right) were performed to visualize α2–3- and α2–6-linked sialic acid residues, respectively. The MAL II-positive bands are indicated on the right with arrowheads, and their molecular weights are given. (B) Flow cytometric analyses of sialic acid contents on the surface of control (top) and 200 mU/ml Nm-treated equine RBC (bottom). The binding activity of MAL II to the RBC was recorded (shaded area) and compared to that of RBC not reacted with MAL II (solid line). Only singly separated cells were gated and 10,000 RBC were analyzed.

with lower concentrations, such as 8 and 40 mU/ml of Nm, the four bands did not completely disappear in the lectin blot with MAL II (data not shown). In contrast, no SNL binding was detected in either PBS- or Nm-treated RBC (Fig. 1A, right). These findings are consistent with the previous report that normal equine RBC contain a large amount of α2–3-linked sialic acid residues but not α2–6-linked ones on the surface (Ito et al., 1997). The four MAL II-positive bands that disappeared by Nm treatment might be a glycophorin HA dimer (63 kDa), an HA-HB hybrid dimer (58 kDa), an HA monomer (29–31 kDa), and an HB monomer (22 kDa) in terms of their molecular weights and sialic acid-rich nature (Murayama et al., 1981).

The removal of α2–3-linked sialic acids was also confirmed on the surface of Nm-treated RBC by flow cytometric analyses. While the PBS-treated RBC revealed a population with a high intensity of MAL II binding (Fig. 1B, top), there were two cell populations with high (67.2%) and low intensities (32.8%) of MAL II binding in the Nm-treated RBC (Fig. 1B, bottom). Since the band of about 8–10 kDa was detected with MAL II in the Nm-treated RBC (Fig. 1A), different susceptibility to Nm might be associated with the band appearing at 8–10 kDa in the lectin blot. However, the origin of the 8–10 kDa band was not clear in the present study. One possibility is that this band might be derived from a higher molecular weight of sialoglycoproteins and/or sialoglycolipids during Nm treatment. Further examination of the origin or function of the lower band is necessary in a future study. In contrast, no difference was observed in SNL binding between the PBS- and Nm-treated RBC (data not shown). Taken together, these results indicate that the Nm treatment clearly but partially removed the α2–3-linked sialic acid residues from at least four membrane sialoglycoproteins on the surface of equine RBC.

The Nm-treated RBC were then used for *B. caballi* cultures to examine how the desialylation of host RBC affects the parasite growth. Pretreatment of RBC with 200 mU/ml Nm resulted in significant inhibition of the parasite growth (<0.1%) as compared to the control parasitemia of 1.90% at 24 h (Fig. 2A). *B. caballi* with pycnotic degradation was more frequently observed outside the RBC in the culture with the Nm-treated RBC (Fig. 2A, bottom inset, arrow) than in the control culture (Fig. 2A, top inset). In addition, *B. caballi* cultured with the Nm-treated RBC showed a much higher percentage of extracellular merozoites, which amounted to 90.7%, than the control culture (15.6%), and 94.8% of these extracellular merozoites were undergoing pycnotic degradation (Fig. 2B). These findings suggested that *B. caballi* could not penetrate the Nm-treated RBC. Pretreatment of RBC with lower concentrations (8 and 40 mU/ml) of Nm also led to a moderate decrease in parasitemia when used in the parasite cultures (data not shown). Therefore, the α2–3-linked sialic acids of four sialoglycoproteins are considered to be, at least in part, responsible for the parasite invasion of equine RBC.

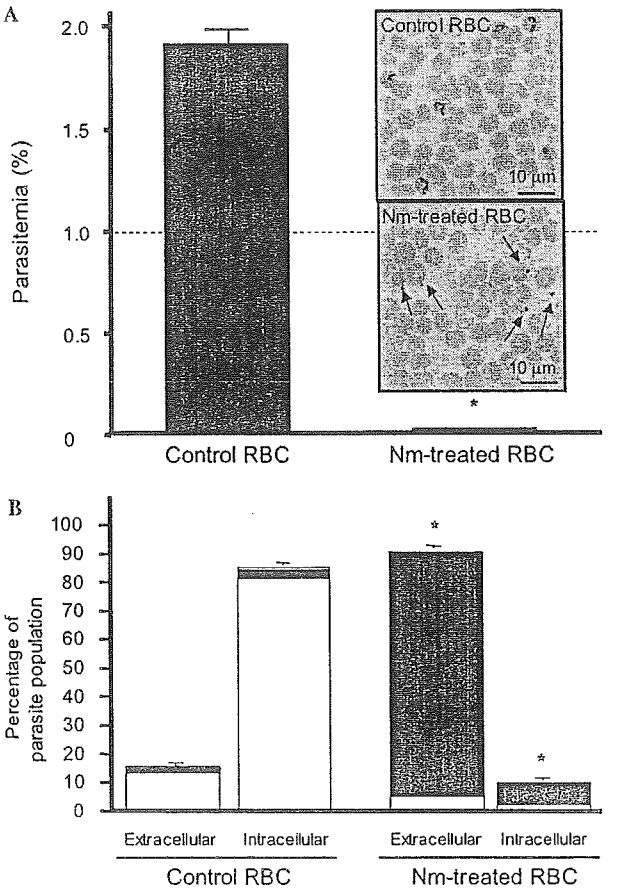

Fig. 2. Growth of *B. caballi* cultured with 200 mU/ml Nm-treated RBC. (A) Parasitemia obtained at 24 h. The cultures were initiated at 1% parasitemia by mixing with PBS- or Nm-treated RBC. Each bar indicates the average value and error bars indicate the standard deviation (SD) of five wells for a representative of three separate experiments. Asterisks indicate significant differences ($P < 0.05$) from the corresponding control growth obtained by the one-way ANOVA with Dunnet's post-test (InStat, GraphPad Software, San Diego, CA). Inset photographs show the typical morphology of the parasites cultured with PBS (control)- (top) and 200 mU/ml Nm-treated RBC (bottom) under a light microscope. Arrows indicate extracellular parasites. (B) Relative percentages of extracellular and intracellular parasites to a whole parasite population. The rates of normal morphology (open part) vs. pycnotic parasites (closed part) are also shown. Error bars indicate the SD of five wells. Asterisks represent significant differences ($P < 0.05$) from the data obtained for the cultures with control RBC.

