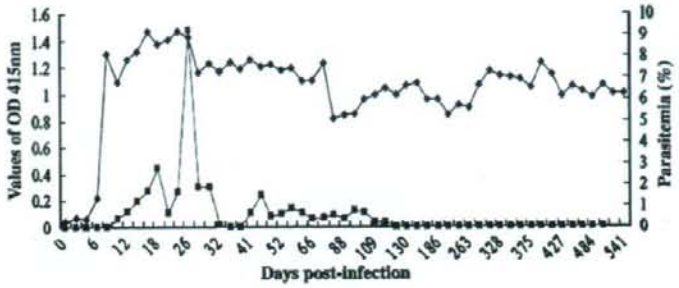

Fig. 2. Antibody response to rBgTRAP in a dog experimentally infected with *B. gibsoni*. The antibody response was detected by the ELISA with rBgTRAP. The parasitemia was determined by microscopic examination.

PCR, while 4 (3.7%) PCR+ samples were ELISA−, and 11 (10.3%) PCR− samples were ELISA+ (Table 1). For four samples that were PCR+/ELISA−, the number of days from onset of symptoms to the date of blood collection was 0–2 days (Table 2). For 11 samples that were PCR−/ELISA+, the number of days from onset of symptoms to the date of blood collection was 200–2465 days (Table 3).

3.3. Comparison of the ELISA with rBgTRAP and previously established ELISAs with rBgP50, rBgSA1, and rBgP32

To evaluate the sensitivity of the ELISA with rBgTRAP, the present ELISA was compared to previously established ELISAs for detection of antibodies to *B. gibsoni* in sera collected from 107 dogs that were diagnosed as having babesiosis. As shown in Table 4, 85 (79.5%), 64 (59.8%), 71 (66.4%), and 65 (60.7%) of the PCR+ samples were positive by the ELISAs with rBgTRAP, rBgP50, rBgSA1, and rBgP32, respectively. In addition, 11 (10.3%), 10 (9.35%), 11 (10.3%), and 12 (11.2%) of the PCR− samples were positive by the ELISAs with rBgTRAP, rBgP50, rBgSA1, and rBgP32, respectively.

Table 1
Diagnosis of *B. gibsoni* infection in dogs that had been identified as having anemic signs at veterinary hospitals in Japan according to the ELISA with rBgTRAP and PCR

PCR	ELISA		Total (%)
	Number (%) positive	Number (%) negative	
Number (%) positive	85 (79.5)	4 (3.7)	89 (83.2)
Number (%) negative	11 (10.3)	7 (6.5)	18 (16.8)
Total (%)	96 (89.7)	11 (10.3)	107 (100)

Table 2
Four PCR-positive samples were negative according to the ELISA with rBgTRAP

	ELISA	PCR	The number of days from onset of symptoms to the date of blood collection (days)
No. 30	−(0.105)	+	1
No. 60	−(0.044)	+	2
No. 66	−(0.005)	+	2
No. 103	−(0.031)	+	0

Table 3
Eleven PCR-negative samples were positive according to the ELISA with rBgTRAP

	ELISA	PCR	The number of days from onset of symptoms to the date of blood collection (days)
No. 13	+(1.434)	−	2002
No. 21	+(1.367)	−	1057
No. 26	+(0.245)	−	598
No. 27	+(1.698)	−	2465
No. 36	+(1.449)	−	200
No. 47	+(0.281)	−	506
No. 55	+(0.214)	−	Unknown
No. 89	+(1.242)	−	1085
No. 93	+(1.251)	−	421
No. 105	+(0.355)	−	382
No. 106	+(1.178)	−	1098

4. Discussion

In this study, the clinical usefulness of the ELISA with rBgTRAP was evaluated using sera collected from dogs that had been diagnosed as having babesiosis at veterinary hospitals in Japan and by comparison to previously established ELISAs.

The ELISA with rBgTRAP clearly differentiated between *B. gibsoni*-infected dog sera and SPF dog sera or sera from dogs in non-endemic areas. In addition, the ELISA detected no cross-reactivity with sera from dogs experimentally infected with closely related parasites, such as *B. canis canis*, *B. canis vogeli*, *B. canis rossi*, and *N. canium*. This result indicated that the BgTRAP is a *B. gibsoni*-specific antigen, although the TRAPs are known as genetically conserved molecules among apicomplexan parasites.

Table 4
Comparison of the present ELISA with rBgTRAP and the previously established ELISAs with rBgP50, rBgSA1, and rBgP32

PCR	ELISA							
	rBgTRAP		rBgP50		rBgSA1		rBgP32	
	Number (%) positive	Number (%) negative	Number (%) positive	Number (%) negative	Number (%) positive	Number (%) negative	Number (%) positive	Number (%)negative
Number (%) positive	85 (79.5)	4 (3.7)	64 (59.8)	23 (21.5)	71 (66.4)	16 (14.9)	65 (60.7)	22 (20.6)
Number (%) negative	11 (10.3)	7 (6.5)	10 (9.35)	10 (9.35)	11 (10.3)	9 (8.4)	12 (11.2)	8 (7.5)

On the other hand, a dog experimentally infected with *B. gibsoni* developed a significant level of antibody titer from eight days post-infection. The significant antibody titer was maintained until 541 days post-infection, even when the dog became chronically infected with a significantly low level of parasitemia. This result indicated that the BgTRAP could induce strong humoral immunity in both the acute and the chronic stages of *B. gibsoni* infections in dogs.

Clinical serum samples collected from dogs that were diagnosed as having babesiosis at veterinary hospitals in Japan were examined for the diagnosis of *B. gibsoni* infection by the ELISA with rBgTRAP. Eighty-five (79.5%) of the tested samples were positive according to both the ELISA and PCR. This data suggests that BgTRAP is a useful antigen to diagnose both past and current infections. However, 4 (3.7%) and 11 (10.3%) of the tested samples revealed ELISA−/PCR+ and ELISA+/PCR−, respectively. Four ELISA−/PCR+ samples were collected from dogs at the significant acute stage, from 0 to 2 days, when the specific antibodies had not been created yet. On the other hand, 11 ELISA+/PCR− samples were collected from dogs at the significantly chronic stage, from 200 to 2465 days, when parasites may have been removed from peripheral blood but antibodies remained. These results suggested that the combined ELISA/PCR approach could greatly improve the clinical diagnosis of canine *B. gibsoni* infection.

To evaluate the sensitivity of the rBgTRAP for the detection of *B. gibsoni* infection, we compared the ELISA with rBgTRAP to the previously established ELISAs with rBgP50, rBgSA1, and rBgP32. Eighty-five (79.5%), 64 (59.8%), 71 (66.4%), and 65 (60.7%) of the PCR-positive samples were positive by ELISAs with rBgTRAP, rBgP50, rBgSA1, and rBgP32, respectively. On the other hand, 11 (10.3%), 10 (9.35%), 11 (10.3%), and 12 (11.2%) of the PCR-negative samples were positive by ELISA with rBgTRAP, rBgP50, rBgSA1, and rBgP32, respectively. These results indicated that the ELISA with rBgTRAP appears to be the most sensitive method. However, it is necessary to confirm using large number of samples.

In clinical sites, the canine anemic symptoms can be caused not only by Babesia infection but also by other diseases, such as autoimmune hemolytic anemia (AIHA) (Inokuma et al., 2005). In the present study, 107 samples collected from dogs with anemic symptoms, 7 (6.5%) samples were negative according to both the ELISA with rBgTRAP and PCR. At the moment, it is unclear whether the samples taken from dogs with anemia caused by a condition other than *B. gibsoni* infection or those taken from *B. gibsoni*-infected dogs had undetectable levels of antibody and parasites. In the latter case, more sensitive serodiagnostic and genetic detection methods would be needed. In addition, the previous study about TRAPs of *Plasmodium* spp. show that TRAPs have the natural sequence variations among strains (Robson et al., 1998). Thus, a further study about a conservation of BgTRAP in field strains is needed so that our data become more definite. In conclusion, the results outlined above demonstrated that the ELISA with rBgTRAP is the most promising serodiagnostic method among ELISAs established to date for the detection of antibodies to *B. gibsoni* in dogs.

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ORIGINAL PAPER

Diagnosis of *Babesia caballi* and *Theileria equi* infections in horses in Sudan using ELISA and PCR

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Abstract The purpose of this study was to estimate the prevalence of equine piroplasmosis in Sudan. The presence of antibodies against *Babesia caballi* and *Theileria equi* was determined in serum samples obtained from 158 horses raised in different locations in Sudan by enzyme-linked immunosorbent assay (ELISA). The *B. caballi* 48-kDa and the *T. equi* EMA-2 purified recombinant proteins were used as antigens in the ELISA test. Results showed that seven (4.4%) were positive for *B. caballi* and 80 (63.5%) were positive for *T. equi*. Polymerase chain reaction (PCR) assays have been applied using primers targeting the *B. caballi* 48-kDa merozoite antigen, the *T. equi* SSUrRNA and the *T. equi* EMA-1 genes. PCR performed on 131 blood spots in filter paper revealed that 33 (25.2%) samples were positive for *T. equi* but no positives were found for *B. caballi*. It is concluded that equine piroplasmosis is endemic in the country. This is the first study on serological and molecular epidemiological diagnosis on equine piroplasmosis in Sudan.

