Age-dependent dynamics of *Theileria equi* and *Babesia caballi* infections in southwest Mongolia based on IFAT and/or PCR prevalence data from domestic horses and ticks

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SUMMARY

Epidemiological factors of tick-borne equine piroplasmoses, caused by *Theileria equi* and *Babesia caballi*, were investigated using logistic regression (GLM) and general additive models (GAM) based on the prevalences determined in 510 domestic horses and in ticks in S.W. Mongolia by indirect immunofluorescence antibody test (IFAT) and/or multiplex PCR. Prevalences of *T. equi* and *B. caballi* in horses were 66·5% (95% CI: 62·1–70·7) and 19·1% (15·6–22·9), respectively by PCR and 78·8% (74·9–82·3) and 65·7% (61·3–69·9) by IFAT. Of 166 ticks analysed from PCR- and IFAT-negative horses 1 was PCR positive for *B. caballi* and none for *T. equi*. GAM demonstrated non-linear increasing proportions of *T. equi*-PCR and -IFAT positive horses with age suggesting persistent infection. In contrast, the *B. caballi*-PCR prevalence decreased with age despite a concurrent increase in the proportion of IFAT-positive animals suggesting parasite elimination. The tick (*Dermacentor nuttalli*) burden of the horses increased with age and decreased with advancing season. Geldings were more likely to be infected with, and seroconvert to, *T. equi*. Neither herd affiliation, date of sample collection nor abundance of tick infestation had a significant influence on parasite prevalence.

Key words: *Theileria equi*, *Babesia caballi*, multiplex PCR, IFAT, horses, ticks, epidemiology, Mongolia, general additive models.

INTRODUCTION

Theileria equi and *Babesia caballi* are intra-erythrocytic protozoa of equids that are transmitted by ixodid ticks in which they undergo sexual reproduction (reviewed by Friedhoff, 1988). These equine piroplasms are probably endemic throughout Asia, with the exception of Japan, and occur in most tropical and subtropical regions of the world. Clinical signs of infection vary from asymptomatic to acute fever, anaemia and dyspnoe, and even death may occur (reviewed by Schein, 1988). Intra-uterine infections with *T. equi* may result in abortion and neonatal death (Potgieter *et al.* 1992; Phipps and Otter, 2004). Horses that recover from acute infection may act as reservoirs for transmission to ticks which subsequently infect other equids. Various authors postulate that *B. caballi* persists for 1–4 years in its equine host whereas *T. equi* remains as a life-long infection (Hourrigan and Knowles, 1979; Schein, 1988; de Waal and van Heerden, 1994).

During the reintroduction of Przewalski's horses (*Equus ferus przewalskii*) to the Gobi B strictly protected area (SPA) in southwest Mongolia, piroplasms have caused mortality in newly introduced

individuals (Robert *et al.* 2005). Therefore, it is important to investigate the epidemiology of these diseases in the local horse population. An essential part of such studies is to reliably detect these parasites using sensitive and specific diagnostic assays and to confirm the identities of the local parasite species and their transmission vectors. Diagnoses of piroplasmoses are achieved by microscopical examination of Giemsa-stained thin blood smears or by the polymerase chain reaction (PCR) (Böse *et al.* 1995; Bruning, 1996). For indirect diagnosis, the immunofluorescence antibody test (IFAT) is the most widely used technique (Gummow *et al.* 1996; Avarzed *et al.* 1997; Heuchert *et al.* 1999). In S.W. Mongolia, 6 tick species of the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* have been reported (Byambaa *et al.* 1994). However, in a study undertaken in 2001 in this region, only *D. nuttalli* was found on horses (Rüegg, 2002). This species has been reported to transmit *B. caballi* (Friedhoff, 1988; Qi *et al.* 1995) and transmission of *T. equi* has been postulated (Pomerantzev, 1959). Indeed, its DNA has been detected in ground-collected specimens (Battsetseg *et al.* 2001) and in progeny of ticks collected from infected horses (Battsetseg *et al.* 2002) but transmission *per se* has never been demonstrated.

