Infections by *Babesia caballi* and *Theileria equi* in Jordanian equids: epidemiology and genetic diversity

MONEEB A. QABLAN[1]*, MIROSLAV OBORNÍK[2], KLÁRA J. PETRŽELKOVÁ[1,3,4], MICHAL SLOBODA[1], MUSTAFA F. SHUDIEFAT[5], PETR HOŘÍN[6,7], JULIUS LUKEŠ[2] *and* DAVID MODRÝ[1,2,7]

[1] *Department of Pathology and Parasitology, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, 612 42 Brno, Czech Republic*
[2] *Institute of Parasitology, Biology Centre, and Faculty of Sciences, University of South Bohemia, 370 05 České Budějovice, Czech Republic*
[3] *Institute of Vertebrate Biology, Czech Academy of Sciences, 603 00 Brno, Czech Republic*
[4] *Liberec Zoo, 460 01 Liberec, Czech Republic*
[5] *National Center for Research and Development, Badia Research Program, Amman, Jordan*
[6] *Institute of Animal Genetics, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, 612 42 Brno, Czech Republic*
[7] *CEITEC – Central European Institute of Technology, University of Veterinary and Pharmaceutical Sciences, 612 42 Brno, Czech Republic*

(*Received 18 December 2012; revised 17 February and 22 March 2013; accepted 26 March 2013*)

SUMMARY

Microscopic diagnosis of equine piroplasmoses, caused by *Theileria equi* and *Babesia caballi*, is hindered by low parasitaemia during the latent phase of the infections. However, this constraint can be overcome by the application of PCR followed by sequencing. Out of 288 animals examined, the piroplasmid DNA was detected in 78 (27·1%). Multiplex PCR indicated that *T. equi* (18·8%) was more prevalent than *B. caballi* (7·3%), while mixed infections were conspicuously absent. Sequences of 69 PCR amplicons obtained by the 'catch-all' PCR were in concordance with those amplified by the multiplex strategy. Computed minimal adequate model analyses for both equine piroplasmid species separately showed a significant effect of host species and age in the case of *T. equi*, while in the *B. caballi* infections only the correlation with host sex was significant. Phylogenetic analyses inferred the occurrence of three genotypes of *T. equi* and *B. caballi*. Moreover, a novel genotype C of *B. caballi* was identified. The dendrogram based on obtained sequences of *T. equi* revealed possible speciation events. The infections with *T. equi* and *B. caballi* are enzootic in all ecozones of Jordan and different genotypes circulate wherever dense horse population exists.

Key words: Jordan, equine, *Theileria equi*, *Babesia caballi*, genetic diversity.

INTRODUCTION

Piroplasmoses are tick-borne diseases caused by protistan parasites of the genera *Theileria* and *Babesia*. For equine piroplasmosis two species are responsible, namely *Theileria equi* and *Babesia caballi*, and both are transmitted by several tick species belonging to the genera *Hyalomma, Rhipicephalus* and *Dermacentor* (De Waal, 1992). Clinically, equine babesiosis and theileriosis vary, but symptoms of both diseases include fever, anaemia, inappetence, oedema and increased respiratory and heart rate (Brüning, 1996; Zobba *et al*. 2008). Diagnosis of the acute phase of the infections in equids is traditionally based on clinical symptoms, confirmed by detection of the intraerythrocytic parasites in Giemsa-stained blood smears (Shkap *et al*. 1998). However, diagnosis of the latent infection, characterized by low parasitaemia, represents a major limitation for intervention in endemic areas (Kumar *et al*. 2008).

