Prevalence of equine piroplasmosis in Central Mongolia

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ABSTRACT

AVARZED, A., DE WAAL, D.T., IGARASHI, I., SAITO, A., OYAMADA, T., TOYODA, Y. & SUZUKI, N. 1997. Prevalence of equine piroplasmosis in Central Mongolia. *Onderstepoort Journal of Veterinary Research*, 64:141–145

Antigen for the indirect fluorescent antibody test (IFAT) was routinely prepared from infected erythrocytes from horses experimentally infected with *Babesia equi* and *Babesia caballi*. With the successful establishment of *in vitro* cultures of *B. equi* and *B. caballi*, it is now possible to employ culture-derived antigens in this test. In this study, *in vitro*-propagated *B. equi-* and *B. caballi*-infected erythrocytes were used as antigen in the IFAT. Various modifications to an established protocol had to be implemented to allow repeatable results. Cultures with 3–4 % parasitized erythrocytes were found to be most suitable. As cross-reactions of control sera on heterologous antigen were observed at serum dilutions of up to 1/40, a reciprocal titre of 80 was considered to be positive. In positive samples, specific fluorescence of *Babesia* parasites and/or erythrocyte membranes was observed. Fifteen sera from *Babesia*-free horses from Japan all tested negative in the IFAT. One hundred and ten field-horse sera from Central Mongolia were investigated in this study. The results indicate that both *B. equi* and *B. caballi* are endemic in horses in Central Mongolia, with 88,2 % and 84,5 % of horses being seropositive to *B. equi* and *B. caballi*, respectively.

Keywords: *Babesia caballi, Babesia equi*, equine piroplasmosis, prevalence

INTRODUCTION

Equine piroplasmosis is caused by *Babesia equi* and *Babesia caballi* and is endemic in equids throughout most of the tropical and subtropical regions of the world (Friedhoff, Tenter & Müller 1990). *B. caballi*, a typical large *Babesia*, infects and multiplies only in erythrocytes and is transmitted transovarially and transstadially by its tick vectors (Friedhoff 1988). In the case of *B. equi*, however, it has been reported that the parasite apparently first multiplies in lymphocytes (Schein, Rehbein, Voigt & Zweygarth 1981; Rehbein, Zweygarth, Voigt & Schein 1982) before invading erythrocytes in which merozoites can frequently be seen arranged in a typical Maltese-cross formation. It is transmitted transstadially only by its tick vectors.

Babesia parasites are usually detected in blood smears only during the acute stage of the infection and animals that recover from the disease remain carriers of these parasites. It has been reported that horses can remain carriers of *B. caballi* parasites for up to 4 years, while they probably remain life-long carriers after infection with *B. equi* (Friedhoff 1982). Identification of these carriers and previously exposed horses can most easily be accomplished by demonstrating specific antibodies in the serum with serological tests (Weiland & Reiter 1988).

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The indirect fluorescent antibody test (IFAT) is the most widely used serological technique to detect specific antibodies in protozoan infections. Ristic & Sibinovic (1964) first reported the use of the IFAT to detect *B. caballi* antibodies in horses. Madden & Holbrook (1968) successfully applied this test to differentiate between *B. equi* and *B. caballi* infection in horses and did not report any cross-reaction. Antibodies detectable by the IFAT start to rise shortly after the first parasites appear in blood smears, and persist for long periods (Donnelly, Joyner & Frank 1980; Weiland 1986; De Waal 1995).

In Mongolia, Dash (1957, 1959, cited by Dash 1967), using microscopic examination of thin blood smears, reported that both *B. equi* and *B. caballi* are widespread in horses. The tick vectors in this region are unknown, but *Dermacentor nuttali* (Olenev 1929, cited by Dash 1967), *Dermacentor salvarum* (Shulth 1933, cited by Dash 1967) and *Hyalomma dromedari* (Svirskaya 1961, cited by Dash 1967) frequently infest horses and all have been reported to be vectors of *B. caballi* elsewhere. However, no serological survey has been conducted to determine the prevalence of these two parasites in horses. In this study *in vitro*-propagated parasites were used as antigen in the IFAT to determine the prevalence of *B. equi* and *B. caballi* antibodies in field horses from Central Mongolia.

MATERIALS AND METHODS

Strains of *Babesia* species

B. equi and *B. caballi*, both USDA strains, were used in this study and were obtained from experimentally infected horses at Tochigi Research Station of the Equine Research Institute, Japan Racing Association.

