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EQUINE PIROPLASMOSES AT THE REINTRODUCTION SITE OF THE PRZEWALSKI'S HORSE (*EQUUS FERUS PRZEWALSKII*) IN MONGOLIA

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ABSTRACT: Piroplasmosis has been identified as a possible cause of mortality in reintroduced Przewalski's horses (*Equus ferus przewalskii*) in the Dsungarian Gobi (Mongolia). A cross-sectional and a longitudinal study were conducted in a representative sample ($n=141$) of the resident domestic horse population and in 23 Przewalski's horses to assess the prevalence of *Theileria equi* and *Babesia caballi*. Piroplasms were detected in blood by light microscopy in 6.7% (95% confidence interval [CI]: 3.6–12.2%) of the domestic horse samples. Antibody prevalence was 88.6% (95% CI: 82.4–92.9%) for *T. equi* and 75.2% (95% CI: 67.4–81.6%) for *B. caballi*. Antibody prevalence did not change over time, but antibody prevalence for both piroplasms were significantly lower in animals less than 1 yr of age. For both piroplasms, the prevalence of presumably maternal antibodies (falling titers) in foals was 100%. Only one of 16 foals seroconverted against *T. equi* during the study period, despite that piroplasms were found in two other individuals. The incidence density (ID) of *T.equi* in foals was therefore 0.0012 seroconversions per horse day (95% CI: 0.00029–0.0057). In contrast, yearlings had an ID of 0.0080 (95% CI: 0.0049–0.010) for *T. equi* and 0.0064 (95% CI: 0.0036–0.0093) for *B. caballi*, and in seven individuals piroplasms were detected. The seroprevalence of both piroplasms rose from 20% in spring to 100% in autumn. Comparison of domestic and Przewalski's horses resulted in a standardized prevalence ratio (SPR) of 0.98 (95% CI: 0.80–1.24, not significant) for *B. caballi*; in contrast, the prevalence of *T. equi* in Przewalski's horses was significantly lower than expected (SPR=0.51, 95% CI: 0.50–0.64).

Key words: *Babesia caballi*, epidemiology, *Equus ferus przewalskii*, Mongolia, *Theileria equi*.

INTRODUCTION

The Przewalski's horse (*Equus ferus przewalskii*), the ancestor of the domestic horse, was endemic to Mongolia. It became extinct in the wild in the 1960s (Van Dierendonck and Wallies De Vries, 1996), but is being reintroduced to its previous home range at three different locations. A retrospective analysis of reports (since 1992) and pathologic samples (since 1999) collected in the project in Takhin Tal in the Dsungarian Gobi showed an elevated mortality rate for reintroduced horses during April and May (Walzer et al., 2000). Pathologic findings in two Przewalski's horses that died in 1999 strongly suggested that equine piroplasms (*Babesia caballi* and *Theileria equi*, formerly *Babesia equi*, Mehlhorn and Schein, 1998) were responsible for these deaths. In addition, a high parasitemia was detected in a stillborn fetus in 2000 (Robert et al., 2005). Serologic analysis of three serum samples indicated that the naïve reintroduced individuals become infected with piroplasms during their first summer (Walzer et al., 2000). No antibodies were detected in the introduced Przewalski's horses prior to their translocation to Mongolia, because they originated from captive breeding programs where piroplasmoses only occur sporadically or are nonendemic (OIE, 2004b).

Babesia caballi and *T. equi* are probably endemic throughout Asia with the exception of Siberia and Japan (Friedhoff and Soule, 1996). Previous studies in northern

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China (Yin et al., 1997) and central Mongolia (Avarzed et al., 1997; Ikadai et al., 2000; Boldbaatar et al., 2005) reported serum antibody prevalences for *T. equi* of between 14.0% and 88.2% and for *B. caballi* of between 46.0% and 84.5%. Possible vectors in the Gobi are the ixodid tick species *Dermacentor nuttalli*, *Dermacentor marginatus* (syn. *Dermacentor niveus, Dermacentor daghestanicus*), *Hyalomma asiaticum asiaticum*, *Hyalomma asiaticum koslovi*, *Hyalomma dromedarii*, and *Rhipicephalus pumilio* (Dash et al., 1988). Both, *D. marginatus* and *D. nuttalli* are known to transmit *B. caballi* (Friedhoff, 1988; Qi et al., 1995) and *T. equi* (Pomerantzev, 1959; Battsetseg et al., 2001; Battsetseg et al., 2002). The aim of this study was to determine the prevalence of equine piroplasmoses among domestic and reintroduced Przewalski's horses in Mongolia and compare prevalences in the two populations.