Application of highly sensitive PCR-based molecular diagnosis not only overcomes the constraints of low parasitaemia during latent infections, but also permits the identification of genetic variants and cryptic species. Various genes have been used as targets for the diagnosis of *T. equi* and *B. caballi* including the genes encoding *EMA* (Battsetseg *et al*. 2002), β-tubulin (Cacciò *et al*. 2000) and the 18S rRNA (Bashiruddin *et al*. 1999; Alhassan *et al*. 2005; Sloboda *et al*. 2011). Due to its low substitution rate, constrained and conserved function and occurrence in multiple copies, the 18S rRNA gene represents the most suitable genetic marker for both diagnosis and phylogenetic studies of piroplasmids (Allsopp and Allsopp, 2006; Hunfeld *et al*. 2008). Neither *Babesia* nor *Theileria* is considered as a separated monophyletic taxonomic unit and previously it has been known that piroplasmids include five major clades (Criado-Fornelio *et al*. 2003; Allsopp and Allsopp,

* Corresponding author: Department of Pathology and Parasitology, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, 612 42 Brno, Czech Republic. E-mail: moneeb_78@hotmail.com

doi:10.1017/S0031182013000486

Table 1. Numbers and origin of sampled animal

		Horse		Donkey		Mule	
Subdistrict	Ecozone	M[a]	F[a]	M	F	M	F
Shuna Al Janubiya	Afrotropical	22	38	8	7	1	0
Wadi Mousa	Irano-Turanian	52	82	44	2	2	1
Wadi Araba	Afrotropical	2	0	3	1	0	0
Azraq	Saharo-Arabian	0	0	0	1	0	0
Dair Alla	Irano-Turanian	1	5	1	0	0	0
Bani Ebaid	Mediterranean	4	11	0	0	0	0
Total		81	136	56	11	3	1

[a] M,males; F,females.

2006; Lack *et al*. 2012). However, recent studies by Lack *et al*. (2012) and Schnittger *et al*. (2012) pointed out that piroplasmids include eight clades and six major monophyletic lineages, respectively. The results by Lack *et al*. (2012) and Schnittger *et al*. (2012) support the placement of *T. equi* and *B. bicornis* in a separated monophyletic group that does not fit in either *Babesia sensu strictu* or *Theileria sensu strictu* groups. Previous 18S rRNA-based phylogenetic analyses indicated a noticeable degree of variation within and among *B. caballi* and *T. equi* isolates from different geographical regions (Bhoora *et al*. 2009; Salim *et al*. 2010; Qablan *et al*. 2012*b*).

The current distribution of these protists depends on the presence of permissive vectors and was probably also influenced by a long and complex history of translocations of horses since their domestication. Infections with *T. equi* and *B. caballi* are endemic in tropical and subtropical zones, with the former piroplasmid being more widespread (De Waal, 1992). In Jordan, it is estimated that the number of horses and donkeys are about 2500 and 19000, respectively (Starkey and Starkey, 2000). So far, only *T. equi* has been reported from equids in Jordan (Hailat *et al*. 1997; Abutarbush *et al*. 2011). However, very recently, both *T. equi* and *B. caballi* were detected in camels and dogs (Qablan *et al*. 2012*a*, *b*). The aim of this study was to survey the distribution of piroplasmid parasites in Jordanian equids and to evaluate their interspecific genetic diversity.

MATERIALS AND METHODS

Study site and sampling

Between 2007 and 2009, a total of 288 equids (217 horses, 67 donkeys and 4 mules) were sampled from the following localities within six subdistricts of Jordan: Shuna Al Janubiya (Suwaymah, Baptism site/Almaghtas, Al Kafrayn), Wadi Mousa (Petra, Um Sayhoon), Wadi Araba (Ghor Al Safi and Ar Rishah), Azraq, Dair Alla and Bani Ebaid (Nu'ayyimah) (Table 1). The age of the inspected animals ranged from 3 months to 20 years. During the last sampling period in 2009, a total of seven and four animals from Petra and Almaghtas, respectively were re-examined. Information about sex, age, locality and host species was obtained using bilingual questionnaires. Blood was collected by puncture of the jugular vein using Hemos H-01 collecting tubes containing EDTA (Gama Group, Czech Republic) equipped with 18G needles. Two thin blood smears were made from each sample; smears were methanol-fixed in the laboratory within the same day. Blood smears were stained with Giemsa solution (Merck, Germany) and examined using light microscopy (Olympus AX 70).