In this cross-sectional epidemiological study on domestic horses in S.W. Mongolia, PCR and IFAT were employed to determine their infection and

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serological status, respectively. Factors affecting the presence of parasites and specific antibodies were investigated using logistic regression and general additive models (GAM). The latter is an appropriate technique to examine potential non-linear and complex risk factor dependencies (Figueiras and Cadarso-Suarez, 2001). The infesting tick species were identified morphologically and evidence of piroplasm infection within these potential vectors was investigated using PCR.

MATERIALS AND METHODS

Study animals and clinical specimens

A total of 510 domestic horses from 22 herds of varying sizes living in the vicinity of the Gobi B SPA in Mongolia were sampled in April and May 2004 (see supplementary data in online version). They were aged between 2 days and 20 years as assessed by dentition and information provided by the owners. Of the 510 horses, 259 were females, 118 males, and 128 geldings. The sex of 5 animals was not determined. Anti-coagulated blood (EDTA) was taken from the jugular vein (from 9 horses no jugular blood could be taken). Blood fractions were separated by overnight sedimentation, and the plasma and the buffy coat including the top erythrocyte layer (containing piroplasm-infected cells, Böse et al. 1995) conserved at $-10\,^{\circ}\mathrm{C}$ for 3 months (during field expedition) and later at $-80\,^{\circ}\mathrm{C}$ in the laboratory. Buffy coat or plasma was not available from 8 and 2 horses, respectively. Further, thin blood smears were prepared from capillary blood, air-dried and fixed in ethanol (no slides were available for light microscopy from 13 animals). Ticks from 1 body side were collected, killed in hot water (approx. 2 min at $80\,^{\circ}\mathrm{C}$) and stored in 70% ethanol.

Microsocopy

From each individual animal, 1 blood smear was Giemsa-stained in the laboratory and examined microscopically. The detected piroplasms were classified as B. caballi if large paired piriform or several large single inclusion bodies were found in the erythrocytes; they were attributed to T. equi if a maltese cross or several small single inclusion bodies were found.

PCR

DNA isolation. DNA was isolated from $100\,\mu\mathrm{l}$ of buffy coat fraction and eluted in $200\,\mu\mathrm{l}$ of buffer using commercial kits (Qiaamp DNA mini kit, Qiagen AG, Basel, Switzerland; Nucleospin blood, Macherey-Nagel, Oensingen, Switzerland) according to the manufacturers' instructions. Ticks were washed twice in sterile phosphate-buffered saline and once in distilled water. After freezing them at $-20\,^{\circ}\mathrm{C}$ they were cut into small pieces using scissors. In order to raise the proportion of salivary gland cells in the sample, fully engorged ticks were divided diagonally from postero-dorsal to antero-ventral and the anterior part was used for further processing. Care was taken not to cross-contaminate the specimens. Cells were lysed after adding $400\,\mu\mathrm{l}$ of 25% Chelex (Bio-Rad, Reinach, Switzerland) by freezing/thawing (liquid nitrogen/$100\,^{\circ}\mathrm{C}$) 3 times. Further, the samples were incubated with proteinase K overnight ($80\,\mu\mathrm{g}$ per sample, $56\,^{\circ}\mathrm{C}$). DNA was isolated by phenol-chloroform-extraction and ethanol precipitation. DNA concentrations were measured using the Nanodrop® ND-1000 spectrometer (Nanodrop Technologies Inc., DE, USA), and $1\,\mu\mathrm{g}$ of DNA was used in the amplification reactions.

PCR conditions and sequencing. Primers targeting the gene of the rhoptry associated protein 1 (RAP-1) of B. caballi were selected from the literature (Battsetseg et al. 2001) and slightly modified (Table 1). For the detection of T. equi, 2 new primers complementary to the 18S rRNA gene were designed (Table 1) using a sequence alignment (Corpet, 1988). Two consensus sites of published T. equi sequences from South Africa (GenBank Accession number Z15105) and Spain (AY534883, AY150063, AY1-50064) were selected that differed from the corresponding sequences of T. buffeli (AF236094), T. sergenti (AB016074), B. microti (AB197940) and B. caballi (Z15104, AY534883, AY309955). The primer pairs were validated on samples with unequivocal light microscopical diagnoses and their specificity evaluated by running the 2 PCR protocols on 5 blood samples from previously unexposed Swiss horses.