Serum samples

Serum samples from horses experimentally infected with *B. equi*, OP isolate (horse no. 460-9) and *B. caballi*, SWA isolate (horse no. 042-9), were obtained from Onderstepoort Veterinary Institute (OVI), South Africa and used as positive control sera. Negative serum was obtained from a horse raised under tick-free conditions at OVI. Blood samples were collected from 15 *Babesia*-free horses in Japan and from 110 horses of the Mongolian native breed, originating from two different herds in the Central Province of Mongolia. These were allowed to clot at room temperature overnight before serum was removed and stored at –20 °C. At the time of blood collection, thin blood smears were also prepared and later stained with Giemsa solution.

Antigen preparation for IFAT

Antigen was prepared from *in vitro*-propagated *B. equi*- or *B. caballi*-infected erythrocytes (Avarzed, Igarashi, Kanemaru, Omata, Saito, Nagasawa, Toyoda & Suzuki 1995). *In vitro*-propagated *Babesia* parasites were transferred from one well of a 6-well culture plate to a 15-mℓ centrifuge tube and centrifuged for 15 min at 700 g at 4 °C. After the first centrifugation, the erythrocyte pellet was washed five times by centrifugation for 5 min at 350 g and after each wash, reconstituted with 10 mℓ of PBS(-)[147 mM NaCl, 5 mM NaH_2PO_4 ,5 mM Na_2HPO_4, pH 7,2]. Any visible white-blood-cell layer was removed after each wash. After the fifth wash, one part packed red-blood cells was reconstituted with two parts of 4 % bovine serum albumin in PBS(-) (1:2, v/v). The blood mixture was dispensed onto previously cleaned glass slides by means of a 1-mℓ syringe and 27-G needle. Fifteen antigen spots, 5 mm in diameter (three rows of five spots), were prepared per slide. The slides were dried for 30 min at 45 °C in a drying oven (forced-convection oven FC-610, Toyo Seisaku, Co., Ltd) and after that for a further 30 min at room temperature. The slides were then wrapped in tissue paper, covered with aluminum foil and stored at –20 °C.

IFAT technique

Antigen slides were removed from storage at –20 °C and placed in a drying oven at 45 °C for 1 h. The antigen spots on the slides were encircled with a pap pen (Kantoukagaku, Japan) and fixed in a large volume of cold acetone (–20 °C) for 5 min. Two-fold serum dilutions were prepared in PBS(-) starting at 1/10–1/5120 dilution. Every test included *B. equi* and

TABLE 1 Summary of IFAT results of Mongolian horse sera

Antigen	Number of horses	
	Seronegative[a]	Seropositive[b]
Babesia equi	13 (11,9%)	97 (88,2%)
Babesia caballi	17 (15,5%)	93 (84,5%)

[a] Reciprocal titre < 80
[b] Reciprocal titre ≥ 80

TABLE 2 Prevalence of *Babesia equi* and *B. caballi* in different age groups of Mongolian horses

Age (years)	Seronegative		Seropositive	
	B. equi	*B. caballi*	*B. equi*	*B. caballi*
1	0	1	16	15
2	1	3	21	19
3	2	3	12	11
4	0	3	13	10
5	0	0	11	11
6–10	6	3	23	26
11–19	4	4	1	1

B. caballi positive and negative control sera as well as a PBS control. Ten microliters of each serum dilution were then placed on the antigen spots. Slides were incubated in a humid chamber at 37 °C for 30 min. The slides were then rinsed in fresh PBS(-) and washed once for 10 min in PBS(-), followed by a further washing with deionized-distilled water for 5 min. Goat anti-horse IgG FITC conjugate (Bethyl Laboratories, Inc., USA.) was diluted 1/80 in 0,01 % Evans blue-PBS(-) (to reduce non-specific fluorescence) and 10 mℓ of diluted conjugate was pipetted onto each spot. The slides were incubated, rinsed and washed as described above. After the last wash, slides were allowed to air dry slightly before being overlaid with 50 % glycerin-PBS(-) (1:1) and covered with a 24 x 60 mm cover slip. The slides were examined under a fluorescent microscope with the use of a 40x objective lens (Microphot EPI-FL, Nikon, Japan). Fluorescence was interpreted as positive (+), trace (±) or negative (−). The highest serum dilution showing positive fluorescence was taken as the titre of the sample.