MATERIAL AND METHODS

Horses

Prevalence of piroplasms and antibodies to *T. equi* and *B. caballi* in the domestic horse population living in vicinity to the reintroduction site of Przewalski's horses in Takhin Tal (45°32′19″N, 093°39′05″E) was assessed in 2001 with a cross-sectional survey. The incidence and the age of the hosts at first infection were assessed with a longitudinal study during the same time.

Cross-sectional study: According to counts of the year 2000, approximately 450 domestic horses were kept in the vicinity of the reintroduction site. Based on previous studies (Avarzed et al., 1997; Ikadai et al., 2000), a sampling fraction of 50% in each of the 19 herds was chosen (median=4/herd, range 1–47). The horses were identified by color, gender, and age. For venipuncture, the subjects were immobilized with a lasso and fixed in lateral recumbence. Blood was drawn from the jugular vein into plain and ethylenediaminetetraacetic acid tubes. Immediately after collecting blood, smears were made from anticoagulated blood. The samples were then left for sedimentation or coagulation overnight. One to 2 days after collection, serum and plasma were separated and stored at −11 C in a solar-powered freezer. Samples from Przewalski's horses were taken over a period of 2 yr (1999–2001) when immobilized for management reasons. No blood smears from Przewalski's horses were available from the samplings prior to 2001, but nine samples taken during this study included blood and smears. At its conclusion, the cross-sectional study included samples from 141 domestic and 23 Przewalski's horses.

Longitudinal study: Sixteen yearlings from a herd of 250 domestic horses belonging to the Mongolian army were marked with subcutaneous microchips (Indexel®, Mérial, Lyon, France). They were sampled as described above at an average interval of 19 days starting 15 April 2001. Because Mongolian herds are free roaming and not all animals could be found in the pastures at each sampling date, two were sampled five times, nine were sampled four times, and three were sampled three times. Two yearlings were sampled only once and were therefore omitted for the calculation of the incidence. Sixteen foals from the same herd were sampled for the first time at an age of 2 to 4 wk. Starting on 2 June 2001, they were sampled four times ($n=10$), three times ($n=4$), or twice ($n=2$). At the first sampling, blood was also drawn from the dams. The latest available sample from each yearling and foal was additionally included into the cross-sectional study.

Laboratory examination

For each individual, one air-dried blood smear was fixed in ethanol, Giemsa-stained and examined microscopically (Böse et al., 1995). Detected piroplasms were classified as *B. caballi* when large paired piriform or several large single inclusion bodies were found in the erythrocytes (Fig. 1); they were attributed to *T. equi* when a Maltese cross or several small single inclusion bodies were found (Fig. 2). For serology, indirect immunofluorescence antibody tests (IFAT) were performed according to the World Organization for Animal Health (OIE) Manual of Standards Diagnostic Tests and Vaccines (Tenter and Friedhoff, 1986). The antigen derived from US Department of Agriculture strains (Böse GmbH, Hildesheim, Germany) and the conjugate (rabbit anti-horse IgG, heavy and light chain; dilution 1:90 in phosphate-buffered saline; Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) were obtained commercially. All samples were diluted 1:20, 1:40,

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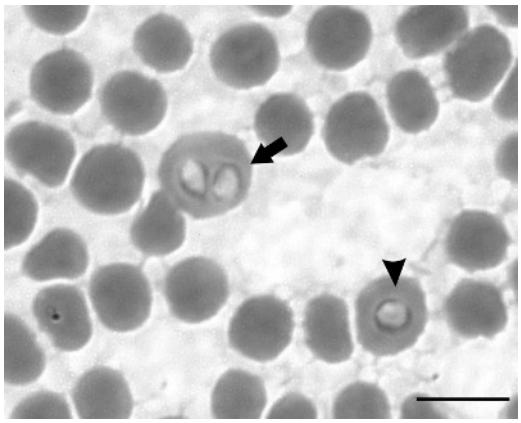


FIGURE 1. *Babesia caballi*, Giemsa stain. Large paired piriform inclusion bodies (arrow) and a large single inclusion body (arrowhead) can be seen.