For single target PCRs, the reaction volume of $100\,\mu\mathrm{l}$ contained PCR buffer (50 mM KCl, 20 mM TRIS-HCl, pH 8·4, 2·5 mM MgCl$_2$, 0·5% Tween 20), 0·2 mM of each dNTP (using dUTP instead of dTTP), 1 μM of each primer and 0·5 U uracil DNA glycosylase (Sigma-Aldrich, Buchs, Switzerland). After a 10-min incubation step at $37\,^{\circ}\mathrm{C}$ and an UDG inactivation step of 12 min at $94\,^{\circ}\mathrm{C}$, 2·5 U Taq polymerase (Sigma-Aldrich, Buchs, Switzerland) were added to the reactions. Cycling was performed according to Table 1 in a thermal cycler (DNA engine; MJ Research, Waltham, USA) with a final extension at $72\,^{\circ}\mathrm{C}$ for 10 min. Amplicons were visualized after electrophoresis of 15 μl of the PCR product on a 1·5% agarose gel stained with ethidium bromide. For each batch of 16 samples, a negative and a size-modified positive control (10^3 molecules) that was created using composite primers (Celi et al. 1993) were added.

Multiplex PCR was perfomed using the Qiagen multiplex PCR kit (Qiagen, Basle, Switzerland) and applying the cycling conditions given in Table 1.

Table 1. Primer sequences and PCR conditions

Target organisms	Primer designation	Sequence (5′–3′)	Amplicon size	Reference	Cycling conditions
B. caballi	BC48F11mod BC48R31	GCGACGTGACTAAGACCTTATTGG GTTCTCAATGTCAGTAGCATCCGC	451 bp	(Battsetseg et al. 2001)	30 sec/94 °C, 30 sec/66 °C, 45 sec/72 °C, 40 cycles (horse sample), 45 cycles (tick sample)
T. equi	TE1323 TE1685	CTAAATAGGRTGYGAGAYTTGG CCCTAGACGTYTCCAAAGAAAAG	362 bp	This publication	30 sec/94 °C, 30 sec/59 °C, 45 sec/72 °C, 40 cycles
B. caballi, T. equi	BC48F11mod BC48R31	See above		Multiplex PCR	Horse sample: 30 sec/94 °C, 90 sec/59 °C, 20 sec/72 °C, 40 cycles
	TE1323F-2 TE1685R-2	TGCTAAATAGGRTGYGAGAYTTGG CTTCCCTAGACGTYTCCAAAGAAAAG	367 bp	This publication	Tick sample: 30 sec/94 °C, 90 sec/60 °C, 20 sec/72 °C, 42 cycles

Notably, the annealing temperature of the *T. equi*-specific primers was raised by adding nucleotides to their 5′ ends to match the temperature of the *B. caballi*-specific primers (TE1323F-2, TE1685R-2, Table 1). All samples and the 5 negative horses were subjected to the multiplex PCR.

The PCR assays were optimized for use with tick-derived DNA regarding annealing temperature and number of cycles (Table 1). Possible inhibition of PCR in the tick-derived specimens was evaluated by spiking 24 samples with 10^3 molecules of the cloned, size-modified target (see above) in parallel control reactions.

For sequencing, the PCR products were purified with a commercial kit (PCR purification kit, Qiagen, Basle, Switzerland) and either sent directly for sequencing (Microsynth, Balgach, Switzerland) or after being cloned (Topo TA cloning, Invitrogen, Basle, Switzerland) if the amplicon concentration was low.

Indirect immunofluorescence antibody test (*IFAT*)

IFAT with antigen derived from USDA (United States Department of Agriculture) strains (Böse, Hildesheim, Germany) and commercial conjugate (rabbit-anti-horse-IgG, heavy and light chains; dilution 1:90 in PBS; Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) were performed as described by Tenter and Friedhoff (1986). All samples were diluted to 1:40, 1:80 and 1:160. Cut-off for seropositivity was a fluorescent signal at 1:40 for *T. equi* and 1:80 for *B. caballi* as recommended by the manufacturer.

Tick identification

All ticks collected on 39 horses randomly selected using the 'simple random sample' function of the Poptools add-in for Excel (Poptools version 2.6.9., http://www.cse.csiro.au/poptools) were morphologically identified. The genus of the ticks was determined based on the key of Wall and Shearer (2001) and the species were morphologically identified with the consolidated keys of Arthur (1960) and Pomerantzev (1959) for Eurasian *Dermacentor* spp. also considering the more recent morphological studies of Filippova and Panova (1985), Voltzit (1991), Estrada-Peña and Estrada-Peña (1991) and Horak *et al.* (2002).