To confirm the role of host sialic acids in erythrocyte invasion by *B. caballi*, the effect of exogenous sialic acids on the parasite growth in an *in vitro* culture was examined using Neu5Ac, Neu5Gc, 3′-sialyllactose, and 6′-sialyllactose. Among these compounds, only Neu5Ac (1.36%) and 3′-sialyllactose (1.06%) showed decreased parasitemia as compared to the others, ranging 1.94–2.12% (Figs. 3A and B, open bars). When the percentages of extracellular parasites to a whole parasite population were calculated, no statistical difference was detected among the control (6.0%), Neu5Ac- (8.9%), or Neu5Gc-treated cultures

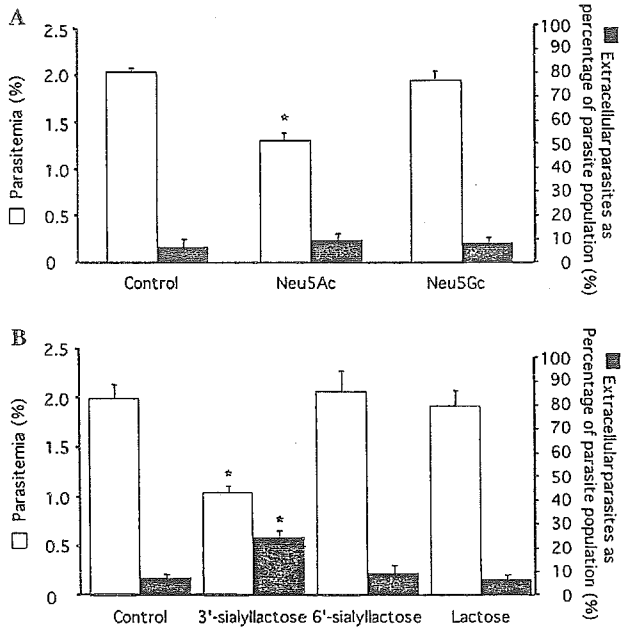

Fig. 3. Parasitemia of *B. caballi* cultured in the presence of exogenous sialic acids (A) or sialyllactoses (B). The results represented were from the assays using a serum-free GIT medium (Ikadai et al., 2001). The cultures were initiated at 1% parasitemia. Parasitemia was checked at 24 h after the addition of each carbohydrate at the concentration of 1 mM (open bars and left *y*-axis). The percentages of extracellular merozoites to a whole parasite population are represented by closed bars and the right *y*-axis. Error bars indicate the SD of five wells. Asterisks indicate significant differences ($P < 0.05$) from the corresponding control growth without any reagents.

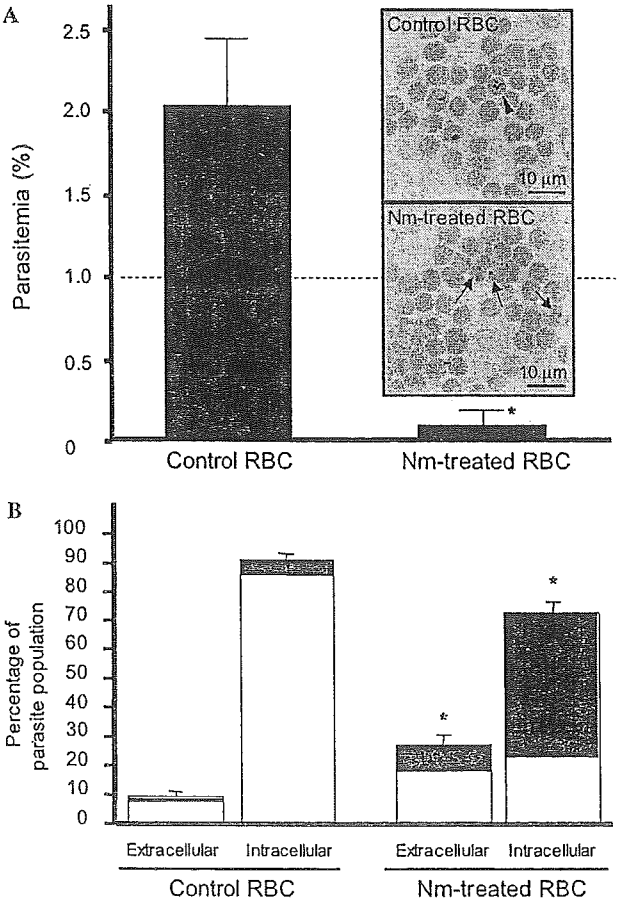

Fig. 4. Growth of *B. equi* cultured with 200 mU/ml Nm-treated RBC. (A and B) Shown in the same way as for *B. caballi* in Fig. 2. The arrowhead indicates the Maltese cross form, and arrows indicate the parasites with pycnotic degradation.

(7.9%) (Fig. 3A, closed bars). These results may suggest that the acetyl base of Neu5Ac should be somehow important for the growth inhibition of *B. caballi* rather than the inhibition of merozoite invasion. On the other hand, a significant increase of extracellular merozoites (24.7%) was observed in the presence of 3′-sialyllactose compared with that in the absence of carbohydrate (7.4%) or in the presence of 6′-sialyllactose (9.4%) and lactose (7.0%) (Fig. 3B, closed bars). The entire parasite populations containing intra- and extracellular parasites were quite similar among treatments, and the increased number of extracellular parasites was correlated with the decreased number of intracellular parasites in the 3′-sialyllactose-treated culture (data not shown). The decreased parasitemia in 3′-sialyllactose-treated RBC is potentially responsible for the increase in extracellular parasites resulting from prevention of merozoite invasion into the erythrocytes. Alternatively, Neu5Ac and and 3′-sialyllactose might have toxic effects against *B. caballi* at 1.0 mM, although there is no data to suggest toxicity due to the differences between the C-5 acetyl and hydroxyl groups or between α2–3 and α2–6 linkage.

The growth of *B. equi* was significantly decreased to 0.12% in the RBC treated with 200 mU/ml Nm as compared to the growth of the control, showing 2.03% (Fig. 4A). Since the Nm pretreatment did not affect the membrane integrity of RBC, the decreased growth of parasites in the Nm-treated RBC is concluded to be specific. Furthermore, *B. equi* with pycnotic degradation, which is associated with reduced viability (Bork et al., 2003), were often observed within host RBC in the culture with Nm-treated RBC (Fig. 4A, bottom inset, arrow) when compared to the control at 24 h (Fig. 4A, top inset). The culture of *B. equi* with the Nm-treated RBC exhibited a significant but slight decrease to 72.8% in the number of intracellular parasites, and 69.3% of the parasites showed abnormal morphology with pycnosis (Fig. 4B). A multi-dividing parasite (Maltese cross form), which is the typical mature form of *B. equi* in the parasitized RBC (Avarzed et al., 1998; Yokoyama et al., 2003), was not detected in the culture with Nm-treated RBC, while 8.0% of intracellular parasites showed the Maltese cross form in the control culture (Fig. 4A, top inset, arrowhead). These results indicate the possibility that *B. equi* can penetrate the membrane of Nm-treated RBC but not proceed to their consequent maturation, demonstrating the significant role(s) of host α2–3-linked sialic acids in the internalization of host RBC and/or intracellular fission in *B. equi*. It is not excluded that the minor

cell population bearing α2–3-linked sialic acids even after Nm treatment might allow parasite invasion or intracellular growth.

Plasmodium falciparum is well known to have multiple ligand–receptor interactions in erythrocyte invasion, including the sialic acid-dependent pathways. Furthermore, the sialic acid-dependent interaction of *P. falciparum* EBA 175 and glycophorin A requires not only the Neu5Acα(2–3)Gal-sequence (Camus and Hadley, 1985; Orlandi et al., 1992; Sim et al., 1990) but also the backboned protein moiety of glycophorin A (Sim et al., 1994). This may be applied to the case of *B. caballi* based on our results indicating that not only the Neu5Ac residues per se but also the α2–3-linkage of terminal sialic acid residues and/or the underlying carbohydrate chains containing lactose contribute to the occurrence of erythrocyte invasion by *B. caballi*. Therefore, the identification and characterization of the sialoglycoproteins of equine RBC that may responsible *B. caballi* invasion would be helpful for the determination of novel babesial ligand(s). In addition, since *B. equi* has been suggested to reclassify as *Theileria equi* because it has several differences compared to the genus *Babesia* (Mehlhorn and Schein, 1998), there may be some differences in how the host sialic acids contribute to erythrocyte infection between these equine *Babesia* parasites. Further elucidation is required to understand the involvement of host sialic acids in the development of *B. equi* within the host RBC.