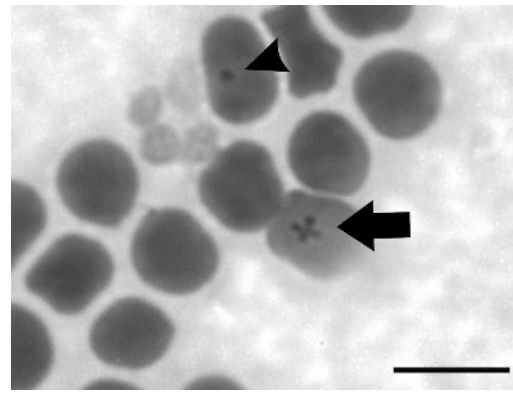


FIGURE 2. *Theileria equi*, Giemsa stain. The typical Maltese cross (arrow) and a single inclusion body (arrowhead) can be seen.

1:80, and 1:160. The positive cut-off was weak fluorescence at 1:40 for *T. equi* and 1:80 for *B. caballi*. Sera were considered questionable for *T. equi* when 1:20 and 1:40 showed weak fluorescence and 1:80 had trace fluorescence, and for *B. caballi*, when 1:20, 1:40, and 1:80 showed weak fluorescence and 1:160 had trace fluorescence. Questionable samples were tested again up to two times. The first nonquestionable result (either positive or negative) was utilized for further analysis. Individuals with a remaining questionable IFAT result after the third testing were considered as seronegative for statistical evaluation. For the longitudinal study, time of seroconversion was considered the date of the first seropositive test result. For foals, loss of detectable antibodies (from maternal antibodies) was demonstrated. The samples from nine foals, which were still seropositive at the last sampling date, were serially diluted from 1:20 to 1:2,560 and the antibody titers determined. The aim was to differentiate falling titers from rising titers because of infection.

Analysis

All statistical procedures were performed with the NCSS 2001 software (Number Cruncher Statistical Systems, Kaysville, Utah, USA) and Microsoft Excel.

Cross-sectional study (domestic horses): Piroplasm prevalence and seroprevalence were determined, and confidence limits calculated with Monte Carlo techniques. For each piroplasm or serologic prevalence, 10,000 random samples from appropriate beta distributions were generated and the 95% confidence limits were determined as the 0.025 and 0.975 quantiles. The horses were then grouped by date of sampling into seven equal-time intervals (18 days). The chi-square test was used to examine differences in antibody prevalence and piroplasm prevalence between genders and date groups. Visually, the first three date groups were assessed to be similar and hence joined into one group, and the remaining intervals were compared to this baseline using the chi-square test where $n>5$ and the Fisher's Exact Test where $n\leq 5$.

For further analysis, the population was age-stratified into five groups: <1 yr (I), 1–5 yr (II), 5–10 yr (III), 10–15 yr (IV), and >15 yr (V). Whether parasitemia was dependent on the age group was evaluated with the chi-square test. The effect of age group on antibody status was evaluated using logistic regression (Kerber et al., 1999). Then, all animals older than 1 yr were grouped and antibody prevalence was compared to the animals <1 yr old with the multinomial logistic regression. The difference between the antibody prevalence of *T. equi* and *B. caballi* within the same age group was evaluated with the Fisher's Exact Test.