Statistical methods

Descriptive statistics were calculated using Excel (Office Excel 2003, Microsoft Corporation, Redmond, WA, USA), and statistical testing and modelling were performed with R 2.2.0 (The R Foundation for Statistical Computing, http://CRAN.R-project.org, source code and data file as

Table 2. *Significant parameters of the generalized linear and additive models used to analyse the dependence of Theileria equi and Babesia caballi PCR and IFAT results and tick infestation on age, herd affiliation, sex, date of sample collection and tick infestation*

(Analyses were performed using generalized linear models (GLM) or generalized additive models (GAM). The more suitable model was selected based on the lower Akaike's information criterion (AIC).)

Dependent factor	Model	Independent factor	Odds ratio	95% confidence interval	*P*-value	Deviance	AIC
T. equi PCR	GLM	Age	1·78	1·66–1·90	<1e-15		
	GLM	Males*	1·73	1·24–2·39	0·091		
	GLM	Geldings	4·28	2·67–6·94	2·3e-3	354·3	406·3
	GAM	Age	Non-parametric (6·33 degrees of freedom)		<2e-16		
	GAM	Males*	1·70	1·20–2·42	0·125		
	GAM	Geldings	3·04	2·60–3·54	0·022	327·2	389·9
T. equi IFAT	GLM	Age	1·61	1·49–1·73	<1e-9		
	GLM	Males*	1·31	0·93–1·83	0·425		
	GLM	Geldings	3·46	2·08–5·74	0·014	302·4	354·4
	GAM	Age	Non-parametric (1·62 degrees of freedom)		2·7e-10		
	GAM	Males*	1·31	0·93–1·84	0·422		
	GAM	Geldings	3·24	1·95–5·39	0·021	300·9	354·1
B. caballi PCR	GLM	Age	0·87	0·83–0·91	6·0e-4	409·9	462·0
	GAM	Age	Non-parametric (1·01 degree of freedom)		8·3e-4	409·9	461·9
B. caballi IFAT	GLM	Age	1·20	1·15–1·24	<1e-5	556·1	511·5
	GAM	Age	Non-parametric (4·35 degrees of freedom)		2·48e-6	445·4	504·1

* Sex is significant, but only geldings differ from the other classes.

supplementary data). The prevalences and seroprevalences of *T. equi* and *B. caballi* were calculated and the exact binomial confidence intervals were computed. Dependencies of PCR and IFAT results on age, herd affiliation, sex, date of sample collection and tick abundance on 1 body side were assessed with logistic regression using the function for generalized linear models (GLM) in R (glm(family = 'binomial')). The abundance of ticks on horses was tested for dependence on the same parameters using the same GLM function, but allowing for an over-dispersed distribution of the ticks (glm(family = 'quasipoisson')). To explore relationships between the response factors and the continuous independent parameters, which are possibly more complex than the logit transformation, generalized additive models (GAM) (Helfenstein *et al.* 1997; Figueiras and Cadarso-Suarez, 2001) were fitted to the data. The GAM function of R was used with default settings using a thin plate regression spline for age, date of sample collection and tick burden. Herd and sex remained as categorical variables. Approximation of all GLMs and GAMs was undertaken with the iteratively reweighted least squares method. The deviance of all models was used to select the better fitting model. To compare models with equal deviance, the Akaike's information criterion (AIC) was calculated based on a maximum likelihood estimate (binomial models) which is additionally penalised for the higher number of parameters in more complex models. To analyse interspecies differences of the PCR and serological results, the Fisher's exact test was applied.

Exact binomial confidence intervals for the prevalence of *T. equi* and *B. caballi* in ticks were computed as described above and compared. The Fisher's exact test was used to verify these results and to verify if the difference of prevalence of piroplasms in ticks from horses potentially harbouring infected erythrocytes in their capillaries to baseline prevalence in ticks was significant.