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Host serum modifies the drug susceptibility of *Babesia bovis in vitro*

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SUMMARY

Babesia parasites generally require a defined percentage of serum in the culture medium for their *in vitro* growth. In this study, we attempted to culture *Babesia bovis* in a serum-free condition. The growth pattern and morphology of *B. bovis* in serum-free (plain) GIT medium were unaltered as compared to those of the standard growth condition containing 40% bovine serum in M199. When exposed to the test drugs, the parasite in plain GIT medium showed clearly lower IC_{50} values than those in 40% serum-containing GIT medium, indicating that several serum components may interfere with the drug bio-availability. Therefore, the serum-free culture system is useful for standardizing drug test protocols and understanding the roles of serum factors in the drug test.

Key words: *Babesia bovis*, *in vitro* cultivation, serum-free medium, drug susceptibility.

INTRODUCTION

The microaerophilous stationary phase system (MASP) was first established as a successful method for the continuous *in vitro* cultivation of *Babesia bovis* (Levy & Ristic, 1980), which is a major aetiological agent of bovine babesiosis in many tropical and subtropical regions of the world (Kuttler & Young, 1984), and further improved by Goff & Yunker (1986). Subsequently, the MASP method has been expanded to the bovine *Babesia bigemina* and *Babesia divergens* (Vega *et al.* 1985; Gorenflot *et al.* 1991), and the equine *Babesia equi* and *Babesia caballi* (Holman *et al.* 1993, 1994; Avarzed *et al.* 1997). In the standard MASP culture method, *Babesia* parasites generally need a 40% volume of host serum in the appropriate media for their growth. However, the host serum represents inconsistent quality (Neves, 1991), which makes a direct comparison of results obtained from similar assays performed in different laboratories very difficult. Furthermore, the requirement for large quantities of serum has several restraints, such as high costs for the procurement and storage of serum (Ofulla *et al.* 1994), and difficulties in the isolation/purification of parasitic proteins in the presence of serum (James, Levy & Ristic, 1981).

To elucidate these problems, several serum-free cultivation systems have been established for *B. divergens* (Schrével, Grellier & Rigomer, 1992; Grande *et al.* 1997), *B. caballi* (Zweygarth, van Niekerk & de Waal, 1999; Ikadai *et al.* 2001), and *Plasmodium falciparum* (Asahi & Kanazawa, 1994). Recently, Jackson *et al.* (2001) successfully replaced the bovine serum by lipid-rich bovine serum albumin for several strains of *B. bovis*. In the present study, we have introduced the novel utility of a plain GIT medium for continuous *in vitro* serum-free culture of *B. bovis*. Subsequently, the serum-free culture system was evaluated by comparing with standard serum conditions in terms of efficacy of several babesiacidal drugs that had been demonstrated to inhibit the *in vitro* growth of *B. bovis* in the standard growth medium (Bork *et al.* 2003*a*, *c*, 2004).

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MATERIALS AND METHODS

In vitro *cultivation of* B. bovis *with various media*

The Texas strain of *B. bovis* was routinely maintained in purified bovine erythrocytes using the standard growth medium consisting of medium M199 (Sigma-Aldrich, St Louis, MO, USA) and 40% bovine serum as described previously (Bork *et al.* 2003*a*). Infected erythrocytes were diluted with non-infected erythrocytes to obtain 1% parasitaemia in a 0·1 ml volume, and the mixture was subsequently suspended in 0·9 ml of the M199 or GIT medium (Nihon Pharmaceutical, Tokyo, Japan) supplemented with the indicated percentage of bovine serum. The suspension was cultured in 24-well culture plates (Nunc, Roskilde, Denmark). During the incubation period, the overlaid culture medium was daily replaced with 0·9 ml of the indicated fresh medium. Experiments were carried out in triplicate wells (for each medium) and in 3 separate trials to obtain conclusive results. Parasitaemia was determined daily as the percentage of parasitized erythrocytes

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(PPE) in Giemsa-stained erythrocyte smears by light microscopy. Differences in the percentage parasitaemia were statistically analysed by the independent Student's t-test, in which $P<0{\cdot}03$ was considered to be of significant difference.

Comparative morphological studies of *B. bovis* cultured in plain GIT, plain M199, and 40% serum-containing M199 media were conducted by light microscopy as described previously (Bork *et al.* 2003*a*).

Drug tests and determination of IC_{50} values

The inhibitory capacities (expressed as IC_{50} values) of the following 5 compounds were evaluated on the growth of *B. bovis* in plain GIT medium as well as in 40% serum-containing GIT and -M199 media (the latter was regarded as a control): triclosan [5-chloro-2-(2,4 dichlorophenoxy)-phenol] (Sigma-Aldrich), clotrimazole (1-[(2-chlorophenyl) diphenylmethyl]-1*H*-imidazole), ketoconazole (cis-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazole-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl] piperazine) (both from Sigma Chemical), clodinafop-propargyl ((2*R*)-2-[4-[(5-chloro-3-fluoro-2-pyridinyl) oxy] phenoxy] propanoic acid-2-propynyl ester) (Riedel-de Haën Laborchemikalien, Germany), and heparin (Wako Chemicals Inc.). Experiments were carried out in triplicate for each drug concentration. The data obtained from 3 separate trials were combined and analysed to determine the IC_{50} values (Bork *et al.* 2003*a*, *b*, *c*, 2004).

RESULTS

In vitro *cultivation of* B. bovis *in a serum-free medium*

Fig. 1 (A) shows the *in vitro* growth patterns of *B. bovis* cultured in GIT and M199 media with or without various concentrations of bovine serum. The growth of *B. bovis* was optimal in the plain and 40% serum-containing GIT media and in the M199 media supplemented with 40 and 20% serum, showing PPE values of 15·8, 16·15, 16·3 and 14·6, respectively, after 3 days of cultivation, followed by M199 media with 10% (PPE 10·9) and 5% (PPE 6·3) bovine serum. M199 medium supplemented with less than 5% serum, and plain M199 did not support the growth, leading to degradation and death of the parasite. Moreover, *B. bovis* cultured in the plain GIT medium was successfully maintained for more than 12 months (data not shown).

Morphologically, *B. bovis* cultured in the plain GIT (Fig. 1B) and 40% serum-containing M199 media (Fig. 1C) exhibited no differences in their characteristics in terms of size and shape as determined in light microscopy. In contrast, the parasites exposed to plain M199 medium (Fig. 1D), which did not support the serum, became degenerated, and died on the third day of cultivation.