Longitudinal study (domestic horses): Antibody and piroplasm prevalences of foals and yearlings at the four and six sampling dates, respectively, were compared with the chi-square test where $n>5$ and the Fisher's Exact Test where $n\leq 5$, with the corresponding Bonferoni adjustment for three and five comparisons. The seroprevalence at each date was compared to the seroprevalence at first sampling and to the one on the consecutive

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date. The four consecutive titers of the nine foals, which were titrated up to 1:2,560, were compared with the paired Student's *t*-test and Bonferoni adjusted for three comparisons (Shkap et al., 1998). The incidence density (ID) in foals and yearlings was determined as number of seroconversions per total observed "horse-days" and the 95% confidence limits were calculated as described for the prevalence (cross-sectional study).

Przewalski's horses: The Przewalski's horses were age-stratified using the same intervals as for the domestic horses. They were additionally stratified according to their time at risk (same intervals), defined as the period between their arrival in Takhin Tal and the date of sampling. Differences in antibody prevalence between groups were evaluated with the Fisher's Exact Test.

To compare the Przewalski's horses to the domestic population, the exposure time-standardized prevalence ratio (SPR) appeared most adequate. The SPR is the ratio of the observed prevalence and the theoretical prevalence assuming the Przewalski's horses had the same stratum-specific prevalences as the corresponding domestic horses. The exposure time is described by time at risk for Przewalski's horses and by age for domestic horses. Age-related immunity could be ignored because juvenile resistance is only reported for young animals and all Przewalski's horses were at least 2 yr of age at introduction; we assume homogenous immunity in the rest of the population. To determine the confidence intervals (CIs) for the SPR, their distribution was simulated by generating 10,000 random variables from the appropriate beta distribution for each prevalence and the corresponding ratios were calculated. The 95% confidence limits were determined as the 0.025 and 0.975 quantile from these 10,000 ratios. The ID was calculated as described above.

RESULTS

Cross-sectional study (domestic horses)

Plasma from all 141 domestic horses (60 female, 81 male) were used for the serological investigation. From seven horses, no blood smear was available; therefore, only 134 samples were included for the analysis of the parasitemic prevalence. No samples were taken between 24 June 2001 and 12 July 2001. Based on microscopic analysis, piroplasms were detected in nine of 134 slides (6.7%, 95% CI: 3.6–12.2%). *Theileria equi* was detected in seven (95% CI: 2.6–10.4%) horses and *B. caballi* was detected in three (95% CI: 0.8–6.4%); one horse was coinfected. In mid-June, there was a significant maximum of three of seven samples with piroplasms present (data not shown). No significant differences were found between genders or between different age groups.

Four IFA test results were questionable and hence considered negative; one in age group I and three in age group IV. Overall, the antibody prevalence was 88.6% (95% CI: 82.4–92.9%) for *T. equi* and 75.2% (95% CI: 67.4–81.6%) for *B. caballi*. It did not vary significantly throughout the season, and neither was there a difference between genders. However, age had a significant influence on antibody prevalence (Table 1). The prevalence of both piroplasms in age group I (<1 yr) was significantly ($P<0.001$) lower than in the older age groups. Young horses had an antibody prevalence of 62.5% and 40.6% compared to the older horses with 96.3% and 85.3% for *T. equi* and *B. caballi*, respectively. Within age groups there was no significant difference between *T. equi* and *B. caballi*.

Longitudinal study (domestic horses)

Foals: The prevalence of antibodies against *T. equi* and *B. caballi* and the prevalence of either piroplasm in foals at different dates throughout the season are given in Table 2. Despite nine foals being seropositive for *T. equi* at the last sampling, the mean antibody titers against both piroplasms of 13 individuals were all decreasing significantly (Fig. 3). Three individuals had titers against both piroplasms, which were rising (number 83) or remained stable above the cut-off (numbers 67 and 81). Only number 83 was considered as a seroconversion. However, piroplasms were detected in smears of two foals (numbers 73 and 75) that had consistently decreasing titers. In conclu-

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TABLE 1. Antibody prevalence (determined by indirect immunofluorescence antibody test [IFAT]) and piroplasm prevalence (light microscopy [LM] of Giemsa-stained blood smear) of *Theileria equi* and *Babesia caballi* in domestic horses in Takhin Tal, Mongolia, 2001. The population is stratified into five age groups. (From seven of the total 141 horses investigated, no blood smear was available.)