RESULTS

Validation of IFA with parasites from *in vitro* culture

In vitro-propagated *B. equi* and *B. caballi* were investigated as antigen source in the IFAT. When the test was performed with *B. equi* or *B. caballi* antigen and homologous positive control sera, strong specific fluorescence of the parasites within the erythrocytes was observed (Fig. 1). Some cross-reactions were observed between the positive-reference serum and the reciprocal antigen at low serum dilutions ≤ 1/40 (data not shown) but no specific fluorescence was observed at 1/80 or higher dilutions. Sera from blood samples collected from the 15 horses born and reared in Japan and regarded as *Babesia*-free,

showed some non-specific fluorescence at low serum dilutions (≤ 1/40) on both *B. equi* and *B. caballi* antigen (Fig. 2). Therefore, a reciprocal titre of 80 with antigens cultured *in vitro* was considered as being positive for the infections.

Screening of Mongolian sera with IFA

Although no blood parasites were detected in the Giemsa-stained blood smears from any of the 110 horses from Central Mongolia, a wide range (1/20–1/2560) of IFA end titres to both *B. equi* and *B. cabali* was found in them (Fig. 3). Based on the criterion for positivity as described above, the prevalence of *B. equi* and *B. caballi* infection in Mongolia was 88,2 % and 84,5 %, respectively (Table 1). The ages of the Mongolian horses varied from 1–19 years and *B. equi* and *B. caballi* antibodies were detected in all age groups, with 90 horses (81,8 %) being seropositive to both infections (Table 2).

DISCUSSION

Until recently, most researchers have performed the IFAT for equine piroplasmosis on infected erythrocyte antigen prepared from experimentally infected horses (Ristic & Sibinovic 1964; Madden & Holbrook 1968; Tenter & Friedhoff 1986; Weiland 1986; De Waal 1995). With the successful establishment of *in vitro* cultures of *Babesia*, it is now possible to employ culture-derived antigens in the IFAT and to avoid the use of experimentally infected animals as antigen sources. Although Böse (1994) reported the use of micro-aerophilous stationary-phase (MASP) cultures as an antigen source for *B. caballi* IFAT, he did not comment on the antigen-preparation procedure. Many difficulties were initially experienced in establishing the IFAT procedure when MASP cultures of *B. equi* or *B. caballi* were used as antigens for the IFAT in comparison with the antigens obtained from
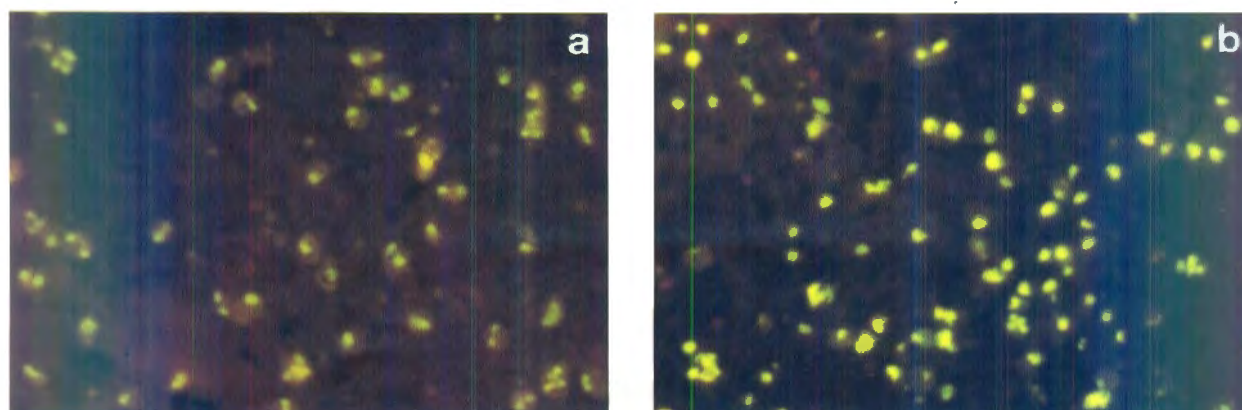