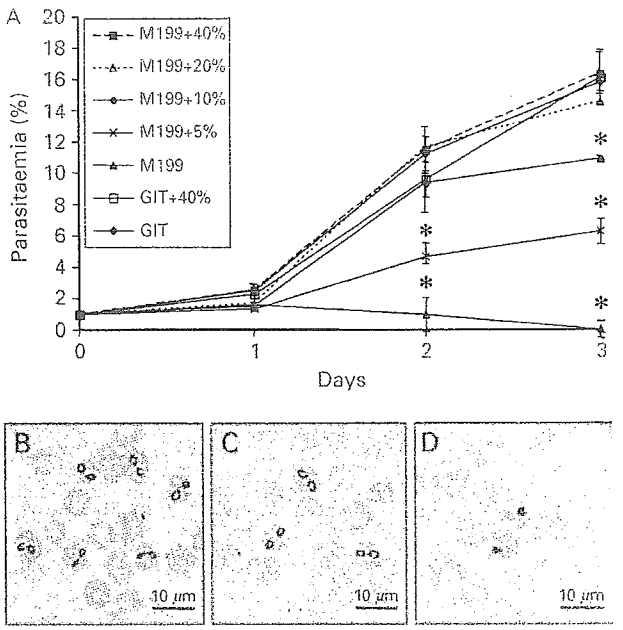

Fig. 1. (A) *In vitro* growth rates of *Babesia bovis* in GIT and M199 media supplemented with different concentrations of serum in cultures for 3 days. Control: 40% bovine serum in M199; M199+20%, 10%, 5%: 20%, 10%, 5% bovine serum in M199; M199: plain M199; GIT: plain GIT. Asterisks indicate P values of $<0{\cdot}03$. (B–D) Light micrographs of *B. bovis*, cultured in plain GIT (B), in M199 with 40% serum (C), and in plain M199 (D). Micrographs were taken on the third day of *in vitro* cultivation. Note the pycnotic appearance of the parasite in (D).

Increased sensitivity of anti-babesial drugs in a serum-free medium

The growth of *B. bovis* was significantly inhibited in the presence of several drug concentrations in standard growth medium and in GIT medium with or without bovine serum. In order to determine the differences in the inhibitory capacity, the IC_{50} values of each drug compound was calculated and summarized in Table 1. The IC_{50} values of clotrimazole, triclosan, and ketoconazole were 7·3-, 6·1-, and 2·1-fold lower in the parasites cultured in the plain GIT medium than those cultured in 40% serum-containing GIT medium. Exposure to clodinafop-propargyl and heparin solely yielded slight reduction of the IC_{50} values of 1·9- and 1·5-fold, respectively, in the plain GIT medium. When comparing the IC_{50} values of the compounds in plain GIT or standard growth medium, CLT, KC and CP in standard growth medium were slightly lower (79, 73 and 86% of the value in plain GIT), while triclosan and heparin remained unaltered. As anticipated, the IC_{50} values of the drugs exposed to 40% bovine serum in M199 medium were virtually identical to those evaluated in previous reports (Bork *et al.* 2003*a*, *b*, 2004).

Table 1. IC_{50} values of different compounds in *Babesia bovis*

	GIT + 40% serum (μg/ml)	Plain GIT (μg/ml)	Fold*	Standard growth medium (μg/ml)
Triclosan	60·0	9·8	6·1	60·0
Clotrimazole	10·24	1·4	7·3	7·99
Ketoconazole	23·27	11·0	2·1	17·0
Clodinafop-propargyl	158·0	82·5	1·9	136·2
Heparin	35·0	22·9	1·5	35·2

* $IC_{50\ (40\%\ serum\ in\ GIT)}/IC_{50\ (plain\ GIT)}$.

DISCUSSION

In the present study, the GIT medium was selected for the replacement of standard growth medium because of its widespread use as a multi-purpose medium for many cells (Ikadai *et al.* 2001). As a result, *B. bovis* was continuously propagated in the plain GIT medium without any supplementations. The growth patterns and the morphological appearance of *B. bovis* cultured in the plain GIT medium were also identical to those cultured in 40% serum-containing M199 medium, indicating that the GIT medium may provide an ingenious source as a basic culture medium for the parasite. Previously, Ikadai *et al.* (2001) also reported the successful cultivation of *B. caballi* in the plain GIT medium. Some components in the GIT medium seem to be essential to the parasite growth and maintenance. On the other hand, Jackson *et al.* (2001) cultured the *B. bovis* by using a lipid-rich bovine serum albumin, AlbuMAX™, as an alternative to bovine serum. Compared to our study, the average percentage of parasitaemia in their experiments was lower, and importantly, the costs for the ingredients were more than 10-fold higher. Further experiments will be required to identify the important growth factors, which can be used instead of host serum for the *in vitro* cultivation. Although Levy, Erp & Ristic (1981) reported the absolute requirement of 40% bovine serum in M199 for the successful growth of *B. bovis*, we demonstrated the unaltered propagation pattern of the parasite cultured in bovine serum as low as 20% in M199. A sufficient explanation of this interesting discrepancy is not possible and remains hypothetical, but it might be due to different parasitic strains used in the two studies, albeit Levy *et al.* (1981) do not mention the parasite's exact specification.

Several authors proved the usefulness of serum-free cultures in the drug susceptibility assays of *P. falciparum* as a reliable alternative standard laboratory practice (Ofulla *et al.* 1994), but similar information has been lacking in *Babesia* parasites. Here, we found that the IC_{50} values of *B. bovis* cultured in the plain GIT with triclosan, clotrimazole, ketoconazole, clodinafop-propargyl, or heparin were relatively lower as compared to those cultured in 40% serum in GIT or 40% serum in M199. This observation might be due to the presence of some serum components including a bovine serum albumin, which is considered to have higher affinity for any drugs, leading to increased IC_{50} values (Ofulla *et al.* 1994). The serum-free culture system used in the present study at least avoids taking into consideration the effects of serum factors on the results of drug tests. Further determination of the component(s) of GIT medium essential for the parasite propagation will provide not only a useful insight into the culture techniques but also a better understanding of the biology of *Babesia* species through the elucidation of the mode of antibabesial drug effects.

In conclusion, the serum-free *in vitro* culture method eliminates the serum effects, and thus will facilitate the standardization of drug test protocols among different laboratories.

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SHORT REPORT: MOLECULAR CLONING AND CHARACTERIZATION OF A PUTATIVE BINDING PROTEIN OF *BABESIA CABALLI*

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Abstract. A composite 2,206 nucleotide DNA sequence encoding a putative immunoglobulin-binding protein (BiP) was constructed from a sequence obtained from *Babesia caballi* cDNA library clones. The 1,962 nucleotide open reading frame predicts a 72 kD protein with extensive homology with BiPs from Apicomplexa parasites. The BiP gene had a predicted N-terminal signal sequence of 18 amino acids and a C-terminal tetrapeptide sequence (Ser-Asp-Glu-Leu) for signaling in the endoplasmic reticulum lumen. The recombinant protein expressed in baculovirus showed an apparent mass of 72 kD, which is identical to that of the native *B. caballi* protein. Monoclonal antibodies (MAbs) against *B. caballi* BiP reacted strongly with extracellular merozoites, but not in early intraerythrocytic stage. Detailed observation showed that the reaction of MAbs against pear-shaped forms was markedly irregular, with either no reaction, or reaction with one or two brightly fluorescent pear-shaped forms (two parasites) of *B. caballi*.