Age group (interval)	Mean age (yr)	*Theileria equi*		*Babesia caballi*	
		No. IFAT positive/ no. investigated (%)	No. LM positive/ no. investigated (%)	No. IFAT positive/ no. investigated (%)	No. LM positive/ no. investigated (%)
I (<1 yr)	0.53	20/32[a] (62.5)	1/31 (3.2)	13/32[a] (40.6)	1/31 (3.2)
II (1–5 yr)	2.10	31/33 (93.9)	3/30[b] (10)	27/33 (81.8)	2/30[b] (6.7)
III (5–10 yr)	6.79	38/39 (97.4)	3/37 (8.1)	34/39 (87.2)	0/37 (0)
IV (10–15 yr)	12.32	25/26 (96.2)	0/25 (0)	22/26 (84.6)	0/11 (0)
V (>15 yr)	16.83	11/11 (100)	0/11 (0)	10/11 (90.9)	0/11 (0)

[a] Significant difference ($P<0.01$) from other age groups.

[b] One double infection.

sion, the ID for *T. equi* was 0.0012 conversions per horse day (95% CI: 0.0003–0.006) with one seroconversion per 847 observed days and horses, but not foals, seroconverted against *B. caballi* (95% CI: 0.0–0.004).

Yearlings: Table 3 shows the antibody and piroplasm prevalences of *T. equi* and *B. caballi* in yearlings throughout the summer season starting on 15 April 2001. Compared to the first sampling, the antibody prevalence of *T. equi* was significantly higher on 22 June ($P=0.001$) and on 14 July ($P<0.001$). Neither the antibody nor the piroplasm prevalences varied significantly on consecutive sampling dates. In seven yearlings, piroplasms were detected, and in over 1,249 observed "horse days", 10 seroconversions were recorded, resulting in an ID for yearlings of 0.008 conversions per horse day for *T. equi* (95% CI: 0.004–0.015).

The seroprevalence of *B. caballi* was significantly lower at the first sampling than on all later dates (15 April vs. 21 May: $P<0.001$; 15 April vs. 22 June: $P=0.0036$; 15 April vs. 14 July: $P=0.0094$). On consecutive dates, this did not vary significantly ($P>0.083$). The ID for *B. caballi* was 0.006 (95% CI: 0.003–0.013) conversions per horse day (eight seroconversions).

Przewalski's horses

None of the 23 sampled animals were at risk for longer than 10 yr. The age of the sampled Przewalski's horses ranged between 22 mo and 13 yr. From the nine animals sampled before March 2001, there were no blood smears available,

TABLE 2. Antibody prevalence (determined by indirect immunofluorescence antibody test [IFAT]) and piroplasm prevalence (light microscopy [LM] of Giemsa-stained blood smear) of *Theileria equi* and *Babesia caballi* in domestic foals in Takhin Tal, Mongolia, 2001.

Date	Mean age (wk)	*Theileria equi*		*Babesia caballi*	
		No. IFAT positive/ no. investigated (%)	No. LM positive/ no. investigated (%)	No. IFAT positive/ no. investigated (%)	No. LM positive/ no. investigated (%)
2 June 2001	1.64	13/13 (100)	0/13 (0)	10/13 (76.9)	0/13 (0)
22 June 2001	7.45	13/16 (81.3)	2/16 (12.5)	9/16 (56.3)	0/16 (0)
14 July 2001	11.16	2/3[a] (66.7)	0/14 (0)	0/3[a] (0)	0/14 (0)
6 August 2001	14.62	9/13 (69.2)	0/13 (0)	1/13 (7.7)	0/13 (0)

[a] Eleven plasma samples were destroyed on 14 July 2001 and therefore not available for calculation of the prevalence.