FIG. 1   Immunofluorescent reactions; (a) *Babesia caballi* antigen with *B. caballi* positive serum, (b) *Babesia equi* antigen with *B. equi* positive serum
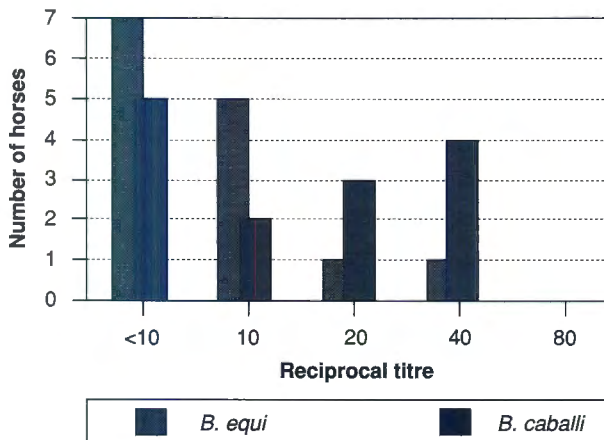
FIG. 2   Distribution of IFAT end titres for *Babesia equi* and *Babesia caballi* in *Babesia*-free horses from Japan
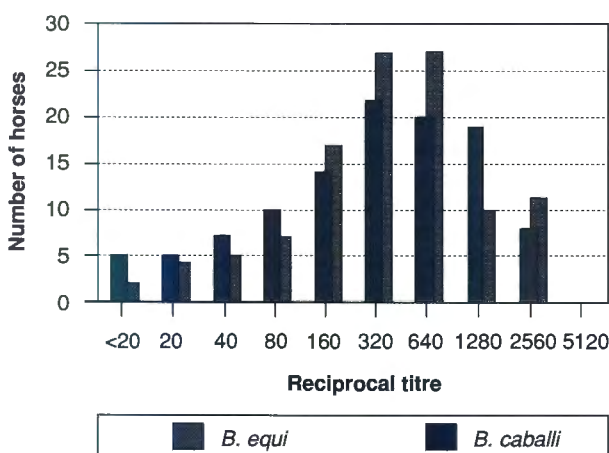

FIG. 3   Distribution of IFAT end titres for *Babesia equi* and *Babesia caballi* in Mongolian horses

infected horses (unpublished observations). Consequently, various modifications had to be implemented to allow repeatable and consistent results. In this study, cultures with 3–4 % parasitized erythrocytes were found to be most suitable. Cross-reactions of control sera with heterologous antigen were observed at serum dilutions of up to 1/40. Similar results were reported by other workers using antigen from experimentally infected horses (Ristic & Sibinovic 1964; Madden & Holbrook 1968; Weiland 1986; Tenter & Friedhoff 1986; De Waal 1995). Although we were unable to completely eliminate the non-specific binding of conjugate to *B. caballi* antigen, it was distinctly different from the positive control and did not interfere with the interpretation of the test.

The Mongolian horse, famous for its hacking qualities, is also used for racing, draught, and milk and meat production. Horse husbandry in Mongolia has

remained a pastoral nomadic system, which includes seasonal migrations and rotations of migration routes. This seems to be a possible reason for re- and mixed infections of horses with *Babesia* parasites. The present study confirmed that both babesial infections occur in horses in Central Mongolia. As shown in Fig. 3, 90 horses had high titres (1/160–1/2560) for *B. equi* and 82 horses for *B. caballi*. The results from this limited survey suggest that equine piroplasmosis is probably endemic in Central Mongolia. Very few clinical cases of equine piroplasmosis are reported in horses in Central Mongolia, probably owing to the fact that most horses in this survey were infected early in life (93,75 % of 1-year-old horses being sera positive to both parasites) when they were protected by colostral immunity and other non-specific factors, and then became carriers (De Waal 1995). However, surprisingly few horses in the 11–19-year age group were seropositive, which is difficult to explain in the light of the high prevalence in the other age groups. The tick vectors of these parasites in Mongolia are still unknown, but *D. nutalli* (Olesnev 1929, cited by Dash 1967), *D. salvarum* (Shulth 1933, cited by Dash 1967) and *H. dromedari* (Svirskaya 1961, cited by Dash 1967) frequently infest horses and all have been reported to be vectors of *B. caballi* in Europe and Russia (Friedhoff 1988). Further surveys and studies on the vectors of equine piroplasmosis are necessary to evaluate the epidemiological status of this disease in Mongolia.

ACKNOWLEDGEMENT

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