Babesia caballi is a tick-borne hemoprotozoan parasite with a life cycle that alternates between an ixodid tick host, and mammalian hosts such as horses, in which it causes economically important diseases worldwide.[1] It is an obligatory intraerythrocytic equine parasite belonging to the Apicomplexa. Although members of the Apicomplexa infect different host and cell types, they have similar host cell invasion processes.[2] Apicomplexa parasites invade their host cells using molecules located at the cell surface and in apical secretory organelles. These organelles are localized at the anterior end of the invasive stages and are named micronemes, rhoptries, and dense granules.[2–4] For intraerythrocytic *Plasmodium* spp., when an extracellular merozoite enters an erythrocyte, it forms an initial reversible attachment that leads to reorientation of the merozoite to bring the anterior apical pole in contact with the plasma membrane of the erythrocyte.[2] A tight junction is formed through which the parasite invades the erythrocyte.

The adaptation of *B. caballi* at different stages of its development within host cells and in the invasive process may involve heat shock or stress proteins. The ubiquitous 70 kD heat shock protein (HSP70) family comprises a diverse group of proteins found in a large number of different organisms.[5,6] The HSP70 family performs an essential molecular chaperone role for the intracellular trafficking of proteins and has other diverse cellular functions.[7–9] The immunoglobulin heavy chain binding protein (BiP) is a member of the HSP70 family of molecular chaperones in eukaryotic cells, and is located in the endoplasmic reticulum (ER).[10,11] It is an abundant and essential protein involved in polypeptide translocation, and it also assists in the folding and assembly of newly synthesized secreted or membrane proteins.[11] However, BiP has not yet been characterized in *B. caballi*. Therefore, we studied the complete cDNA sequence of a novel BiP gene isolated from *B. caballi* to characterize the BiP gene and its product.

United States Department of Agriculture strains of *B. caballi* were maintained in purified horse erythrocytes in continuous cultures as previously described.[12,13] A *B. caballi* merozoite cDNA library constructed in the λZAP II (Stratagene, La Jolla, CA) was screened with anti-*B. caballi* mouse serum according to the method of according to the method of Ikadai and others.[14,15] Phagemids were excised from the clones and sequencing of the DNA insert of the pBluescript SK (+) plasmid was performed on both strands using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with six primers: T3 (5′-AATTAACCCTCACTAAAGGG-3′), T7 (5′-GTAATACGACTCACTATAGGGC-3′), F1 (5′-CGAAATGGGAAACCGTATCA-3′), F2 (5′-AACATCCTGGTGTACGATCT-3′), F3 (5′-CCCCAAGATCAGGAAAATGA-3′), and F4 (5′-GAAGCGCAACATCGTCATTA-3′). Electrophoresis was carried out on an ABI PRISM 310 DNA sequencer (Applied Biosystems). The sequencing analysis was performed using the computer program GENETYX-MAC version 10.1 (Software Development, Tokyo, Japan).

Several positive clones were obtained and two cDNA clones showed the BiP homolog sequence (GenBank accession no. AB159783). Analysis of the cDNA insert sequence showed that the constructed 2,206 nucleotide fragment encoded BiP with a single open reading frame (ORF) of 1,962 nucleotides starting with methionine at position 189. The ORF encoded a polypeptide of 654 amino acid residues with a size of 72.1 kD. Comparison of the deduced amino acid sequence was performed using the GenBank database and the FASTA program (European Molecular Biology Organization Institute-European Bioinformatics Institute, Heidelberg, Germany). The *B. caballi* ORF encoded a protein of 654 amino acids that showed 64.3% identity with BiP of *Toxoplasma gondii*[16] (GenBank accession no. AF110397), 64.8% identity with BiP of *Eimeria tenella*[17] (GenBank accession no. Z66492), 62.9% identity with the heat shock protein of *Plasmodium falciparum*[18] (GenBank accession no. X69121), 56.1% identity with BiP of *Trypanosoma brucei*[19] (GenBank accession no. L14477), 55.5% identity with BiP of *Saccharomyces cerevisiae*[20] (GenBank accession no. M31006), and 62.3% identity with BiP of *Rattus norvegicus*[21] (GenBank accession no. M14050).

Using the algorithm described by von Heijne,[22] we predicted that the *B. caballi* BiP signal sequence was the first 18

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N-terminal amino acids ([1]MYAKKLVTALVTFLFGQA[18]) of the peptide. The C-terminal peptides Ser-Asp-Glu-Leu ([651]SDEL[654]) of the putative *B. caballi* BiP may function as an anchor to the ER. In *T. gondii* and *E. tenella*, the ER-retention signals are C-terminal peptides composed of Lys-Asp-Glu-Leu (KDEL) and His-Asp-Glu-Leu (HDEL), respectively.[16,17,23-25] Moreover, the C-terminal peptides in *P. falciparum* are SDEL.[18,26] The results establish the generality of the *X*DEL targeting signal throughout the broad range of eukaryotic phylogenetics. Presumably, this conservation extends to the mechanism that mediates ER localization.

DNA was extracted from *B. caballi* and horse blood by a standard method.[27] *Babesia caballi* genomic DNA was amplified by a polymerase chain reaction (PCR) with oligonucleotide primers bipF (5′-AAAGTGTGTGTTGTGCAGAC-3′) and bipR (5′-ATTAGACTGGCTTACAGCTC-3′). The positions of the two primers on the cDNA were nucleotides 70–89 and 2,164–2,145, respectively. The resulting DNA fragment was approximately 2,100 nucleotides. Moreover, horse genomic DNA was not amplified by the PCR with these two primers. The amplified DNA was cloned into a pCR 2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). The plasmid containing the gene was isolated and subjected to DNA sequence analysis. The completed DNA sequence of the BiP gene was analyzed and contained a single intron of 36 nucleotides (Figure 1A).

Recombinant BiP was expressed in insect cells by using a baculovirus expression system. The BiP gene was amplified by PCR with a set of oligonucleotide primers: bipF-orf, which included the ATG initiation codon (5′-GCAAC*ATG*TACG-CCAAAAAG-3′) and Age-bipR, which included the *Age* I restriction enzyme site (5′-AT*ACCGGT*CAGCTCGTC-GCTGTA-3′). This amplified DNA was ligated into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) with the transfer vector pBlueBac4.5/V5-His TOPO TA expression kit (Invitrogen), and digested with *Age* I and self-ligated. The resulting plasmid was designated pBlueBac4.5/V5-His-BiP, and was sequenced using these amplification primers. *Spodoptera frugiperda* (Sf9) cells were co-transfected with the recombinant transfer vector pBlueBac4.5/V5-His-BiP and linear AcNPV Bac-N-Blue DNA (Invitrogen) using Cellfectin reagent (Invitrogen). After six days of incubation at 27°C, recombinant plaques were purified using a blue color selection system in which 5-bromo-4-chloro-3-indolyl-β-D-galactosidase was present in the agarose overlay. Positive blue plaques were selected, and recombinant baculovirus (AcBiP) was obtained after three cycles of purification.

The Sf9 insect cells were infected with the recombinant baculovirus AcBiP in protein-free Sf-900 medium (Invitrogen) at a multiplicity of infection of 5 plaque-forming units/cell. At four days post-infection, infected cells (SfBiP) were harvested and washed three times with cold phosphate-buffered saline (PBS). Infected cells (5×10^6) in Freud's complete adjuvant (Difco Laboratories, Detroit, MI) were injected intraperitoneally into seven-week old BALB/c mice. The same antigen in Freud's incomplete adjuvant (Difco Laboratories) was injected intraperitoneally into the mice on day 14 and day 28. Sera were collected from immunized mice 10 days after the last immunization.