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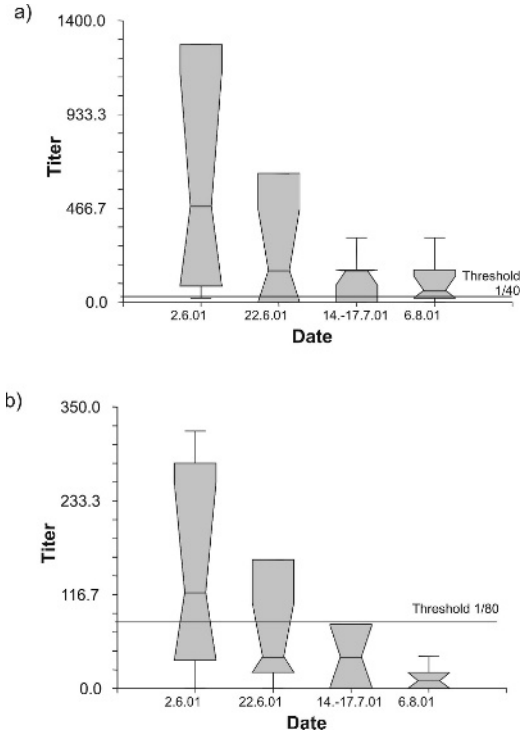


FIGURE 3. Median (horizontal line) antibody titers of foals against *Theileria equi* (a) and *Babesia caballi* (b) on subsequent sampling dates. The top and bottom of the box are the 25th and 75th percentiles. The whisker above the box (upper adjacent value) is the largest observation that is less than or equal to the 50th percentile plus 1.5 times the interquartile range (IQR=length of the box). The lower adjacent value is the smallest observation that is greater than or equal to the 50th percentile minus 1.5 times IQR (compare results of the longitudinal study).

and no piroplasms were detected in 14 samples taken in April 2001. The antibody prevalence was 44.0% (95% CI: 26.1–65.2%) for *T. equi* and 72.0% (95% CI: 52.2–91.3%) for *B. caballi*. The antibody prevalence for each stratum by time at risk and age is presented in Table 4. No significant differences were found between any strata. The SPR was 0.51 (95% CI: 0.50–0.64) for *T. equi* and 0.98 (95% CI: 0.80–1.24) for *B. caballi* (Przewalski's horses : domestic).

DISCUSSION

In the present cross-sectional study of domestic horses, we found *T. equi* or *B. caballi* in nine of 134 blood smears, which is in contrast to results from previous studies in central Mongolia where no parasites were found with microscopic examination (Avarzed et al., 1997; Boldbaatar et al., 2005). Although it is generally believed that they are mainly found during acute infections (Schein, 1988), none of these horses showed clinical signs. In other studies, similar situations were observed under endemically stable conditions (Mahoney and Ross, 1972; Joyner and Donnelly, 1979; Pfeifer Barbosa et al., 1995). The antibody prevalence in adult horses, around 90% for both piroplasms, and the 100% prevalence of maternal antibodies in foals at birth provide additional evidence for endemic stability in Takhin Tal. We could, however, not confirm the higher antibody prevalence of *T. equi* compared to *B. caballi* found in previous studies (Donnelly et al., 1980; Pfeifer Barbosa et al., 1995).

For this study, the latest available sample of each of the 16 foals from the longitudinal study was included. Despite 9/13 seropositive foals for *T. equi* and 1/13 for *B. caballi* at the end of the investigation, only one foal developed antibodies against *T. equi* during this season. All others were seropositive because of maternal antibodies. Nevertheless, the prevalence in animals younger than 1 yr, which included other additional foals, was significantly lower than in older horses. Using the presence of antibodies (not from maternal antibodies) as an indicator for infection with piroplasms, most of the animals appear to become infected only in their second summer. The ID of 0.001 (*T. equi*) in foals compared to 0.008 (*T. equi*) and 0.006 (*B. caballi*) seroconversions per horse per day in yearlings, as well as the seroprevalence of 3/15 and 4/15, respectively, in yearlings at the beginning of the season support this. Under endemically stable conditions it is,

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TABLE 3. Antibody prevalence (determined by indirect immunofluorescence antibody test [IFAT]) and piroplasm prevalence (light microscopy [LM] of Giemsa-stained blood smear) of *Theileria equi* and *Babesia caballi* in domestic yearlings in Takhin Tal, Mongolia, 2001.