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Introduction

Equine piroplasmosis, caused by *Theileria equi* and *Babesia caballi*, is globally distributed and poses a serious threat to the horse raising industry and international movement of horses (Friedhoff et al. 1990; Avarzed et al. 1997). The disease is widely distributed in the tropical and subtropical areas (Preston 2001; Uilenberg 2001). *T. equi* is considered more pathogenic and more consistent cause of hemoglobinuria and death in equines, while *B. caballi* causes a more persistent syndrome characterized by fever and anemia (Zaugg and Lane 1992). *T. equi* infection may be suppressed by chemotherapy but it cannot be completely eliminated (de Waal and van Heerden 1994). Equine piroplasmosis was firstly reported in Sudan by Oliver (1907) cited in Abdoon (1984), who studied the epidemiology of the disease in Khartoum State using blood smears and the complement fixation test (CFT). Of the investigated 80 horses, 16% showed typical clinical signs of piroplasmosis, 20% showed *Babesia* parasites in blood smears, and 70% were positive by CFT. Oliver's findings also illustrated that *T. equi* was more common than *B. caballi* with a prevalence of 53.4% in the examined population.

It is not possible to differentiate between *T. equi* and *B. caballi* infections on the basis of clinical signs (de Waal 1992). Definitive diagnosis depends on the identification of *B. caballi* and *T. equi* in blood smears stained by Giemsa or by acridine orange (Ali et al. 1996). Although the method is simple, it is insufficient for accurate detection and identification of *B. caballi* and *T. equi* during mixed infections and in particular in carrier statuses with low parasitemia (Seifi et al. 2000; Krause 2003). For serological detection of equine piroplasmosis, CFT and the indirect immunofluorescent antibody test (IFAT) have been used as standard tests for the detection of equine piroplasmosis. Many

problems have been reported for the CFT including low sensitivity and the yield of false-positive and false-negative results for *B. caballi* (Friedhoff and Soulé 1996; Ikadai et al. 2001). Thus, in several studies, the CFT has been replaced by the more sensitive IFAT, which has been used in serosurveillance for *Theileria* and *Babesia* infections in many countries. Researchers, however, have also reported on the inherent problems of IFAT that include cross reactivity, subjectivity, and impracticability especially for testing large numbers of samples (Brüning 1996; Papadopoulos et al.1996). Various forms of enzyme-linked immunosorbent assay (ELISA) have been standardized and reported to be more sensitive than the CFT in the diagnosis of equine piroplasmosis (Brüning et al. 1997; Bakheit et al. 2007). Several *T. equi* proteins have demonstrated immunoreactive antigens and used in ELISA, of which the merozoite surface proteins EMA-1 and EMA-2 have been identified as the most common immunodominant molecules of *T. equi* (Knowles et al. 1992; Kappmeyer et al.1993) and the 48-kDa rhoptry protein of *B. caballi* (Ikadai et al. 1999; Xuan et al. 2001). Moreover, it has been demonstrated that EMA-1 is geographically conserved among all *T. equi* isolates (Knowles et al. 1991), thus could provide a good diagnostic tool when used in ELISA. Besides the application of microscopy and serology in the diagnosis of equine piroplasmosis, in vitro culture of blood was used for the detection of carrier host animals with *T. equi* (Holman et al. 1997). Molecular techniques have been proven useful for the detection of equine piroplasmosis. These methods are based on species-specific polymerase chain reaction (PCR) assays, which mainly target the 18S rRNA gene (Caccio et al. 2000; Birkenheuer et al. 2003; Criado-Fornelio et al. 2003; Rampersad et al. 2003). PCR has been found sensitive enough to detect parasite DNA from 2.5 µl blood sample with parasitemia of 0.000001% (Xuan et al. 1998; Alhassan et al. 2007).

The objective of this study was to apply ELISA and PCR methods to determine the prevalence and distribution of *T. equi* and *B. caballi* infections in the equine population in Sudan.

Materials and methods

Description of study area

Samples were collected from different eco-climatic zones which included Khartoum, Khartoum North, and Omdurman (15°40′ N 32′28′ E), Atbara (17°42′ N 34′ E), Kosti (13°09′ N 32°40′ E), El Obied (13°11′ N 30°13′ E), and Gadarif (14° N 35°30′ E; Fig. 1). Whole blood was taken by jugular venipuncture using vacutainer tubes, and thereafter, serum samples were separated and stored at

Fig. 1 Map of Sudan. Localities from where samples were collected are marked with *filled circles*

−20°C till use. Blood spots on filter papers were collected for PCR amplification. The blood spots were made in circles of 1/2 in. diameter, air dried, labeled, and fixed with 70% ethanol before being stored at 4°C.

ELISA

The presence of antibodies against *T. equi* and *B. caballi* was determined in serum samples from 158 horses by ELISA. The *B. caballi* 48-kDa and the *T. equi* EMA-2 purified recombinant proteins were obtained from the National Research Centre for Protozoan Diseases, Obihiro, Japan and used as antigens in ELISA. Recombinant antigens were diluted to 7 µg/ml in carbonate bicarbonate buffer, pH 9.6 and 50 µl used to coat the wells of 96 wells micro-titer ELISA plate. The plates were kept at 4°C overnight. The plates were then washed six times with phosphate-buffered saline (PBS) pH 7.4, supplemented with 0.05% Tween-20 (PBS-T). The unoccupied sites in each well were blocked by adding 200 µl in each well of a blocking buffer composed of PBS, pH 7.4, 3% skimmed milk (PBS-SM) and incubated for 1 h at 37°C. The plates were again washed six times with PBS-T. Sera were diluted at 1:100 in PBS-SM and pipetted in a volume of 100 µl/well. Each plate was used to test 40 serum samples, with the first two columns used for controls including blank control (two wells), positive controls (four wells), and negative controls (ten wells). Plates were

incubated for 1 h at 37°C then washed with PBS-T. Goat anti-horse IgG antibody, conjugated to horse radish peroxidase was added at a dilution of 1:10,000 in PBS-SM and incubated for 1 h at 37°C. Finally, a substrate solution [composed of 0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, 0.3 mg of 2, 2′-azino-di-(3-ethylbenzthiazoline sulfonate) per milliliter] was freshly prepared and added in a volume of 100 µl/well and allowed to stand in dark at room temperature for 1 h. The development of a green color was obtained from positive control and positive samples. The plates were read at 450 nm in an ELISA plate reader (Titertek multiskan reader, Labsystems multiskan RC) and the mean (OD) of the negative control was calculated. Any sample showing an OD above the mean + (3×standard deviation) of ten negative wells was considered positive.

DNA extraction and PCR amplification

Under clean conditions, the filter papers were put on a dry clean rapper pad cleaned with 70% ethanol. A 3-mm diameter from each spot was punched-out into 1.5 ml microcentrifuge tube using puncture as previously described (Alhassan et al. 2007). DNA of *B. caballi* and *T. equi* were separately extracted from the blood spots on filter paper using QIAamp DNA mini kit (Qiagen, Germany) as described by the manufacturer. The obtained DNA was then stored at −20°C till used.

Two sets of oligonucleotides were used to amplify *T. equi* DNA. The first PCR assay was performed according to Alhassan et al. (2005). This assay utilized a universal forward primer sequence (Bec-UF2) with the sequence 5′-TCGAAGACGATCAGATACCGTCG and a *T. equi*-specific reverse primer (Bec-R) with the sequence 5′-ATCGCAAGGAAGTTTAAGGCA. The second set of specific primers was designed based on the sequence of the *T. equi* merozoite antigen 1(EMA-1) gene with a forward primer EMA-1F: 5′-GCATCCATTGCCATTTC-GAG and a reverse primer EMA-1R: 5′-GCTTCT CCGTCTATGGCGCA. To amplify DNA of *B. caballi*, a forward primer BC48-F: 5′-GGCTCCCAGCGACTCTG and reverse primer BC48-R: 5′-GCATCAAGAGGGC ACTTAAG was used to amplify 610 bp from the *B. caballi* BC48 gene. EMA-1 is encoded by a single copy gene in *T. equi* (Knowles et al. 1997; Kappmeyer et al. 1993), while BC48 is a multi-copy gene encoding the 48-kDa rhoptry protein of *B. caballi* (Ikadai et al. 1999). PCR was performed in a final volume of 25 µl, which contained 3 µl template DNA, 2.5 µl of 10× PCR reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 800 nM of each primer, and 1.25 U of *Taq* DNA polymerase (AmpliTaq Gold, Applied Biosystems, Japan). PCR cycling included an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s for both primer pairs used to amplify *T. equi* DNA, and 55°C of the primer pair to amplify *B. caballi*, extension at 72°C for 1 min. This was followed by a final extension step at 72°C for 7 min. PCR products were electrophoresed in 1.5% agarose in TBE buffer before being visualized under UV light. *B. caballi*-positive samples showed an expected band of 610 bp, while *T. equi*-positive samples showed a band of 435 bp for PCR amplifying the SSU rRNA and 744 bp for PCR amplifying the EMA-1 gene fragments.