DNA isolation and PCR assay

Blood was preserved in plastic tubes containing 10 mM EDTA, frozen and analysed upon transport to the laboratory, whereas total DNA extracted using the DNAeasy blood and tissue kit (Qiagen, Germany) was subject to two diagnostic PCR-assays. Detailed information about methodology and primer design has been published elsewhere (Sloboda *et al*. 2011). The first PCR reaction aimed to identify the animals positive for all possible *Babesia* and *Theileria* species using the universal 'catch-all' primers TB-F (5′-CTTCAGCACCTTGAGAGAAAT-3′) and TB-R (5′-TCDATCCCCRWCACGATGCRBAC-3′), amplifying 496 bp region of 18S rRNA. To distinguish between *T. equi* and *B. caballi*, a single multiplex PCR reaction targeting 18S rRNA employed a mixture of primers composed of a single forward primer (TBM; 5′-CTTCAGCACCTTGAGAGAAATC-3′), and two reverse primers (Equi-R; 5′-TGCCTTAAACTTCCTTGCGAT-3′ and BC-R; 5′-GATTCGTCGGTTTT GCCTTGG-3′) with expected 360 bp- and 650 bp-long amplicons for *T. equi* and *B. caballi*, respectively (Sloboda *et al*. 2011). PCR reactions were conducted in a total volume of 25 μL, composed of 12·5 μL of commercial Master Mix (TopBio, Slovakia), 10 μM of each primer and ~20 ng of genomic DNA. Genomic DNA

Table 2. Prevalence of piroplasmids in equines as revealed by the 'catch all' and multiplex PCR assays

		Primers					
		Catch-all		*T. equi*		*B. caballi*	
Host	Total number	*n*	%	*n*	%	*n*	%
Horse	217	62	28·6	48	22	13	6
Donkey	67	13	19·4	3	4·5	8	11·9
Mule	4	3	75	3	75	ND[a]	ND[a]
Total	288	78	27·1	54	18·8	21	7·3

[a] ND,not determined.

isolated from a donkey naturally infected with *T. equi* and *B. caballi* in Italy, with microscopically detected parasites and which exhibited clinical symptoms, was used as a positive control. DNA isolated from the blood of a piroplasmid-free horse maintained at the clinic at the University of Veterinary and Pharmaceutical Sciences was used as a negative control. The amplified PCR products were subjected to 1·5% agarose gel electrophoresis stained with Gold-View and documented using the Vilber Lourmat system (France).

Sequencing and phylogenetic analyses

Amplicons from the PCR with 'catch-all' primers were purified using Qiaquick Gel Extraction kit (Qiagen, Germany) and sequenced in both directions by Macrogen (South Korea). The obtained sequences were aligned separately for each species with homologues available in the GenBank™ using Kalign (Lassmann and Sonnhammer, 2005) and consequently manually edited in Bioedit (Hall, 1999). Phylogenetic trees were constructed by maximum likelihood (ML) with the GTR model for nucleotide substitutions and discrete gamma distribution in four categories (PhyML; Guindon and Gascuel, 2003) and maximum parsimony (MP) as implemented in PAUP* 4b10 (Swofford, 2002).

Statistical analyses

We fitted several general linear models (GLMs) with binomial distribution in order to explore the effect of 'host species', 'sex', 'age' and 'locality' on the occurrence of (i) piroplasmids (*T. equi* and *B. caballi* tandemly detected by universal primers), (ii) *T. equi* and (iii) *B. caballi*. For the analyses, data from 253 animals with complete information regarding all the examined criteria were used. Animals were classified into groups by host species (horses, donkeys), sex (males, females) and subdistricts (Shuna Al Janubiya, Wadi Mousa, Wadi Araba and Bani Ebaid). The age of each animal was used as a numerical explanatory variable. Due to low number of examined animals, the data obtained from horses from two subdistricts (Azraq and Dair Alla) and from mules were excluded. In the case of re-examined animals, only one sample from each animal was analysed. Moreover, only one interaction (sex × age) into the all-maximum model was included. Using model simplification, from each maximum model the least significant terms were gradually removed to obtain a minimal adequate model (Crawley, 2007).