Date	Mean age (wk)	*Theileria equi*		*Babesia caballi*	
		No. IFAT positive/ no. investigated (%)	No. LM positive/ no. investigated (%)	No. IFAT positive/ no. investigated (%)	No. LM positive/ no. investigated (%)
15 April 2001	45.43	3/15 (20)	0/10 (0)	4/15[a] (26.7)	0/10 (0)
7 May 2001	48.57	1/5 (20)	0/2 (0)	3/5 (60)	0/2 (0)
21 May 2001	50.57	6/11 (54.6)	1/2 (50)	11/11 (100)	0/2 (0)
22 June 2001	55.14	9/10[b] (90)	4/8 (50)	9/10 (90)	1/8 (12.5)
14 July 2001	58.48	9/9[b] (100)	3/9[a] (33.4)	8/9 (88.9)	1/9[c] (11.1)
6 August 2001	62.25	2/3 (66.7)	0/2 (0)	1/3 (33.4)	0/2 (0)

[a] Significantly lower at the first sampling than on all later dates (15 April vs. 21 May 21: $P<0.001$; 15 April vs. 22 June: $P=0.0036$; 15 April vs. 14 July: $P=0.0094$).

[b] Significantly higher on these two dates ($P\leq0.001$).

[c] One double infection.

however, contradictory that foals avoid infection. To explain this phenomenon, further investigations on the transmission are needed; the vector species and stage and the prevalence of piroplasms in this population especially need to be elucidated. So far, the main activity season of the transmitting vector can be deduced from the prevalence data. In the longitudinal study, the piroplasms were observed only between mid-May and mid-July, and primarily in yearlings, which corresponds to the maximum of prevalence found in the cross-sectional study in mid-June.

The confidence interval of the SPR includes one (95% CI: 0.71–1.34) indicating that the seroprevalence of *B. caballi* is equivalent in the Przewalski's horse population and in the domestic horse population. In contrast, the SPR for *T. equi* (95% CI: 0.30–0.76) shows that the Przewalski's horses have a significantly lower antibody prevalence. Either a higher transmission rate of *B. caballi* to Przewalski's horses or slower humoral immune response of Przewalski's horses to *T. equi* than to *B. caballi* could explain this observation. Also, a higher susceptibility to *T. equi* of Przewalski's than domestic horses would cause a higher mortality and eliminate infected animals, resulting in a lower piroplasm and antibody prevalence. To address the humoral immune response and susceptibility of Przewalski's horses, clinical trials with this species are needed. However, to elucidate details of the transmission rate, primarily the vector would need to be investigated.

TABLE 4. Antibody prevalence (determined by indirect immunofluorescence antibody test [IFAT]) of *Theileria equi* and *Babesia caballi* in the Przewalski's horse population in Takhin Tal, Mongolia, 2001. The population is stratified into groups either according to the time exposed to piroplasms (risk groups) or according to age (age groups).

Group	No. IFAT positive/no. investigated	
	Theileria equi	*Babesia caballi*
Risk group I (<1 yr)	1/2	2/2
Risk group II (1–5 yr)	5/15	10/15
Risk group III (5–10 yr)	5/6	6/6
Age group II (1–5 yr)	5/14	10/14
Age group III (5–10 yr)	4/6	5/6
Age group IV (10–15 yr)	2/3	3/3

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Losses in Przewalski's horses directly affect the outcome of the reintroduction project (Robert et al., 2005). In addition to possible higher mortality rates, it is reported that *T. equi* plays an important role in reproduction failure in endemic areas (Neitz, 1956; Donnelly et al., 1982; Potgieter et al., 1992). During the reintroduction, two main factors are responsible for the high susceptibility of Przewalski's horses to clinical piroplasmosis. Because they originate from Australia and northern Europe, where equine piroplasms are not present, or only sporadically occurring, they have no acquired immunity against the two protozoa (OIE, 2004a). Secondly, they are transported from their original countries to Takhin Tal at an age of 2 yr or more (Kaczensky and Walzer, 2002). At this age possible juvenile innate resistance factors are lost (Sippel et al., 1962; James, 1988).

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