Results

Serum antibodies detection

A total number of 80 out of 126 serum horse samples (63.5%) were found positive for *T. equi* antibodies. The highest prevalence was found in Khartoum North (100%) and Atbara (100%) followed by Omdurman (84.4%). Prevalence was lower in Kosti (45.5%), Khartoum (44.4%), and El Obied (39%; Table 1). Out of 158 samples, only seven (4.4%) were positive for *B. caballi* in Atbara (10%), Omdurman (8.2%), and El Obied (2.5%), and no positives were detected in Khartoum, Khartoum North, and Kosti areas (Table 1).

PCR detection of *T. equi* and *B. caballi*

One hundred thirty-one blood spots from horses were subjected to DNA isolation and PCR. All PCR assays used to amplify *T. equi* or *B. caballi* performed well in our hands, and expected bands of 435 bp (for the *T. equi* 18S gene), 744 bp (for the *T. equi* EMA-1 gene), and 610 bp (for *B. caballi* 48-kDa gene) were detected (shown exemplarily for *T. equi* in Fig. 2). Out of the 131 samples tested, none was positive for *B. caballi*, but 33 (25.2%)

Table 1 Detection of *Theileria equi* and *Babesia caballi* antibodies in serum samples of horses from Sudan as determined by indirect ELISA in the period of 2006–2007

Location	*Theileria equi*		*Babesia caballi*	
	No. examined	Positive (%)	No. examined	Positive (%)
Khartoum	18	8 (44.4)	6	0
Khartoum North	19	19 (100)	18	0
Omdurman	32	27 (84.4)	49	4 (8.2)
Kosti	11	5 (45.5)	25	0
Atbara	5	5 (100)	20	2 (10)
El Obied	41	16 (39)	40	1 (2.5)
Total	126	80 (63.5)	150	7 (4.4)

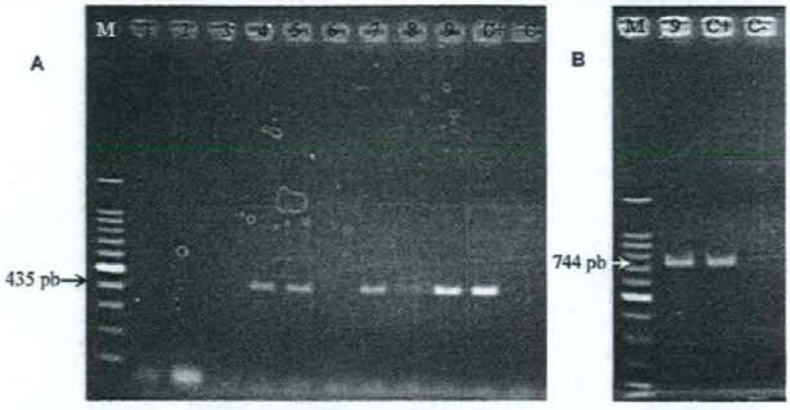

Fig. 2 Amplification of *Theileria equi* DNA using two primer sets: A primer set to amplify a fragment of 435 bp of the 18S gene (**a**) and another set to amplify a fragment of 744 bp of the *T. equi* EMA-1 gene (**b**). *Lane M* is a 100-bp ladder. *Lanes 1–9* in (**a**) and *lane 9* in (**b**) represent field samples obtained from apparently healthy horses in El Khwai District, Sudan. *Lane C+* is a control positive DNA. *Lane C−* is a negative DNA control isolated from horse blood

were positive for *T. equi* (Table 2). The highest prevalence was found in Kosti (62.5%), followed by Atbara (52.6%), Khartoum North (15.8%), Khartoum (15%), El Obied (14%), and Omdurman (0%).

Discussion

The objective of this study was to estimate the prevalence of equine piroplasmosis in Sudan using serological and molecular tools. Several reasons account for the application of these techniques for equine piroplasmosis compared to the microscopic detection. Microscopic examination has been shown insensitive to detect low parasitemia, especially in endemic areas of the disease (Calder et al. 1996). Moreover, microscopic detection is subjective; distinguishing *Babesia* species on the basis of host specificity appears to be less useful than once thought when *B. microti* has been shown to have broader host specificity (Edelhofer et al. 1998). Thus, in many instances, it has been pointed out that serological and molecular techniques represent a more objective tool for the diagnosis of equine piroplasmosis (Persing and Conrad 1995).

Table 2 Detection of *Theileria equi* and *Babesia caballi* DNA in blood of horses as determined by PCR

Location	Total number examined	*Theileria equi* positive (%)	*Babesia caballi* positive (%)
Khartoum	20	3 (15)	0
Khartoum North	19	3 (15.8)	0
Omdurman	7	0	0
Kosti	16	10 (62.5)	0
Atbara	19	10 (52.6)	0
El Obied	50	7 (14)	0
Total	131	33 (25.2)	0

In this study, we applied recombinant protein-based ELISA to detect circulating antibodies against *T. equi* and *B. caballi* infections. These ELISAs utilized the EMA of *T. equi* and the 48-kDa rhoptry protein of *B. caballi*, their suitability for serodiagnosis has been shown before (Ikadai et al. 1999; Xuan et al. 2001; Knowles et al. 1992). Our findings indicated a high prevalence of antibodies against these parasites in all investigated areas, an indication that equine piroplasmosis is widespread in Sudan. On the other hand, the antibody prevalence of *T. equi* was found to be higher than that of *B. caballi*. This could possibly be attributed to the vector distribution. The most abundant ticks associated to horses are *Hyalomma anatolicum anatolicum* and they might be more potentially important in the transmission of *T. equi* than *B. caballi* in Sudan. Another possible reason for the low prevalence of *B. caballi* could be the earlier elimination of the parasite after a short period of infection (Frerichs et al. 1969). This is also supported by the findings that *B. caballi* is difficult to detect in blood smears at any stage of the disease except the early acute phase of infection (Todorovic and Carson 1981). In a previous study based on CF test alone, Abdoon (1984) found 54.3% *T. equi* and 45.7% *B. caballi*. The positive samples of *B. caballi* reported by Abdoon (1984) using blood smears may not reflect the natural situation as the samples were taken from clinical cases of animals brought to the veterinary clinics.

During this study, we noticed differences in the prevalence of equine piroplasms between the individual study areas. In Omdurman, for instance, there was a high titer of antibodies against both parasites, and 27 out of 32 serum samples (84.4%) were positive for *T. equi* and four out of 49 serum samples (8.2%) were positive for *B. caballi*. This is probably due to differences in the management of the sample animals pertaining to their nutrition and healthcare with tick control.

Using molecular tools on the other hand, *T. equi* was shown again to be more prevalent than *B. caballi* though the overall prevalence of both parasites by PCR was lesser than that detected by ELISA. Molecular detection of the parasites requires DNA isolation from parasites that are physically present in the blood sample to a detectable level above the sensitivity threshold of the particular detection method used. Usually, *T. equi* parasites are not completely eliminated from the blood of horses after treatment or natural recovery (de Waal and van Heerden 1994) as compared to *B. caballi*. In endemic countries, horses are sometimes known to adapt to infections, but stress and other factors that cause severe immuno-suppression may result in sub-clinical infections becoming overt and detectable. This perhaps explains the lesser prevalence of *T. equi* using PCR. In addition, there is evidence that animals infected with *T. equi* become lifelong carriers (Brüning 1996), while that of *B. caballi* may also persist in subclinical form for at least 1–4 years before being eliminated. Therefore, failure to detect *B. caballi* by PCR is most probably due to the parasites clearance from the circulating blood by the host or reduction to a level beyond the detection sensitivity of the PCR method used. In addition, all samples collected during this study were obtained from adult horses (4 years old and above) and this might be age dependent as observed by Rüegg et al. (2007).

To our knowledge, this is the first report on epidemiology of equine piroplasms using molecular techniques in Sudan. Accurate diagnosis of equine piroplasmosis is essential for providing baseline information about its epidemiology, distribution, and prevalence in the affected equine population and for effective control measures. However, considering the land mass of Sudan and the sample size used, further investigations on equine piroplasms are required.