RESULTS

PCR validation

Blood samples from all individuals with blood smears showing morphologically unequivocal *T. equi* (*n* = 28) or *B. caballi* (*n* = 8) infections by microscopy were positive in the monospecific as well as in the multiplex PCRs, whereas the 5 samples from horses without piroplasm exposure were negative. Sequencing of the 2 amplicons from samples microscopically negative for *T. equi* and *B. caballi*, respectively, were 99% and 100% (AY534882) and twice 93% (AB017700) identical with the targeted organisms published in GenBank.

Infections in horses

By light microscopy, an overall prevalence of *T. equi* of 5·7% (95% confidence interval: 3·7–8·1) was determined (*n* = 495 horses), whereas the prevalence as analysed by multiplex PCR (*n* = 493 horses) was 66·5% (95% CI: 62·1–70·7). The logistic regression model indicated that the prevalence increased with age and was higher in geldings (Table 2).
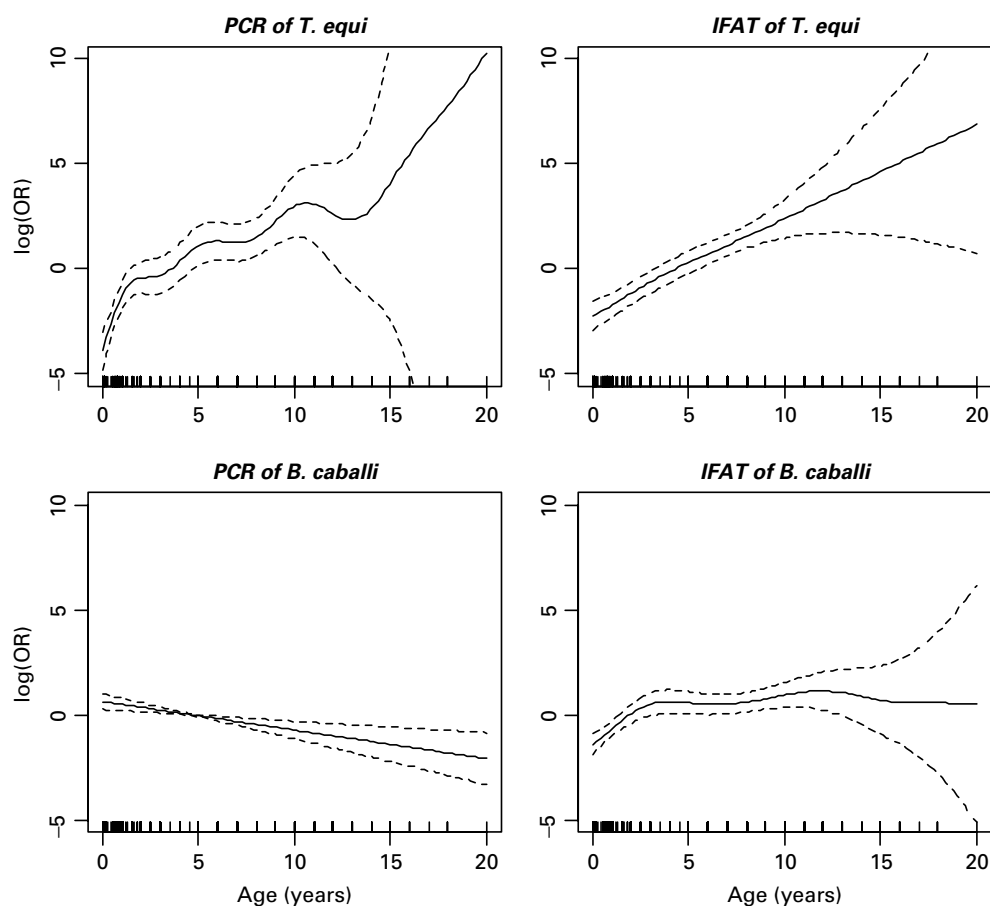
Fig. 1. Graphical representation of the smoothing function of age (i.e. the relation of the log of the odds ratio (logistic regression), adjusted for other significant variables in the model, to age (black line) and the 95% confidence limits (broken lines)) of PCR and IFAT assays from 493 and 499 domestic horses, respectively, for equine piroplasmoses performed in S.W. Mongolia in 2004. The tick marks above the x-axis represent the count of samples at the corresponding age.