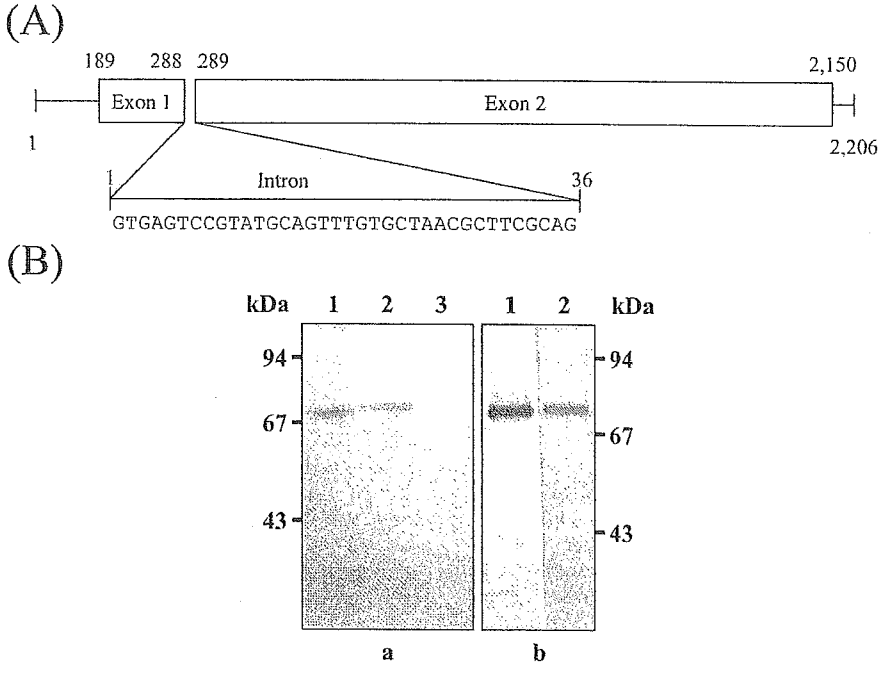

FIGURE 1. **A**, Structure and nucleotide sequence of the genomic immunoglobulin-binding protein (BiP) gene of *Babesia caballi*. The nucleotide sequence of the intron is shown. **B**, Western blotting analysis. **a**, Native *B. caballi* and recombinant SfBiP expressed in insect cells and culture media using monoclonal antibody (MAb) 3B2. Lane 1, native *B. caballi*-infected erythrocytes; lane 2, AcBiP-infected cells; lane 3, AcBiP-infected cell culture media. **b**, Native *B. caballi*-infected erythrocytes with MAb 3B2 and mouse antibodies against SfBiP. Lane 1, MAb 3B2; lane 2, mouse antibodies against SfBiP. The sizes of the molecular mass standards in kilodaltons (kDa) are shown outside the blots. SfBiP = BiP expressed in *Spodoptera frugiperda* cells; AcBiP = BiP expressed in a recombinant baculovirus.

Development of monoclonal antibody (MAb) 3B2 for *B. caballi* BiP was conducted in this study as described previously.[14] Hybridoma supernatants were screened by an indirect immunofluorescence test (IFAT). A mouse MAb isotyping kit (Amersham Bioscience, Branchburg, NJ) was used to classify MAb 3B2 as an IgM antibody. The Sf9 cells infected with AcBiP were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting[14] to determine whether the SfBiP protein was expressed. Cell lysate and culture media were tested using Western blotting with MAb 3B2. A single band of SfBiP protein was observed in the cell lysate, and the molecular mass of the SfBiP protein was the same as that of native *B. caballi* 72 kD protein by western blotting (Figure 1B). This indicates that the ORF observed in the BiP gene was complete. In contrast, no band was detected in the culture medium or uninfected Sf9 cells. Moreover, antibodies against SfBiP, which were obtained from mice, recognized only the 72 kD native protein, as observed with MAb 3B2. These results indicate that the antibodies against SfBiP and MAb 3B2 reacted with the same *B. caballi* 72 kD protein.

The localization of BiP was examined using thin films of *B. caballi*-infected erythrocytes and extracellular merozoites. Thin blood smear films of cultured *B. caballi*- infected erythrocytes were fixed in cold methanol:acetone (1:1) for 20 minutes and incubated in undiluted culture supernatant containing MAb 3B2 or mouse antibodies against SfBiP at 37°C for one hour. Slides were washed with PBS for 10 minutes and incubated with fluorescein-conjugated goat anti-mouse IgM plus IgG plus IgA (heavy and light chains) (Southern Biotechnology, Birmingham, AL) with 5 μg/mL of Hoechst 33258 (Polysciences, Warrington, PA) at 37°C for one hour. The slides were then washed with PBS for 10 minutes and mounted in 90% glycerol for microscopic observation. Different patterns of reactivity with MAb 3B2 were observed in the cold methanol:acetone–fixed preparations of *B. caballi* in the IFAT (Figure 2). Monoclonal antibody 3B2 reacted strongly with extracellular merozoites, but did not react with the early intraerythrocytic stage and horse erythrocytes. Detailed observation showed that the reactivity of the MAb against pear-shaped forms was markedly irregular, with either no reactivity or reactivity with one or two brightly fluorescent pear-shaped forms (two parasites) (Figure 2). This result suggests that the maturation of merozoites after binary fission may not be synchronous, and that the rate of maturation may differ among individual merozoites.

In co-precipitation experiments, BiP transiently associates with newly synthesized secretory proteins, including variant surface glycoproteins, which confirm its role as a molecular chaperone in *T. brucei*.[28] Further characterization of the babesial secretory pathway is required for complete understanding of the cell biology of these important pathogenic organisms. This study provides a foundation for future studies of these proteins, particularly those concerned with the development of secretory reporters.

In conclusion, we report the complete cDNA sequence of a novel BiP gene isolated from *B. caballi* in this study. The high degree of homology with BiP proteins from other species suggests that the *B. caballi* BiP is localized in the ER of this protozoan, where it may play an important role in polypeptide translocation into and through the ER by ensuring that secretory or membrane proteins are correctly folded and assembled.

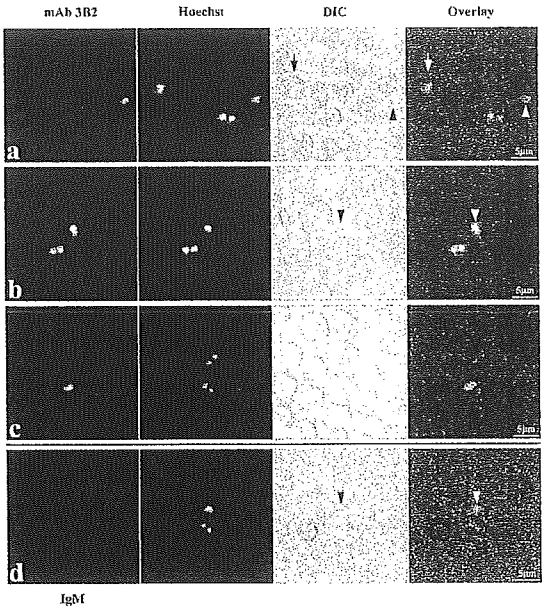

FIGURE 2. Analysis of *Babesia caballi* immunoglobulin binding protein by an indirect immunofluorescent test with monoclonal antibody (mAb) (**a**, **b**, and **c**) and mouse IgM as a negative control (**d**) incubated with cold methanol:acetone–fixed preparations of *Babesia caballi* (a, b, c, and d). Panels in the left column show staining with mAb 3B2 or mouse IgM; panels in the left of center column show Hoechst staining; panels in the right of center column show differential interference contrast (DIC); panels in the right column show overlaid images. **Arrowheads** indicate extracellular merozoites and the **arrow** indicates the early intraerythrocytic stage. Fluorescence microscopy and digital image collection were performed using an eclipse E600 fluorescence-DIC microscope (Nikon, Tokyo, Japan) and a cooled Penguin 600CL charge coupled device camera (Pixera Corporation, Los Gatos, CA) equipped with InStudio software (Pixera Corporation). This figure appears in color at www.ajtmh.org.