RESULTS

Prevalence and distribution of T. equi *and* B. caballi

Although we recorded various health disorders, such as laminitis, external wounds or general weakness, no clinical signs usually associated with piroplasmoses were observed. Microscopic examination of blood smears did not reveal any erythrocytic stages typical for *Babesia* or *Theileria* species.

The PCR results with 'catch-all' primers revealed that 78 out of 288 animals (27·1%) were positive for piroplasmids. The highest prevalence was detected among mules followed by horses and donkeys (Table 2). The prevalence of *T. equi* and *B. caballi* was 18·8 and 7·3%, respectively. *T. equi* was more prevalent in horses and mules, while donkeys were more frequent hosts of *B. caballi* (Table 2). From the group of re-sampled animals only a single horse was infected with *T. equi*, being positive in 2 consecutive years. The infection was detected in three out of six subdistricts; Wadi Mousa, Shuna Al-Janubiya and Bani Ebaid with prevalences of 20·4%, 5·9% and 0·7%, respectively. Although in all these localities both piroplasmid species occurred, no mixed infections were detected. The total prevalence of piroplasmids, established with the 'catch-all' primers, was higher among males than among females (31·4% *vs* 23%) (Table 3). A similar trend was revealed with multiplex primers for both *T. equi* and *B. caballi* (Table 3).

Data analyses

The minimal adequate model that fits the occurrence of piroplasmids included the significant factors, of

Table 3. Prevalence of piroplasmids in males (M) and females (F) based on PCR assay results

		Primers					
		Catch-all		*T. equi*		*B. caballi*	
Host	Total number	*N*	%	*n*	%	*n*	%
Horse							
M	81	30	37	22	27·2	8	9·9
F	136	32	23·5	26	19·1	5	3·7
Donkey							
M	56	12	21·4	2	3·6	8	14·3
F	11	1	9·1	1	9·1	ND[a]	ND[a]
Mule							
M	3	2	66·6	2	66·6	ND[a]	ND[a]
F	1	1	100	1	100	ND[a]	ND[a]
Total							
M	140	44	31·4	26	18·6	16	11·4
F	148	34	23	28	18·9	5	3·4

[a] ND,not determined.

age ($\chi^2 = 18{\cdot}084$; $P << 0{\cdot}001$), species ($\chi^2 = 5{\cdot}792$; $P = 0{\cdot}016$) and sex ($\chi^2 = 4{\cdot}753$; $P = 0{\cdot}029$). Piroplasmids were more prevalent in horses and males and the prevalence increased with the age of the animal. For *T. equi*, the minimal adequate model included species ($\chi^2 = 12{\cdot}612$, $P << 0{\cdot}001$) and age ($\chi^2 = 13{\cdot}673$, $P << 0{\cdot}001$), whilst the minimal adequate model for *B. caballi* included a single significant factor – sex ($\chi^2 = 4{\cdot}447$, $P = 0{\cdot}034$). The prevalence of *T. equi* increased with age, and the infection was more prevalent in horses. More males were infected by *B. caballi* than females.