Neither herd, date of sample collection nor abundance of tick infestation had a significant influence. The overall seroprevalence of *T. equi* ($n = 499$ horses) was 78·8% (95% CI: 74·9–82·3). Again, the seroprevalence increased with age and was higher in geldings (Table 2). The lower AIC of the GAMs and degrees of freedom for age above 1 indicated that PCR- and IFAT-positive results had a complex dependence on age (Table 2, Fig. 1). However, the deviance from the linear model for the IFAT was relatively minor. In both the logistic model and the GAM, the continuous variables of date of sample collection and tick burden were non-significant.

The prevalence of *B. caballi* was 1·6% (95% CI: 0·7–3·1) as determined by light microscopy ($n = 495$ horses) and 19·1% (95% CI: 15·6–22·9) by multiplex PCR ($n = 493$ horses). The logistic regression model indicated that only age had an influence on the prevalence of this parasite (Table 2). Overall seroprevalence ($n = 499$ horses) was 65·7% (95% CI: 61·3–69·9) and was age dependent (Table 2). No other of the investigated parameters had a significant

impact on the IFAT result. The lower AIC of the GAMs and degrees of freedom for age above 1 indicated that PCR and IFAT had a complex dependence on age (Table 2, Fig. 1). However, the deviance from the linear model for the PCR results, although significant, was relatively small (Fig. 1). Other continuous variables were not significant in the GAM. The non-homogeneous age distribution of the sample is reflected by the large confidence intervals at high age (Fig. 1). Seven samples were IFAT negative and PCR positive for *T. equi*, whereas for *B. caballi* this was the case for 19 samples. The Fisher's exact test showed significant differences for both the PCR ($P < 2·2e-16$) and the serological results ($P = 1·742e-05$) between the 2 parasite species.

Tick identification

From a randomly selected subsample of 556 of a total of 7534 collected adult ticks, all were identified as *Dermacentor* spp. All males (279) were identified as *D. nuttalli*. Of the 277 females, 189 were attributed to

Table 3. *Significant parameters of the generalized linear and additive models used to analyse the dependence of the tick infestation on age, herd affiliation, sex and date of sample collection*

(Analyses were performed using generalized linear models (GLM) or generalized additive models (GAM). The more suitable model was selected based on the lower deviance.)

Dependent factor	Model	Independent factor	Regression coefficient	S.E.	*P*-value	Deviance
Tick infestation	GLM	Age	0·036	0·008	<1e-4	
	GLM	Males*	−0·140	0·090	0·118	
	GLM	Geldings	−0·381	0·097	<1e-3	
	GLM	Date of sample collection	−0·078	0·039	0·047	4093·8
	GAM	Age	Non parametric (8·04 degrees of freedom)		<2e-16	
	GAM	Males*	−0·089	0·078	0·254	
	GAM	Geldings	−0·391	0·083	<1e-8	
	GAM	Date of sample collection	Non parametric (5·38 degrees of freedom)		2·7e-12	2834·0

* Sex is significant, but only geldings differ from the other classes.

the same species, whereas 88 could not unequivocally be identified.

Tick infestation

The infestation of horses with adult ticks varied from 0 to 161 ticks per horse side (*n* = 510 horses). The variance of 503·7 being much larger than the mean of 14·8 indicates an aggregated distribution. The abundance of ticks increased with age, but decreased with the date of collection (Table 3). Geldings had significantly lower tick burdens. The herd affiliation had no influence. The deviance of the GAM was much lower than of the GLM, and age and date had a much higher degree of significance (Table 3) indicating a complex dependence of the tick infestation on both age of horse and date of sampling. The variation of tick infestation with sex remained significant in the GAM (Table 3).

Piroplasm prevalences in ticks

Of 166 investigated ticks from 31 both IFAT- and multiplex PCR-negative horses originating from 8 herds, 1 was PCR-positive for *B. caballi* (95% CI: 0·1–1·7%), but none for *T. equi* (95% CI: 0·0–0·4%). Hence, there is no significant difference (*P* = 0·99) in their prevalences. The sequence of the *B. caballi* isolate, which has 93% identity with known sequences (AB017700), is published in GeneBank (Accession number DQ987620). One of 47 ticks collected from 14 horses assumed to harbour piroplasms in their capillary beds despite showing no sign of infection in the jugular blood (IFAT positive, PCR negative, >6 months of age) was positive in the *B. caballi*-PCR (95% CI: 0·6–5·7%), and thus there was no significant difference to the baseline prevalence (*P* = 0·39). No inhibition by tick-derived specimens was observed using the commercial multiplex PCR kit or the single-target PCRs.