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Molecular characterization of a putative protein disulfide isomerase from *Babesia caballi*

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SUMMARY

We produced a mAb against the *Babesia caballi* extracellular merozoite termed mAb 2H2 and used it to screen a cDNA expression library prepared from *B. caballi* merozoite mRNA for highly expressed proteins. The complete nucleotide sequence of the cloned gene had 1547 nucleotides and contained a 36-nucleotide intron. The 1398 nucleotide open reading frame predicts a 51 kDa protein showing similarity to protein disulfide isomerase (PDI) from other species. The PDI gene had a predicted N-terminal signal sequence of 19 amino acids and a C-terminal tetrapeptide sequence (His-Thr-Glu-Leu; HTEL) for retention in lumen of the endoplasmic reticulum (ER). The recombinant protein expressed in baculovirus showed an apparent mass of 51 kDa, identical to that the native *B. caballi* protein. Moreover, the ER retention signal site (HTEL) of the recombinant protein retained its function in ER of insect cells. This 51 kDa protein was strongly expressed by extracelluar *B. caballi* merozoites in indirect immunofluorescence antibody tests, and was not expressed in the early phase of trophozoite development. Interestingly, detailed observation showed that the reaction of anti-P51 antibody and mAb 2H2 against pear-shaped forms was very erratic, some displaying one or two brightly fluorescent patterns.

Key words: *Babesia caballi*, protein disulfide isomerase, cDNA, mAb, indirect immunofluorescence antibody test, endoplasmic reticulum.

INTRODUCTION

Babesia caballi, a member of the phylum Apicomplexa, is a tick-borne haemoprotozoan parasite with a life-cycle that alternates between an ixodid tick host, and mammalian hosts such as the horse, in which it causes economically important diseases worldwide. In the horse, this parasite causes destruction of erythrocytes following invasion, and then induces fever, anaemia, jaundice and haemoglobulinuria. Complete prevention of *B. caballi* infection by drug therapy or vaccination is not currently possible (Schein, 1985; Knowles, 1988; Brüning, 1996).

B. caballi is an obligatory intraerythrocytic equine parasite. Although members of the Apicomplexa phylum infect different host and cell types, they have similar host cell invasion processes. Specifically, when an extracellular merozoite makes contact with an erythrocyte, it forms an initial reversible attachment, which leads to reorientation of the merozoite to bring the anterior apical pole in contact with the plasma membrane of the erythrocyte (Dubremetz *et al.* 1998; Soldati, Dubremetz and Lebrun, 2001). This in turn leads to the formation of a tight junction, through which the parasite invades the erythrocyte. Central to the invasion of host cells by Apicomplexan parasites is their employment of molecules located at the cell surface and in apical secretory organelles. These organelles are localized at the anterior end in the invasive state, at which time they are termed micronemes, rhoptries and dense granules (Dubremetz *et al.* 1998; Preiser *et al.* 2000; Blackman and Bannister, 2001).

These findings suggest that the *B. caballi* extracellular merozoite expresses some proteins that are important for their adhesion to and invasion of erythrocytes. To identify one of these proteins, the object of this study was to establish a mAb against a highly expressed protein of the *B. caballi* extracellular merozoite, to use this mAb to isolate the protein by immunoscreening of the *B. caballi* cDNA expression library, and to characterize the gene and its product, including its localization.

MATERIALS AND METHODS

Parasite

The United States Department of Agriculture strains of *B. caballi* were maintained in purified horse erythrocytes in continuous culture, as

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previously described (Avarazed *et al.* 1997; Ikadai *et al.* 2001).

Production of monoclonal antibody (mAb) 2H2

MAb 2H2 was produced by using a previously described method (Ikadai *et al.* 1999*a*). Briefly, 7-week-old female BALB/c mice (Clea Japan, Inc., Tokyo, Japan) were injected i.p. with 5×10^5 merozoites suspended in 0·1 ml of PBS emulsified with 0·1 ml of Freund's Complete Adjuvant (Difco, Michigan, USA). At 2-week intervals, 7 additional stimulations with the same amount of merozoites emulsified with 0·1 ml of Freund's Incomplete Adjuvant (Difco) were given. These mice were boosted with an inoculation of 5×10^5 merozoites in PBS into the caudal tail vein 2 weeks after their final immunization. The mice were sacrificed 3 days later, and their spleen cells were fused with Sp-2 mouse myeloma cells in polyethylene glycol 1500 (Roche Diagnostics, Mannheim, Germany). Hybridoma cells were selected in a GIT medium (Nihonseiyaku, Tokyo, Japan) supplemented with hypoxanthine-aminopterin-thymidine (ICN Pharmaceuticals, Ohio, USA) and maintained in GIT medium supplemented with BriClone (BioResearch, Dublin, Ireland). MAb 2H2 against a *B. caballi* 51 kDa protein was prepared by screening hybridoma supernatants with the indirect immunofluorescence antibody test (IFAT) and selecting a mAb 2H2 reacting with extracellular merozoite. The mAb 2H2's class and subclass was identified as IgG2a using a mouse mAb isotyping kit (Amersham Bioscience, Branchburg, NJ).

Western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as described previously (Ikadai *et al.* 1999*a*).

Immunoscreening of a cDNA expression library and cDNA sequencing

A *B. caballi* merozoite cDNA library constructed in the λ Zap II phage gene expression vector (Stratagene, La Jolla, USA) was screened with culture supernatant containing the mAb 2H2 according to the method of Ikadai *et al.* (1999*b*). Phagemids were excised from the clones and sequencing of the insert DNA of pBluescript SK (+) plasmid was performed on both strands using a Dye Terminator Cycle Sequencing Kit supplied by Applied Biosystems (Foster City, USA) with the 4 primers M13F (5′-GTAAAACGACGGCCAGT-3′), M13R (5′-GGAAACAGCTATGACCATG-3′), Bcp51F1 (5′-TACCCTCAAGTTCTTCCG-3′) and Bcp51R1 (5′-GCATGAACTTCTTGCAGTG-3′). Analysis was done with an ABI PRISM 310 DNA sequencer (Applied Biosystems) and sequencing analysis with the GENETYX-MAC Ver. 10 software (Genetyx Corp., Tokyo, Japan). The sequenced cDNA was designated as the *p51* gene. Nucleotide sequence data are available in the GenBank database under Accession number AB201253.