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Isolation and pathogenic characterization of an OB1 variant of *Babesia rodhaini* which has a glycophorin A-independent pathway to murine red blood cells

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ABSTRACT

Recent studies using several *Babesia* spp. have demonstrated that these species commonly recognize host sialic acids of red blood cells (RBCs) for their invasion. Glycophorin A (GPA), which is a major carrier of the sialic acids on RBCs, is a possible invasive receptor for *Babesia* parasites. In the present study, a variant of *Babesia rodhaini* was successfully isolated from a GPA homozygous knockout ($GPA^{-/-}$) mouse infected with an Australian strain of *B. rodhaini* which had originally been unable to replicate in $GPA^{-/-}$ mice. The isolated parasite (designated as an OB1 variant) caused lethal infection to wild-type mice, as in the case of the parent Australian strain. However, although the growth of the OB1 variant in $GPA^{-/-}$ mice was comparable with that in wild-type mice at 1–4 days after infection, the growth was significantly inhibited from day 5 onward, leading to the eventual survival of the $GPA^{-/-}$ mice. Resistance of $GPA^{-/-}$ mice against OB1 infection was lost by splenectomy, although the cytokine responses to the infection in the sera of $GPA^{-/-}$ mice were similar to those of wild-type mice. The autoantibody levels to GPA-defective RBCs in the sera of $GPA^{-/-}$ mice were depressed at a lower level at 0–2 days after infection than those of wild-type mice, while the levels of $GPA^{-/-}$ mice progressively increased and reached comparable levels to those of wild-type mice at day 3 or later. These results indicate that the isolated OB1 variant has a GPA-independent invasion pathway into murine RBCs and suggest that the resistance of $GPA^{-/-}$ mice against infection with the OB1 variant may be attributed to the effective clearance of the parasitized RBCs lacking GPA in the spleen, possibly mediated by preferential autoantibody binding to the RBC membrane.

1. Introduction

Babesiosis, caused by hemoprotozoan parasites of the genus *Babesia*, is one of the most common infections of pastured animals and is responsible for remarkable economic losses to the livestock industry worldwide (Brown and Palmer, 1999). The disease has also received increased attention as a worldwide-distributed zoonosis in humans (Homer et al., 2000). The intraerythrocytic asexual development of *Babesia* spp., which is a critical part of their life cycle and is responsible for the majority of the clinical signs relating to babesiosis, is initiated by parasite invasion of host red blood cells (RBCs) (Homer et al., 2000). Therefore, a better understanding of the molecular mechanism of parasite invasion can contribute to the successful development of effective strategies against babesiosis.

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Recent studies have reported that the *in vitro* invasion or growth of several *Babesia* spp. is compromised when the host RBCs are treated with neuraminidase (Gaffar et al., 2003; Lobo, 2005; Okamura et al., 2005; Takabatake et al., 2007b), indicating that these species commonly use the host sialic acids on RBCs as their invasive receptor. Glycophorin A (GPA) is an erythroid-specific membrane protein conserved in mammals and is also a major carrier of sialic acids on RBCs (Matsui et al., 1989; Murayama et al., 1989). In our previous study, GPA-homozygous knockout ($^{-/-}$) mice were shown to be completely resistant against infection with *B. rodhaini* that causes lethal infection in wild-type mice (Takabatake et al., 2007a). Taken together, these results have suggested that *B. rodhaini* is dependent on the sialic acids of GPA for the invasion of murine RBCs. On the other hand, *in vitro* growth of *B. divergens* is only mildly inhibited when the host RBCs lack the GPA (Lobo, 2005), suggesting that *B. divergens* has an alternative GPA-independent invasion pathway. This implies the possibility that *B. rodhaini* also has an alternative GPA-independent invasion pathway.

In the present study, a variant of *B. rodhaini* that can proliferate in GPA$^{-/-}$ mice was successfully isolated. The pathogenicity of the cloned parasite (designated as the OB1 variant) to both wild-type and GPA-knockout mice was examined. The present findings indicate that *B. rodhaini* also has a GPA-independent invasion pathway into murine RBCs.

2. Materials and methods

2.1. Parasites

An Australian strain of *B. rodhaini* (kindly provided by the Kyusyu Branch of the National Institute of Animal Health, Japan) and a Munich strain of *B. microti* (kindly provided by the Free University of Berlin, Germany) have been maintained by intraperitoneal passage with the parasitized RBCs in mice at the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan. Parasitemia was determined by counting the percentage of parasitized RBCs in a total of 300–10,000 RBCs at the center of Giemsa-stained thin blood smears (Igarashi et al., 2000).

2.2. Mice

Eight- to 12-week-old female mice were used for all infectious experiments. GPA-homozygous knockout ($^{-/-}$) mice (Arimitsu et al., 2003) had been back-crossed into C57BL/6J mice (Nihon CLEA, Tokyo, Japan) for 12 generations at the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan (Takabatake et al., 2007a). C57BL/6J mice were used as the control wild-type mice. Genotyping of the mice was performed by PCR as previously described (Arimitsu et al., 2003).

2.3. Parasite cloning

The limited dilution cloning of OB1 was conducted as described below. The OB1 variant-infected blood was diluted with phosphate-buffered saline (PBS), pH 7.4, to 5 parasitized RBCs/ml. Into a splenectomized GPA$^{-/-}$ mouse, 0.2 ml of the diluted blood containing one parasitized RBC was inoculated intravascularly (I.V.). After the inoculation, parasitized blood was obtained from the mouse. The cloning procedure described above was repeated twice more. Parent Australian strain was also cloned as indicated above using splenectomized wild-type mice.

2.4. Experimental infections of mice

GPA$^{-/-}$ and wild-type mice were respectively inoculated intraperitoneally (I.P.) with 1×10^7 parasitized RBCs with the OB1 variant and the Australian strain cloned as above. After the infection, the parasitemia dynamics and survival rate of each group were monitored until the mouse died or the parasitemia reached an undetectable level. All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan.

2.5. DNA sequencing of the parasites' 18S ribosomal RNA (rRNA) gene

Blood from the OB1 variant, the cloned Australian strain, or *B. microti*-infected mice was obtained by cardiac puncture and anticoagulated with citrate-phosphate-dextrose with adenine (Sigma, St. Louis, MO, USA). RBCs were collected by centrifugation at 500 × *g* for 10 min at 4 °C and washed three times in PBS. The RBCs were lysed with an RBC lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) for 10 min at room temperature and then centrifuged at 15,000 × *g* for 10 min at 4 °C. Parasite DNAs were extracted from the pellet using proteinase K and phenol-chloroform-isoamyl alcohol (Sigma) as previously described (Dalrymple, 1990). The DNA sequences of 18S rRNA from *B. rodhaini* (Genebank accession number AB049999) and *B. microti* (Genebank accession numbers AB190459 and AB119446) were aligned with the CLUSTAL W Multiple Sequence Alignment Program (version 1.83, http://align.genome.jp/clustalw/) (Thompson et al., 1994). Based on the alignment, Buni1 (forward primer, 5′-gtcatatgcttgtcttaaagattaagcc-3′) and Buni2 (reverse primer, 5′-gccaataccctaccgtcca-3′) were designed on the conserved regions between *B. rodhaini* and *B. microti*. Partial 18S rRNA genes were amplified from each parasite DNA using Buni1 and Buni2 with a high-fidelity DNA polymerase (PrimeSTAR HS DNA Polymerase; Takara Bio, Otsu, Japan) according to the manufacturer's instructions. The thermal conditions of the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) were 30 cycles at 98 °C for 10 s, 55 °C for 5 s, and 72 °C for 1 min. The amplified DNA products were purified with a DNA fragment purification kit (MagExtractor; Toyobo, Osaka, Japan), sequenced directly using the primers above with an ABI PRISM BigDye TM Terminator Cycle Sequencing Kit (Applied Biosystems), and then analyzed on an ABI 3100 genetic analyzer (Applied Biosystems) according to the

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manufacturer's instructions. These DNA sequences were aligned with the CLUSTAL W Multiple Sequence Alignment Program.

2.6. Cytokine profiling

Serum samples were collected from the GPA$^{-/-}$ and wild-type mice at day 0 just before inoculation of 1×10^7 OB1-parasitized RBCs and, subsequently, at days 1–4 (every 24 h) and day 25 after the inoculation. Sera were stored at −80 °C until use. Gamma-interferon (IFN-γ), interleukin (IL)-2, IL-4, and IL-10 were quantified in the collected sera using mice Th1/Th2 cytokine and inflammatory cytometric bead array kits (BD Bioscience Pharmingen, San Diego, CA, USA) with a FACSCalibur flow cytometer (BD Bioscience Pharmingen) according to the manufacturer's instructions.

2.7. Preparation of RBC membrane extracts

The RBCs obtained from uninfected GPA$^{-/-}$ mice were treated with the RBC lysis buffer as above, centrifuged at 15,000 × g for 10 min at 4 °C, and then washed three times with PBS. The pellet was extracted with the same volume of PBS containing 1% (w/v) Triton X-100 for 10 min on ice. The suspension was centrifuged at 15,000 × g for 20 min at 4 °C, and the obtained supernatant was regarded as an RBC membrane extract. A parasitized RBC membrane extract was also prepared from OB1-infected GPA$^{-/-}$ mice (parasitemia, about 30%) in the same way. The protein concentrations of the extracts were determined with a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. These membrane extracts were stored at −80 °C until use.