DISCUSSION

In this study, we extensively investigated the infection status of piroplasms of horses by IFAT and PCR and explored the relationship between the animals' age, herd affiliation, sex, date of sample collection, tick infestation and the presence of antibodies or of the piroplasms in blood. GAMs and GLMs detected the same significant independent parameters. The presence of parasites and parasite-specific antibodies were significantly related to age. The additional flexibility of GAMs allowed the detection of a complex age-dependency of the log odds ratio of the presence of *T. equi* and of the presence of antibodies against *B. caballi* as depicted in Fig. 1. Besides the graphical presentation, the almost linear dependence of the other two dependent variables can be interpreted from the very small difference in the AIC between the GLM and the GAM analysis as well as the degrees of freedom close to unity in the GAM anlysis. Complex age-related variations in the prevalence of piroplasms have also been observed previously with *B. bovis* and *B. bigemina* in cattle (Mahoney, 1962).

The observed prevalence of the parasites in the blood is a consequence of the relationship between the corresponding acquisition and elimination rate. The elimination rate depends on the effectiveness of the immune response of the host to the piroplasm infection. The presence of specific antibodies is epidemiologically useful to measure this immune response although it does not explicitly reflect the protective immunity. The prevalence of antibodies against both parasites increased with rising age, but the seroprevalence of *B. caballi* was significantly lower than that of *T. equi*. The high antibody titres determined in our study appear to correspond to what has been found in previous comprehensive sero-epidemiological studies from Central Asia, but the variance in age and the significant difference

between the 2 parasites are contradictory. In Central Mongolia, sero-prevalences of 88·2% and 84·5% for *T. equi* and *B. caballi*, respectively, were observed (Avarzed *et al.* 1997). In contrast to the present study, these authors found a significant decrease in prevalence in animals between 11 and 16 years, which may be due to the large stratification intervals (1, 2, 3, 4, 5 years, 6–10 years, 11–16 years) and the small sample size in this particular stratum ($n=5$). The previous study in the Gobi B SPA determined seroprevalences of 88·6% (*T. equi*) and 75·2% (*B. caballi*) (Rüegg *et al.* 2006). Boldbaatar *et al.* (2005) determined the seroprevalences of the 2 protozoa using ELISA on sera from 254 horses from 6 Mongolian provinces in different climatic zones revealing high variations from 39 to 96% for *T. equi* and between 17 and 63% for *B. caballi*, which, given the age and sex-dependence of the seroprevalence, may be due to sampling differences between the provinces. In China, prevalences of 14 and 81·6% were observed in endemic areas for *T. equi* and *B. caballi*, respectively (Wang cited by Yin *et al.* 1997), but more comprehensive studies are not available from this neighbouring country.

Therefore, we assume that there is an increasing immunity against *T. equi* and *B. caballi* with rising age, which should result in an effective elimination of the parasites. Although this is the case with *B. caballi*, the prevalence of *T. equi* rises with age. Several authors have postulated that *T. equi* remains as a life-long infection, whereas *B. caballi* is eliminated after 1–4 years (Hourrigan and Knowles, 1979; Schein, 1988; de Waal and van Heerden, 1994). However, the longest investigations with experimental infections with equine piroplasms lasted 16 months and included only serological analysis (Tenter and Friedhoff, 1986). The time of persistence of equine piroplasms has thus never been verified experimentally or epidemiologically. Our results for the first time explicitly show the persistence of *T. equi* and the elimination of *B. caballi* with increasing age.

The parasite prevalences are further influenced by the rate of acquisition which depends on the tick infestation, prevalence of piroplasms in ticks and the proportion of infective tick bites resulting in infection. The regression analysis shows that there is a rise of tick infestation with age, and the decreasing tick burden with progressing date confirms the observation that tick numbers peak at the beginning of the season in this area (Byambaa *et al.* 1994; Rüegg, 2002).