Isolation of the p51 *genomic clone*

Total DNA was extracted from *B. caballi* by the standard method (Sambrook, Fritsch and Maniatis, 1989). *B. caballi* genomic DNA was amplified by PCR using one set of oligonucleotide primers, Bcp51F (5′-CATCACTTTTAACGCACCC-3′) and Bcp51R (5′-AGAGTACTCAGAGCTCAGT-3′). The primers' corresponding positions on the cDNA were 17–35 and 1460–1479, respectively, PCR was performed with Bcp51F and Bcp51R using AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 1 cycle at 95 °C for 10 min, 30 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and 1 cycle at 72 °C for 7 min. This amplified DNA was cloned into a pCR 2.1-TOPO vector using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, USA). The entire ligation reaction was used to transform *Escherichia coli* DH5 α competent cells. Plasmid DNA from 2 positive transformants was used for DNA sequencing of the insert. The plasmid containing the gene was then isolated and subjected to DNA sequence analysis.

Northern and Southern blotting analysis

Formaldehyde-denatured total RNA (10 μg) was fractionated on a 1·2% formaldehyde–agarose gel, transferred to a nylon membrane (Hybond-N, Amersham Biosciences) and hybridized with a ^{32}P-labelled probe derived from the *p51* cDNA using the random primer DNA synthesis method in the presence of [^{32}P]dCTP (Amersham Biosciences) (Feinberg and Vogelstein, 1983). Pre-hybridization and hybridization were performed overnight at 42 °C. Membranes were washed 3 times with 0·1 × SSC (0·3 M NaCl plus 0·03 M trisodium citrate, pH 7·0) containing 0·1% sodium dodecyl sulfate (SDS) at 42 °C for 15 min. Bands hybridizing to the probe were detected by standard techniques. For Southern blotting analysis, total DNA was extracted from *B. caballi* by the standard method (Sambrook *et al.* 1989). Restriction enzyme-digested *B. caballi* genomic DNA was run on a 0·7% agarose gel, and the DNA was transferred onto a nylon membrane as described above. The membrane was processed and probed in the same way as for Northern blotting analysis.

Expression of p51 *gene in* E. coli *and production of anti-GST-P51 serum*

The *p51* gene was amplified by PCR using one set of oligonucleotide primers, Bcp51F2 (5′-TACGCTCTCAGCCACATTT-3′) and Bcp51R (5′-AGAGTACTCAGAGCTCAGT-3′). These amplified DNAs were ligated into a cloning vector, pCR2.1-TOPO, using a TOPO TA Cloning kit (Invitrogen). The entire ligation reaction was used to transform *E. coli* DH5 α competent cells. The inserted *p51* gene in pCR2.1-TOPO vector was subcloned into the pGEX4T plasmid (Amersham Biosciences) of the *E. coli* expression vector after digestion with *Eco*RI. The resulting plasmid pGEX-P51 was checked by sequence analysis. The pGEX-P51 was used to transform *E. coli* (BL21; Stratagene) by the standard technique (Sambrook *et al.* 1989) The recombinant protein was expressed as glutathione S-transferase (GST) fusion protein, designated GST-P51 protein.

Antiserum against the GST-P51 protein was produced in mice as anti-P51 antibody. The GST-P51 protein was purified with a MicroSpin GST Purification Module (Amersham Biosciences) after lysis of the collected bacteria by sonication in TNE (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA) containing 1% Triton-X 100. Purified GST-P51 protein (50 μg/animal) in Freund's complete adjuvant (Difco Laboratories, Detroit, USA) was intraperitoneally injected into 2 BALB/c mice aged 7 weeks. The same antigen in Freund's incomplete adjuvant (Difco Laboratories) was intraperitoneally injected into the mice on days 14, 28 and 42. Sera from immunized mice were collected 14 days after the last immunization.

IFAT

The IFAT was performed as follows. Smears of *B. caballi*-infected erythrocytes were prepared on slides, dried, and fixed in a 50% acetone–50% methanol solution for 5 min at −20 °C. MAb 2H2 and anti-P51 antibody were applied as first antibody on the fixed erythrocytes and incubated for 30 min at 37 °C. After 3 washes with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (ICN Pharmaceuticals, Aurora, USA) was applied as second antibody and incubated for 30 min at 37 °C. The slides were washed 3 times with PBS, incubated with 25 μg of propidium iodide (PI) per ml (Molecular Probes, Eugene, USA) and 50 μg of RNase A per ml for 10 min at 37 °C, and then mounted in 50% glycerol–PBS. The slides were photographed using confocal laser scanning microscopy (CLM) (TCS NT, Leica, Germany), and imaging was done using Photoshop, Version 5.0 (Adobe Systems).

Expression of p51 *gene in insect cells*

The *p51* gene was amplified by PCR using 2 sets of oligonucleotide primers, BamBcp51, including the *Bam*HI restriction enzyme site and ATG initiation codon (5′-AC*GGATCC*AGTTGCAAC-GatgG-3′), and Bcp51Age, including the *Age* I restriction enzyme site (5′-TG*ACCGGT*GAG-CTCAGTGTGAGGC-3′); and BamBcp51 and Bcp51stopAge, including a stop codon (5′-TG*ACCGGT*tcaGAGCTCAGTGTGAGGC-3′). One set of BamBcp51 and Bcp51Age oligonucleotide primers for PCR was used for the expressed recombinant P51 protein containing His-tag, while the second set of BamBcp51 and Bcp51stopAge was used for recombinant P51 protein without His-tag. These amplified DNAs were ligated into a cloning vector, pCR2.1-TOPO, using the TOPO TA Cloning kit (Invitrogen). The entire ligation reaction was used to transform *E. coli* DH5 α competent cells. The inserted *p51* gene in the pCR2.1-TOPO vector was subcloned into the *Autographa californica* nuclear plyhedrosis virus (AcNPV) transfer vector pBlueBac4.5/V5-His plasmid (Invitrogen) after digestion with *Bam*HI and *Age*I. The resulting plasmids were designated pBlueBac 4.5/V5-*p51*-His and pBlueBac4.5/V5-*p51*-stop, respectively. Both plasmids were completely sequenced using the above-mentioned primers.

Spodoptera frugiperda (Sf9) cells were co-transfected with the recombinant transfer vectors pBlueBac4.5/V5-*p51*-His and pBlueBac4.5/V5-*p51*-stop, and linear AcNPV Bac-N-Blue DNA (Invitrogen) using the Cellfectin reagent (Invitrogen). After 6 days of incubation at 27 °C, positive blue plaques containing recominant virus were selected by a blue colour selection system. Moreover, recombinant baculovirus (Ac *p51*-His and Ac *p51*-stop) were obtained after 3 cycles of purification, respectively.

RESULTS

Cloning and sequencing of p51 *cDNA clones*

Screening of a cDNA expression library with mAb 2H2 against *B. caballi* extracellular merozoite to identify a highly expressed protein produced 5 positive clones. The nucleotide sequence of the total 1547 nucleotide cDNA was determined. Starting with methionine at position 75, a single open reading frame (ORF) of 1398 nucleotides was present. The ORF encoded a polypeptide of 465 amino acid residues with a size of 50·8 kDa. The ORF signal sequence was predicted by the algorithm of von Heijne (1986) to be the first 19 N-terminal amino acids (^{1}MDFLAPLAFLFSVASVSFA^{19}) of the peptide (Fig. 1A). Comparison of the deduced amino acid sequence with the GenBank database using the FASTA program showed that the *B. caballi*