2.8. Detection of antibody response by enzyme-linked immunosorbent assay (ELISA)

The serum samples described above were also examined to detect antibody binding to the RBC membrane extracts obtained from OB1-infected or uninfected GPA$^{-/-}$ mice by ELISA. Briefly, wells were coated with 50 μg of the indicated RBC membrane extract as above in a sodium carbonate buffer, pH 9.6, at 4 °C overnight and then incubated with a blocking solution in PBS for the immunoassay, pH 7.2 (Nacalai Tesque, Kyoto, Japan), for 1 h at 37 °C. The antigen-coated wells were incubated for 1 h at 37 °C with 100 μl of the indicated serum samples diluted in the blocking solution. Sera were tested over a range of serial twofold dilutions from 1:50. All sera were tested in duplicate, and the results were averaged. After washing with PBS containing 0.05% (w/v) Tween 20 (Sigma), 100 μl of goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (DAKO, Glostrup, Denmark) diluted at 1:1000 was added to each well and then incubated for 1 h at 37 °C. The enzyme reaction was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) (TBNP-0100-01; BioFX, Owings Mills, MD, USA). The reaction was terminated with 0.1 N HCl, and the absorbance was recorded at OD450 nm using an ELISA microplate reader (MTP-500; Corona, Hitachinaka, Japan). To detect the antibody response to parasite antigens, sera were incubated with the uninfected RBC membrane extract (1 μl of sera/50 μg of extract) for 1 h at 37 °C in the blocking solution and then subjected to ELISA as above.

2.9. Statistical analyses

The significance of differences between values in parasitemia, cytokine levels, and autoantibody levels was examined by the Mann–Whitney *U*-test. Survival estimates were calculated by the Kaplan–Meier method, and the significance of differences between values in survival rate was analyzed by the log–rank test. Differences giving $P < 0.05$ were regarded as significant.

3. Results

3.1. Isolation of the OB1 variant from a GPA$^{-/-}$ mouse infected with the parent Australian strain of *B. rodhaini*

To examine whether *B. rodhaini* has an alternative GPA-independent invasion pathway to murine RBCs, we repeated infectious experiments in which GPA$^{-/-}$ mice were inoculated I.P. with 1×10^7 RBCs parasitized with an Australian strain of *B. rodhaini*. From a total of 26 GPA$^{-/-}$ mice, only one mouse showed proliferation of the *Babesia* parasite in the blood circulation. The parasite was initially detected at day 11 after infection, and the parasitemia subsequently increased and peaked (27.9%) at day 14. It gradually decreased and reached an undetectable level on day 28 (Fig. 1). Blood was collected from the tail vein of the infected mouse and serially passaged I.P. in other GPA$^{-/-}$ mice seven times. Eventually, the parasite grew well in all of the inoculated GPA$^{-/-}$ mice. For further characterization, we cloned the parasite and the parent Australian strain by limiting dilution of the infected blood and subsequent inoculation into mice. The cloned parasite capable of proliferating in GPA$^{-/-}$ mice was designated as the OB1 variant. There was no morphological difference between the OB1 variant and the cloned Australian strain

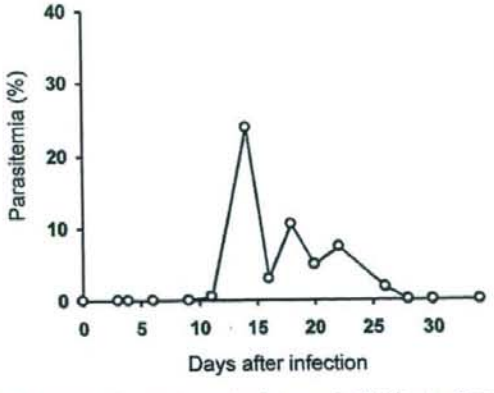

Fig. 1. Isolation of a *Babesia* parasite that grows in GPA$^{-/-}$ mice. From a total of 26 GPA$^{-/-}$ mice inoculated with 1×10^7 Australian strain of *B. rodhaini*-parasitized RBCs, only one showed proliferation of *Babesia* parasites in the blood circulation. The data indicate the dynamics of the parasitemia (%) in this mouse.

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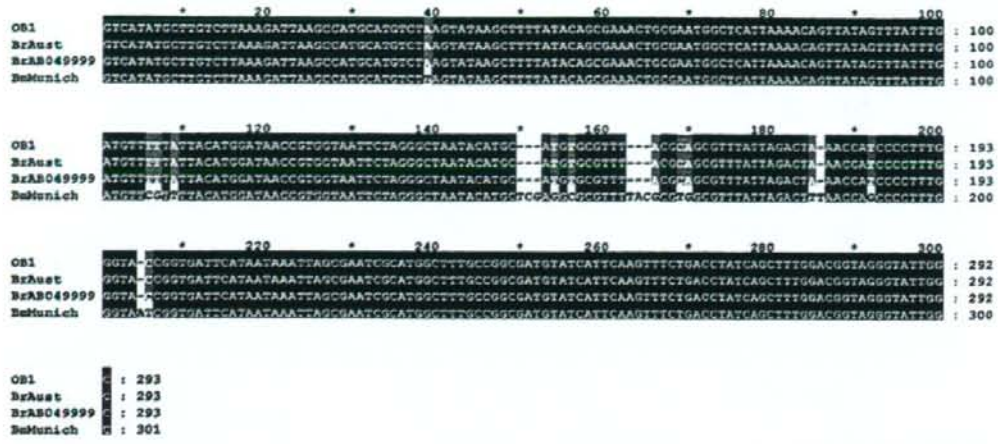

Fig. 2. Comparison of the partial DNA sequences of 18S rRNA genes from the OB1 variant, *B. rodhaini*, and *B. microti*. The multiple sequence alignment was generated using the ClustalW program. BrAust, the Australian strain of *B. rodhaini* cloned in our laboratory; Br AB04999, *B. rodhaini* (GenBank accession no. AB04999); BmMunich, the Munich strain of *B. microti* maintained in our laboratory; dashes, gaps introduced for optimal alignment. Residues shaded in black and gray are the conserved regions in four and three sequences, respectively. The determined sequences of BmMunich, BrAust, and OB1 were registered in the DDBJ database under accession numbers AB366158, AB366159, and AB366160, respectively.

(data not shown). To confirm that the OB1 variant was *B. rodhaini*, the partial DNA sequences of 18S rRNA genes from the OB1 variant, the cloned Australian strain of *B. rodhaini*, and a Munich strain of *B. microti* were determined and then aligned (Fig. 2). The aligned scores of the sequence of OB1 versus those of the cloned Australian strain of *B. rodhaini*, *B. rodhaini* (GenBank accession number AB049999), and the Munich strain of *B. microti* were 100, 100, and 96, respectively (Fig. 2). The data showed that the OB1 variant is *B. rodhaini* but not a contaminant derived from another rodent *Babesia* parasite.

3.2. Pathogeneses of the OB1 variant and the cloned Australian strain to wild-type and GPA$^{-/-}$ mice

The OB1 variant and the cloned Australian strain infections in wild-type mice were examined by comparing the parasitemia dynamics and survival rate in the two groups (5 mice in each group). The parasitemia rapidly increased in the mice infected with Australian strain, eventually leading to the death of hosts at day 6 after infection. The mice infected with OB1 variant also showed a rapid elevation of parasitemia and finally died at day 6 or 7 after infection (Fig. 3). No significant difference in parasitemia ($P > 0.347$) and survival rate ($P = 0.518$) was found between the OB1 variant and the cloned Australian strain in wild-type mice.

Next, the infectious course of the OB1 variant in GPA$^{-/-}$ infected mice was examined by comparing the parasitemia dynamics and survival rate with those of wild-type mice (5 mice in each group). In GPA$^{-/-}$ mice, although the parasitemia increased at a similar pace to that in wild-type mice by day 4 after infection, it peaked (40.7 ± 9.3%, mean ± standard deviation [SD]) at day 5, and the peak was significantly lower ($P = 0.037$) than that in wild-type mice (69.6 ± 19.4%) (Fig. 4). It subsequently decreased and reached an undetectable level at day 22, and all OB1-infected GPA$^{-/-}$ mice eventually survived (Fig. 4). In contrast, the growth of the cloned Australian strain was completely inhibited in the inoculated GPA$^{-/-}$ mice as reported before (Takabatake et al., 2007a). These data indicated that invasion and development of the OB1 variant

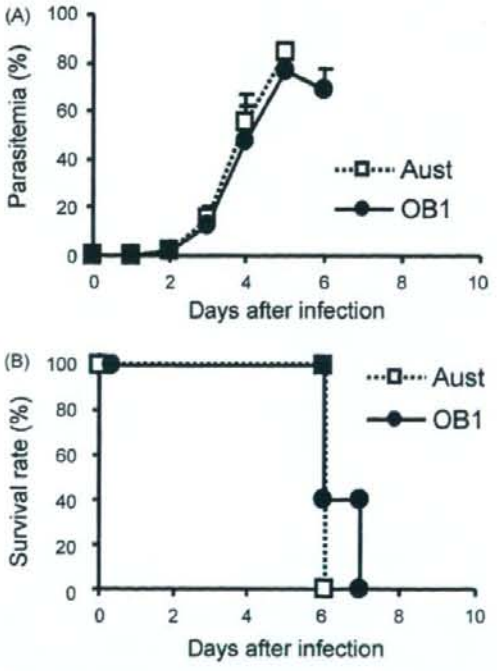

Fig. 3. Comparison of the pathogeneses of the cloned OB1 variant and the cloned Australian strain against wild-type mice. Wild-type mice ($n = 5$ in each group) were inoculated I.P. with 1×10^7 OB1 variant- or Australian strain-parasitized RBCs. (A) Dynamics of the parasitemia. Aust, Australian strain; Bar, SD. (B) Survival rate. Data are representative of two separate experiments.