Phylogenetic analyses

In total, 58 amplicons obtained with the PCR 'catch-all' primers were sequenced (*T. equi* = 45 and *B. caballi* = 13). Ten sequences of *T. equi* and 10 sequences of *B. caballi*, representing the obtained sequence variability, were indexed in the GenBank™ (JQ417242-61) and subsequently used for phylogenetic analyses. Alignments containing 442 and 453 nt positions of the 18S rRNA genes of *T. equi* and *B. caballi*, respectively, were analysed using ML and MP. Both methods resulted in virtually identical topologies. The tree topology of *B. caballi* (Fig. 1) shows the known two genotypes A and B. One of the newly identified sequences (JQ417253) clusters together with sequences JF827602 from a Jordanian dog and EU642514 of unknown origin from South Africa, forming a separate clade referred to here as novel genotype C (Fig. 1). The tree of *T. equi* shows five major clades representing the known genotypes of *T. equi* (Fig. 2). With the exception of genotypes C and E, all other *T. equi* genotypes were found in the present study, although genotypes B and D were represented by single sequences (JQ417251 from a horse and JQ417247 from a mule). At the same time, genotypes C and D appear to be closely related. The clustering of *T. equi* isolates revealed the existence of three genetically distinct groups represented by clade (A), clades (C and D) and clades (E and B).

DISCUSSION

Equine theileriosis was previously reported from Jordan based on blood smear microscopy and serology (Hailat *et al.* 1997; Abutarbush *et al.* 2011). PCR-based diagnosis followed by sequencing reported herein proved the wide occurrence of *T. equi*, and also documented the presence of *B. caballi* in Jordanian equines for the first time. In general, the prevalence of *T. equi* is usually higher than that of *B. caballi* (Shkap *et al.* 1998; Rüegg *et al.* 2007; Bhoora *et al.* 2009; Kouam *et al.* 2010*b*; Abutarbush *et al.* 2011). The lower rate of *B. caballi* infections in studies based on microscopic diagnosis has been ascribed to low parasitaemia during the chronic phase (Kumar *et al.* 2008). However, since PCR-based detection should overcome these constraints, the different prevalence of *B. caballi* and *T. equi* is likely to be a consequence of the more efficient elimination of *B. caballi* by the host immune system, in contrast to the life-long persistence of *T. equi* (Brüning, 1996). Recently, 14·6% seropositivity for *T. equi* among horses in Jordan was reported, yet the authors were unable to detect the piroplasmid DNA (Abutarbush *et al.* 2011). This was likely a technical issue, since PCR-based diagnosis of both species appears to be relatively straightforward (Qablan *et al.* 2012*a*, *b*; this work). The overall prevalence of the infection established with the 'catch-all' primers was rather high (27%). However, all cases were apparently asymptomatic, which is typical for endemic areas (De Waal, 1992; Brüning, 1996).