The prevalence of *T. equi* parasites and specific antibodies against this parasite as well as tick burden was significantly related to sex. Geldings appeared approximately 3 times more likely to be infected with *T. equi* than other horses and had lower tick burdens. This is in contrast to previous experimental reports with mice, showing that elevated testosterone levels both increased the likelihood of infections with piroplasms (Hughes and Randolph, 2001*b*) and depressed their resistance to tick infestation (Hughes and Randolph, 2001*a*). The present results may be due to sex-specific husbandry techniques and underline the importance of verifying laboratory results with field studies (Randolph and Nuttall, 1994).

No other independent factors had a significant effect on parasite or antibody prevalence. This could have been suspected for herd affiliation because, due to the transhumance of Mongolian herders, exposure to tick habitats was probably quite homogeneous. The date of sample collection did not influence the probability of infection. This is likely to be related to the persistence of the parasites in the host. Finally, as expected, tick infestation had no effect on the probability of piroplasm infection as transmitting ticks have left the host when serological evidence of piroplasm infection can be detected.

The multiplex PCR assay developed in this study correctly identified the microscopically unequivocal samples and we therefore assume that it will demonstrate *T. equi* and *B. caballi* if they are present in the blood. The prevalence determined with this assay showed an age-dependent increase in the case of *T. equi* but a decrease in the case of *B. caballi*. Such a decrease could be due to increased sequestration of the parasites in the microvasculature with rising age of infection. If this were the case, the true sensitivity of our PCR would depend on the time of blood collection in relation to the period of the parasitaemia. However, sequestration is known to occur, to a variable extent, in only a few haemoparasites (*Plasmodium falciparum*, *P. chabaudi*, *P. knowlesi*, *B. canis* and perhaps *Babesia* WA-1 isolate) but nothing is known for *T. equi* or *B. caballi* (Allred and Al-Khedery, 2004). To evaluate the possibility of a decreased sensitivity of the *B. caballi*-PCR due to sequestration we assessed the infectivity of *B. caballi*-IFAT-positive and PCR-negative horses for ticks. According to Donnelly and Peirce (1975), who performed a similar assessment for *B. divergens* using blood smears to evaluate the infection status of cattle, these animals would be "premune carriers" and infective for ticks. Unfortunately, too few ticks were collected from these horses to confirm or disprove this result with adequate power ($\beta=0·12$).

The use of morphological keys unequivocally identified all investigated male ticks as *D. nuttalli*. In contrast, species identification of 88 of 277 investigated females was not possible. The key separates *Dermacentor* females according to the presence or absence of cornua at the posterior margin of the basis capituli. In these 88 cases, rudimentary projections were present which would attribute these ticks to the species *D. everestianus*. However, because all males were identified as *D. nuttalli*, we postulate that this criterion is not reliable for the differentiation

of these two species and that these females should be attributed to *D. nuttalli*. The exclusive infestation with *D. nuttalli* confirms previous findings in Mongolia where over 90% of the recovered ticks from horses were *D. nuttalli*, and this species was predominant in the Gobi steppe at altitudes of 1500 to 3200 m above sea level (Byambaa *et al.* 1994). These authors did not report *D. everestianus*. Tick larvae and nymphs were very difficult to collect systematically due to their small size and the thick winter coat of the horses. However, few collected nymphs were attributed to *Dermacentor* but could not be attributed to a species as no keys are available for *Dermacentor* nymphs of Central Asia.

The presence of *T. equi* and *B. caballi* was demonstrated in adult *D. nuttalli* and their progeny by Battsetseg *et al.* (2002) and we therefore assume that both piroplasms are transmitted by these ticks. Thus, because the prevalence of both protozoa in ticks was not significantly different in our study, the diverging development of the two parasite prevalences in the mammalian host must be due to other factors. In the case of *T. equi*, it is evident that persistent infection leads to persistent antibody titres. Humoral immunity for *B. caballi*, on the other hand, persists despite the apparent decrease of the parasite's presence. As expected for a disease with a high transmission rate with consecutive seroconversion, 13 of 19 horses which tested IFAT negative/PCR positive for *B. caballi* were younger than 1 year of age. In contrast, for *T. equi*, the equivalent samples originated predominantly from older horses (5/7 were >5 years). Unfortunately the analysis with GAMs is descriptive and does not allow any inference on underlying mechanisms. Thus, more mechanistic mathematical models will be applied to better understand the true nature of these non-linear interactions.

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