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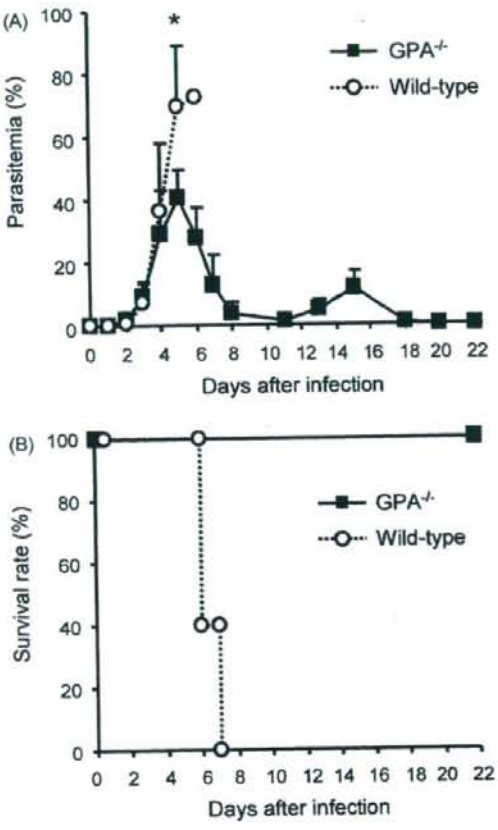

Fig. 4. Susceptibility of GPA$^{-/-}$ and wild-type mice to infection with the OB1 variant. GPA$^{-/-}$ and wild-type mice ($n = 5$ in each group) were inoculated I.P. with 1×10^7 of the parasitized RBCs. (A) Dynamics of the parasitemia. Bar, SD; $^*P = 0.007$. (B) Survival rate. Data are representative of three separate experiments.

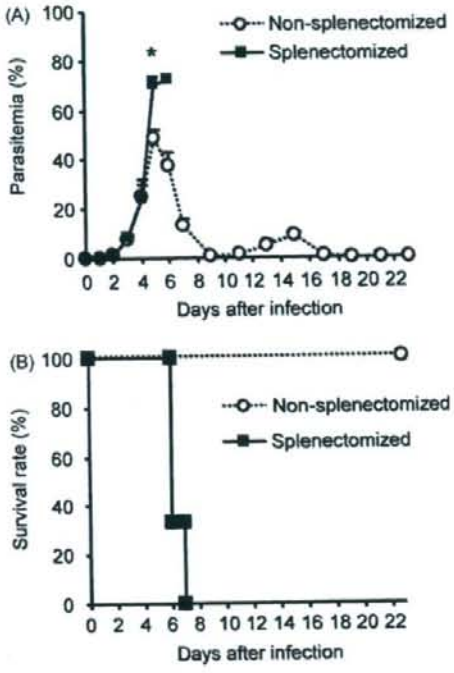

Fig. 5. Effect of splenectomy on the resistance of GPA$^{-/-}$ mice against infection with the OB1 variant. Non-splenectomized and splenectomized GPA$^{-/-}$ mice ($n = 3$ in each group) were inoculated I.P. with 1×10^7 parasitized RBCs. (A) Dynamics of the parasitemia. Bar, SD; $^*P = 0.049$. (B) Survival rate. Data are representative of two separate experiments.

within the GPA-defective RBCs were equal to those within wild-type RBCs, but GPA$^{-/-}$ mice inhibited the growth of the OB1 variant from day 5 onward.

3.3. Roles of the spleen and cytokine responses in the resistance of GPA$^{-/-}$ mice against OB1 infection

To assess the contribution of the spleen to the resistance of GPA$^{-/-}$ mice against OB1 infection, the parasitemia dynamics and survival rate of splenectomized GPA$^{-/-}$ mice were compared with those of non-splenectomized GPA$^{-/-}$ mice (3 mice in each group). The parasitemia in splenectomized GPA$^{-/-}$ mice acutely increased at a similar rate to that in wild-type mice (Figs. 3 and 4) at days 1–4, and all splenectomized mice were dead by day 6 or 7 (Fig. 5). Next, to evaluate the role of cytokine responses in the resistance of GPA$^{-/-}$ mice against OB1 infection (5 mice in each group), serum cytokine levels in GPA$^{-/-}$ and wild-type mice were monitored from day 0–4 after infection (Fig. 6). A spike in IFN-γ secretion at day 1 after infection and progressive increases of IL-2, IL-4, and IL-10 secretions were observed in GPA$^{-/-}$ and wild-type mice. There was no significant difference ($P > 0.066$) between the two groups (Fig. 6).

3.4. Antibody responses to RBC membrane extracts

To evaluate the contribution of the antibody responses to the resistance of GPA$^{-/-}$ mice against the OB1 variant infection, the levels of antibody recognizing the OB1-parasitized (Fig. 7A) or uninfected RBC membrane extract (Fig. 7B) were compared between serum samples periodically collected from GPA$^{-/-}$ and wild-type mice (5 mice for each group). Progressive increases in the antibody responses were observed in the groups against the parasitized and uninfected RBC membrane extracts, but the levels of GPA$^{-/-}$ mice were significantly lower ($P < 0.017$) than those of wild-type mice at 0–2 days after infection (Fig. 7A and B). There was no significant difference in antibody levels to the parasite antigens between the two groups by 4 days after infection (Fig. 7A and B). These results show that (1) the autoantibody level to host RBC membrane proteins was depressed in uninfected GPA$^{-/-}$ mice, (2) the level increased progressively and became comparable with that of wild-type mice after infection, and (3) specific antibody responses to the parasite antigens were not different between the GPA$^{-/-}$ and wild-type mice by 4 days after infection.

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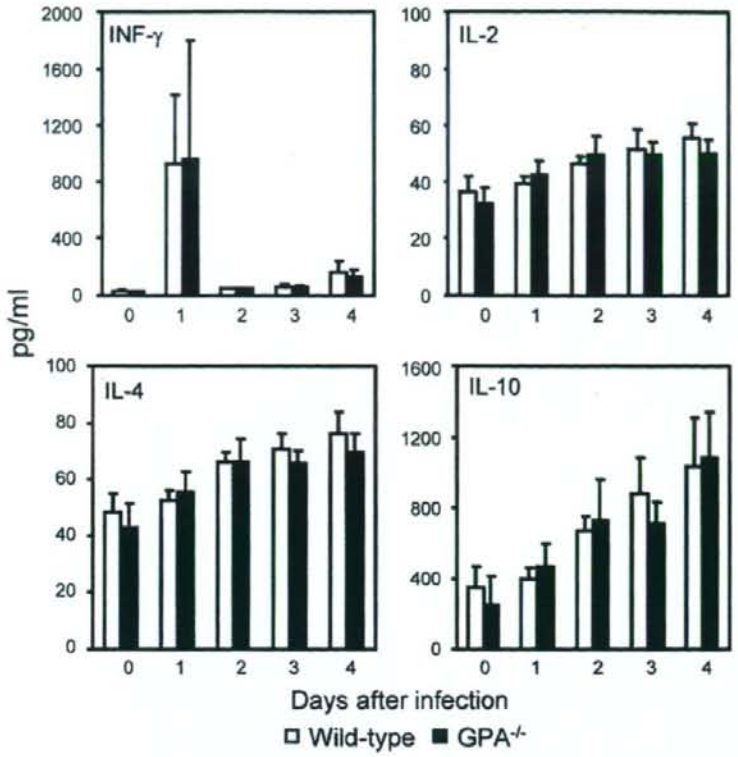

Fig. 6. Serum cytokine profiles in GPA$^{-/-}$ and wild-type mice after OB1 infection. Serum cytokine levels in GPA$^{-/-}$ and wild-type mice ($n = 5$ in each group) following inoculation of 1×10^7 OB1 variant-parasitized RBCs are presented as the mean ± SD at each sampling point. Data are representative of three separate experiments.

4. Discussion

The results in the present study showed that the growth of the isolated OB1 variant in GPA$^{-/-}$ mice was comparable to that in wild-type mice in the early phase of infection, indicating that the asexual growth cycle of the OB1 variant in the blood stage is independent of GPA on murine RBCs. While our previous study suggested the inability of the parent Australian strain of *B. rodhaini* to invade GPA-defective RBCs (Takabatake et al., 2007a), the results of the present study demonstrate that the OB1 variant of *B. rodhaini* has an alternative invasion pathway that is independent of GPA.

The parent stock of an Australian strain of *B. rodhaini* may contain a mixture of multiple parasite variants that have GPA-dependent or -independent invasion pathways. Because *B. rodhaini* undergoes an antigenic variation under host immune pressure (Allred, 2001), the parent population of the Australian strain maintained in mice for many years in our laboratory may have achieved multiple invasion pathways by antigenic variation. Consequently, GPA-deficient RBCs possibly selected the OB1 variant from the mixture of parasites that exhibit multiple invasion pathways. In *Plasmodium falciparum*, the polymorphism of the RBC binding ligand, BAEBL/EBA-140, leads to changes in its receptor specificity on human RBCs (Mayer et al., 2004). Alternatively, *B. rodhaini* may be able to originally vary in its dependency on GPA for invasion, as *P. falciparum* is known to have the ability to switch the receptor used for invasion from sialic acid-dependent to acid-independent pathways by differential expression of the ligand genes (Stubbs et al., 2005). By the present time, however, no parasite growth has been detected in the GPA$^{-/-}$ mice infected with the cloned Australian strain, not even in the splenectomized mice (data not shown).

We previously reported that intracellular parasites of *B. equi* cannot grow in neuraminidase-treated RBCs (Okamura et al., 2005) and that intracellular *B. bovis* incorporates the host sialic acids during asexual development, suggesting that the host sialic acids on RBCs are required not only for RBC invasion but also for the intraerythrocytic maturation of *Babesia* spp. (Okamura et al., 2007). In the present study, *B. rodhaini* could not be detected to incorporate the host GPA within the RBCs of infected wild-type mice as assessed by confocal microscopy with antiserum against mouse GPA as previously reported (Takabatake et al., 2007a), and no morphological abnormality was observed in the intracellular development of the OB1 variant. Further study is required to understand the involvement of the host sialic acids and GPA in the asexual growth cycle of *B. rodhaini*.

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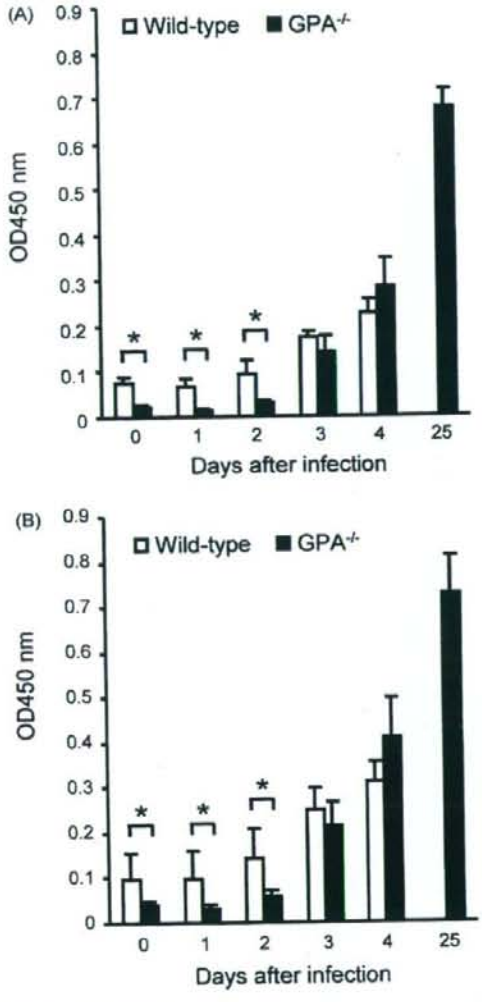

Fig. 7. Antibody responses in GPA$^{-/-}$ and wild-type mice against OB1-parasitized (A) or uninfected RBC membrane extract (B) after infection with the OB1 variant. Antibody levels in GPA$^{-/-}$ and wild-type mice ($n = 5$ in each group) following inoculation of 1×10^7 OB1 variant-parasitized RBCs against extract from the OB1 variant-infected or uninfected GPA-defective RBCs are presented as the mean ± SD at each sampling point. *$P < 0.016$ (wild-type vs. GPA$^{-/-}$ mice). The dilution fold of sample sera is 1:100. Data are representative of three separate experiments.

The present study also showed that GPA$^{-/-}$ mice could inhibit the growth of the OB1 variant in the later phase and eventually survived the lethal infection. The spleen is central to the innate and acquired immune responses to babesiosis (Brown et al., 2006). The spleen was indeed shown to be essential for growth inhibition in the present study, although the cytokine responses of GPA$^{-/-}$ mice were similar to those of wild-type mice after infection. The binding of naturally occurring autoantibodies to the host RBC membranes is known to mediate the clearance of aging RBCs by macrophage phagocytosis in the spleen (Ensinck et al., 2006), and the loss of RBC membrane constituents, such as sialic acids, enhances spleen clearance (Biondi et al., 2002). GPA$^{-/-}$ mice originally lose a large quantity of sialic acids from the surface of RBCs but never present with anemia (Arimitsu et al., 2003; Takabatake et al., 2007a). The lower level of autoantibody against the uninfected-RBC in GPA$^{-/-}$ mice should reflect the depression of the excessive activation of RBC clearance. After OB1 infection, the amount of the autoantibody progressively increased in GPA$^{-/-}$ mice and reached a comparable level with that of wild-type mice. Therefore, GPA-defective RBCs may become more susceptible to the clearance than wild-type RBCs under the increased appearance of autoantibodies following OB1 infection.

The presence of anti-RBC autoantibodies has been reported in infected mice with *B. rodhaini* (Matsuda et al., 1987), dogs with *B. gibsoni* (Adachi and Makimura, 1992), and cattle with *B. bigemina* (Goes et al., 2007). Although we agree that the presence of autoantibodies in the infected animals may be relevant to hemolytic anemia caused by the incidental destruction of uninfected RBCs under low parasitemia (Adachi and Makimura, 1992; Goes et al., 2007), the results in the present study suggest that the autoantibodies also contribute to the resistance of animals against *Babesia* infection. In particular, we postulate that an unknown alteration in the parasitized RBC membrane enhances the preferential binding of the autoantibodies to the parasitized RBC and induces the systematic clearance of the parasitized RBCs in the spleen. *P. falciparum* promotes oxidative alterations in the RBC membrane, which lead to the exposure of antigenic sites recognized by anti-band 3 autoantibodies and enhance the host phagocytosis of parasitized RBCs (Giribaldi et al., 2001; Turrini et al., 2003). Interestingly, the levels of parasite-induced oxidative modification and phagocytosis induction are remarkably higher in humans with mutant RBCs, which are widespread in geographic regions with a high incidence of *P. falciparum* malaria (Ayi et al., 2004).

In summary, the isolated OB1 variant of *B. rodhaini* has a GPA-independent invasion pathway to murine RBCs, and the resistance of GPA$^{-/-}$ mice against infection with the OB1 variant, which causes lethal infection in wild-type mice, may be attributed to the effective clearance of the parasitized RBCs lacking GPA in the spleen, possibly mediated by autoantibody binding to the RBC membrane. The OB1 variant and GPA will provide considerable insight into the molecular functions involved in the parasite invasion of host RBCs and the pathological mechanisms of babesiosis in the future.

Acknowledgments

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ORIGINAL PAPER

Evolutionary Analysis of Synteny and Gene Fusion for Pyrimidine Biosynthetic Enzymes in Euglenozoa: An Extraordinary Gap between Kinetoplastids and Diplonemids

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A unique feature of the genome architecture in the parasitic trypanosomatid protists is large-scale synteny. We addressed the evolutionary trait of synteny in the eukaryotic group, Euglenozoa, which consists of euglenoids (earliest branching), diplonemids, and kinetoplastids (trypanosomatids and bodonids). Synteny of the pyrimidine biosynthetic (*pyr*) gene cluster, which constitutes part of a large syntenic cluster in trypanosomatids and includes four separate genes (*pyr1—pyr4*) and one fused gene (*pyr6/pyr5* fusion), was conserved in the bodonid, *Parabodo caudatus*. In the diplonemid, *Diplonema papillatum*, we identified *pyr4* and *pyr6* genes. Phylogenetic analyses of pyr4 and pyr6 showed the separate origin of each in kinetoplastids and euglenoids/diplonemids and suggested that kinetoplastids have acquired these genes via lateral gene transfer (LGT). Because replacement of genes by non-orthologs within the syntenic cluster is highly unlikely, we concluded that, after separation of the line leading to diplonemids, the syntenic *pyr* gene cluster was established in the common ancestor of kinetoplastids, preceded by their acquisition via LGT. Notably, we found that diplonemid *pyr6* is a stand-alone gene, inconsistent with both euglenoid *pyr5/pyr6* and kinetoplastid *pyr6/pyr5* fusions. Our findings provide insights into the evolutionary gaps within Euglenozoa and the evolutionary trait of rearrangement of gene fusion in this lineage.

Key words: Euglenozoa; gene cluster; kinetoplastids; lateral gene transfer; pyrimidine biosynthetic enzymes; synteny.

Introduction

Trypanosomatids are flagellated parasitic protists and include medically important pathogens, such as those causing Chagas' disease, African sleeping sickness, and leishmaniasis. The phylogenetic position of trypanosomatids has been extensively studied using molecular phylogeny. Species of trypanosomatids are monophyletic, and this clade is nested in the kinetoplastid clade with bodonids, the sister group of trypanosomatids.

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Kinetoplastids, together with euglenoids and diplonemids, are assembled into a large monophyletic group, the Euglenozoa, which is characterized by distinctive mitochondria with discoid cristae (Cavalier-Smith 1981). Of the three branches of Euglenozoa, the euglenoids constitute the earliest branch, followed by separation of the diplonemid and kinetoplastid lineages (Simpson and Roger 2004; Simpson et al. 2002).

Synteny, the preserved order of genes, is often observed in the genomes of phylogenetically related eukaryotic species. Conserved synteny is used as an evolutionary marker, which can not only indicate the descent of different species from a common ancestor but also functional and/or evolutionary relationships of the clustered genes (Bennetzen and Freeling 1997; Nadeau 1989).

Comparative genomics of three trypanosomatids, *Trypanosoma cruzi*, *T. brucei*, and *Leishmania major*, have highlighted large-scale synteny of polycistronic gene clusters as a feature of their unique genome architecture (El-Sayed et al. 2005). That is, protein-encoding genes, most of which are functionally unrelated, are tandemly arrayed on either strand of DNA as syntenic gene clusters and constitute polycistronic transcription units (Bonen 1993; Liang et al. 2003; Martínez-Calvillo et al. 2004). Due largely to the lack of genomic information on other euglenozoan groups, however, the origin of conserved synteny and gene clustering in trypanosomatids has not yet been determined (Dávila and Lukeš 2003; Jackson et al. 2006).

The de novo pyrimidine biosynthetic pathway is one of the essential catalytic activities in organisms, which produces uridine-5′-monophosphate (UMP) for incorporation into DNA and RNA. This pathway consists of six enzymes: pyr1 (EC 6.3.5.5, carbamoyl-phosphate synthetase II), pyr2 (EC 2.1.3.2, aspartate carbamoyltransferase), pyr3 (EC 3.5.2.3, dihydroorotase), pyr4 (EC 1.3.3.1, dihydroorotate dehydrogenase), pyr5 (EC 2.4.2.10, orotate phosphoribosyltransferase), and pyr6 (EC 4.1.1.23, orotidine-5′-monophosphate decarboxylase), in their order of reaction.

We previously showed that all *pyr* genes are clustered in the genomes of two trypanosomatid species, *T. cruzi* (Gao et al. 1999) and *L. mexicana* (GenBank™ Accession number AB029444), in both of which five genes, *pyr1, pyr3, pyr6/pyr5* fused gene, *pyr2,* and *pyr4* are juxtaposed in this order on chromosomal DNA. Similarly, *T. brucei* possesses the *pyr* gene cluster, although *pyr3* is not annotated in this cluster (Berriman et al. 2005). The *pyr* gene cluster is the only known clustering of genes that encode all enzymes in an essential metabolic pathway in eukaryotes, while there are examples in filamentous fungi of the clustering of genes encoding enzymes catalyzing secondary metabolites (Saikia et al. 2007; Young et al. 2006).

Clustering of genes coding for a metabolic pathway is structurally similar to bacterial operons, which may be advantageous for concerted expression of functionally related enzymes at the appropriate times in these bacteria. However, in trypanosomatids, no regulatory mechanism of transcription initiation is found and regulation of expression seems to occur at the post-transcriptional level. Thus, the biological significance of the occurrence of the *pyr* gene cluster in trypanosomatids is still unknown.

The *pyr* gene cluster appears to constitute part of a large polycistronic gene cluster in trypanosomatids and, importantly, includes genes acquired via lateral gene transfer (LGT). Indeed, trypanosomatid genomes contain large numbers of genes thought to have been acquired via LGT (Opperdoes and Michels 2007). Phylogenetic analyses of pyr4 and pyr6 have shown that both genes have a prokaryotic origin, not only in trypanosomatids but also in bodonids (Annoura et al. 2005; Makiuchi et al. 2007; Nara et al. 2000). Thus, LGT events are likely to have preceded the establishment of the *pyr* gene cluster, as well as contributing to it.

In the present study, we regarded the *pyr* gene cluster as a model synteny and addressed whether this cluster is present in non-trypanosomatid kinetoplastids, i.e. bodonids. We demonstrate clustering of the *pyr* genes in the bodonids, *Parabodo caudatus* (formerly *Bodo caudatus*) and *Neobodo saliens* (formerly *B. saliens*), suggesting that this gene synteny emerged in a common ancestor of kinetoplastids. In addition, we found that *pyr4* and *pyr6* genes in the diplonemid, *Diplonema papillatum*, had a different origin from the kinetoplastid genes. Phylogenetic and gene organization analyses suggested that the standalone *pyr6* in diplonemids might represent a transitional status from the fused *pyr5/pyr6* in euglenoids to the inversely fused *pyr6/pyr5* in kinetoplastids. Our findings provide insights into an evolutionary gap between kinetoplastid and non-kinetoplastid groups in the Euglenozoa.

Results

The *pyr* Gene Cluster in the *P. caudatus* Genome

The *pyr1* and *pyr4* genes are the 5′- and 3′-terminal genes, respectively, of the *pyr* gene

cluster in trypanosomatids (Gao et al. 1999). Therefore, sequence analysis of the regions downstream to *pyr1* and upstream to *pyr4* would allow us to detect the putative *pyr* gene cluster in the bodonid genome.

By a series of PCR amplifications using *P. caudatus pyr*-specific primers and the genomic DNA as templates, we identified the *pyr* gene cluster in the *P. caudatus* genome (Fig. 1. See also Supplementary Fig. S1). The *P. caudatus pyr* gene cluster consists of 11,285 bp and shows the same gene order and organization as the trypanosomatid ones. We found that, compared with the parasitic trypanosomatids, free-living *P. caudatus* has shorter untranslated regions between the *pyr* genes. Southern blot analysis showed that all *pyr* genes are single-copy genes and are juxtaposed on chromosomal DNA (Supplementary Fig. S1).

We also obtained a partial *pyr* gene cluster in *N. saliens* by screening the genomic DNA library

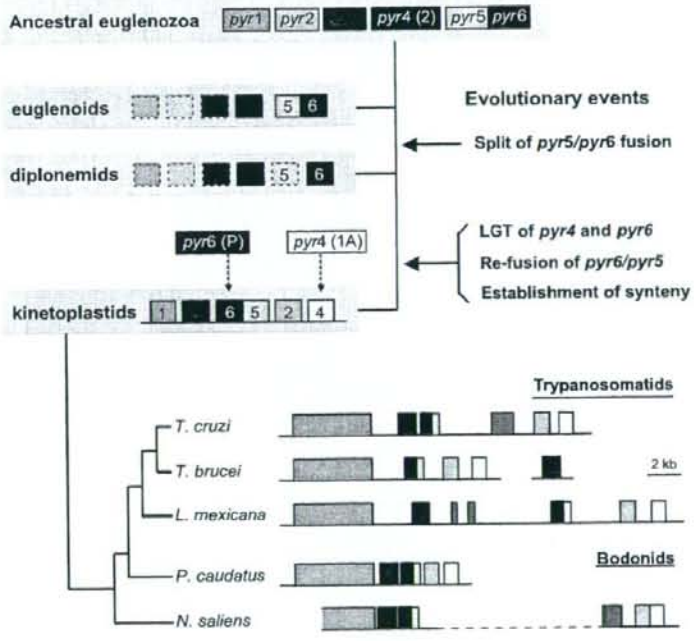

Figure 1. The evolutionary scenario of synteny and gene fusion of pyrimidine biosynthetic (*pyr*) genes in Euglenozoa. The euglenozoan tree is based on a recently proposed model, in which euglenoids constitute the earliest branch, followed by separation of the diplonemid and kinetoplastid lineages (Simpson et al. 2006b). The de novo pyrimidine biosynthetic pathway is composed of six enzymes and encoded by *pyr1—6* genes. Genes for the first three enzymes, pyr1, pyr2, and pyr3, are fused in animals, Fungi, and Amoebozoa (called unikonts), while they are assumed to be independent in the other eukaryotic groups (bikonts), including Euglenozoa (Stechmann and Cavalier-Smith 2003). In this scheme, an ancestral euglenozoa is assumed to have independent *pyr1*, *pyr2*, *pyr3* genes, family-2 type *pyr4* gene, and *pyr5/pyr6* fused gene. Boxes with a dotted line indicate as yet unidentified genes in euglenoids and diplonemids. The stand-alone *pyr6* gene in diplonemids was derived by secondary split of *pyr5/pyr6*. An ancestral kinetoplastid acquired both family-1A *pyr4* and *pyr6* of a prokaryotic origin, as indicated by dashed arrows (Annoura et al. 2005; Makiuchi et al. 2007). The gene re-fusion event occurred subsequently between the stand-alone *pyr5* and the acquired prokaryotic *pyr6*. Following these events, the syntenic *pyr* gene cluster was established. The *pyr* gene cluster in the kinetoplastid species (lower panel) is illustrated along with their organismal tree. It is unclear whether all *pyr* genes cluster in *N. saliens*. Gray boxes indicate genes unrelated to pyrimidine synthesis. Note: *pyr4* (2), family-2 *pyr4*; *pyr4* (1A), family-1A *pyr4*; *pyr6* (P), *pyr6* of a possible prokaryotic origin.