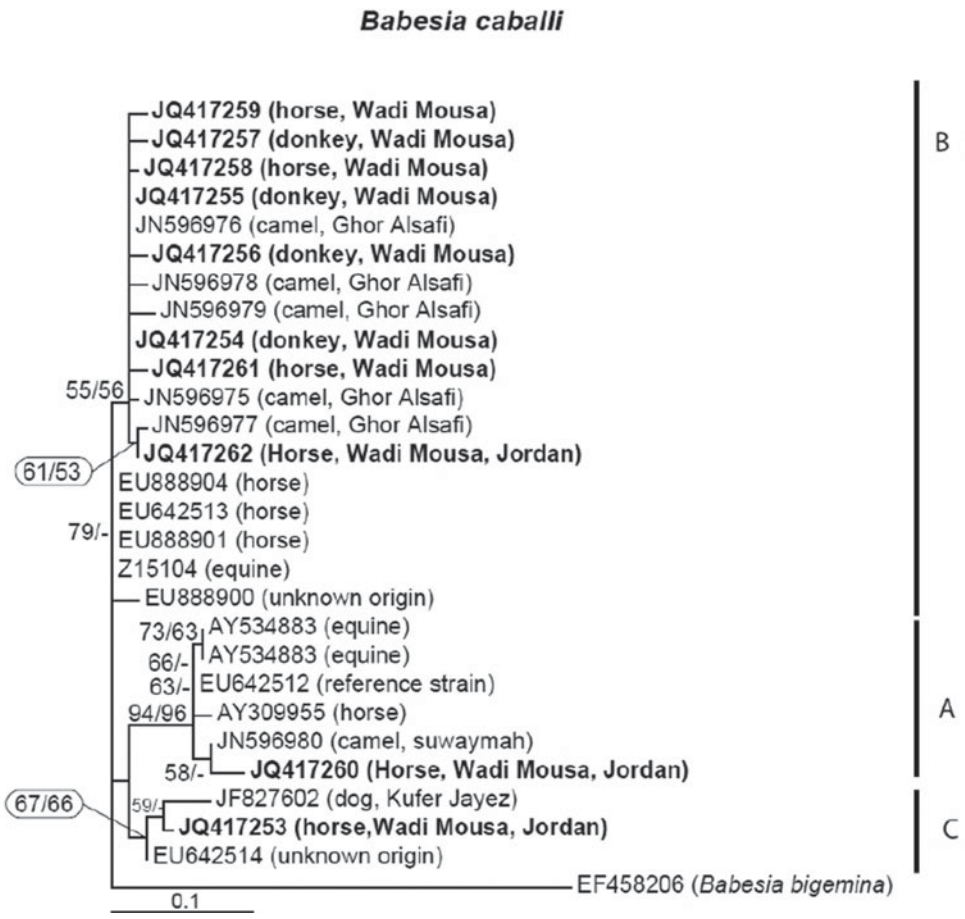

Fig. 1. Phylogenetic tree of *Babesia caballi* as inferred from partial sequences of the 18S rRNA gene. The numbers above branches indicate maximum likelihood/maximum parsimony bootstrap supports (1000/1000 replicates). Sequences in bold refer to those obtained in this study. Hosts and localities are in parentheses. A, B and C denote genotypes.

Jordan is located within the eastern Mediterranean climate zone with moderately rainy winters and hot summers. Biogeographically, the country is divided into four major ecozones; the Mediterranean, Irano-Turanian, Saharo-Arabian and Afrotropical (Al-Eisawi, 1985). Most sampling sites in this study were from the densely inhabited western part of the country where the borders between ecozones are narrow and affected by altitude and humidity. In this study, the *T. equi* and *B. caballi* infections were identified in equids from the Mediterranean, Irano-Turanian and Afrotropical ecozones, while previously we have also detected *T. equi* in camels from the Saharo-Arabian ecozone (Qablan *et al.* 2012*b*). Our results are in agreement with previous seroprevalence data reported from Jordan (Abutarbush *et al.* 2011). Apparently, equine piroplasmids are enzootic in Jordan and their distribution pattern is likely affected by the presence and densities of suitable hosts rather than by ecological conditions, which is in accordance with a recent study of these pathogens from Greece (Kouam *et al.* 2010*a*).

Using the results of 'catch-all' PCR, host species, age and sex were consistently recognized as significant risk factors for piroplasmid infection. Analogous analyses computed for both species separately indicate that only host species and age have a significant effect on the distribution of *T. equi*. In our dataset, horses appear to be more susceptible to *T. equi*, which is in agreement with serological studies from Turkey (Balkaya *et al.* 2010) and relative scarcity of the infections in donkeys (Pearson *et al.* 2000). Interestingly, mules were found to be the most susceptible hosts in Greece (Kouam *et al.* 2010*a*) and Brazil (Dos Santos *et al.* 2011) and also in our study we found three out of four animals to be positive for *T. equi*. However, due to the low number examined, mules have been excluded from the statistical analyses. No influence of host species was proven for *B. caballi*.

Horses usually reach sexual maturity at the age of three years. While some serological surveys show that age is a significant factor for *T. equi* infection (Kouam *et al.* 2010*a*; Grandi *et al.* 2011), others suggest the opposite (Shkap *et al.* 1998). To our knowledge, so far only two studies have used PCR-diagnosis data to evaluate the effect of age on the *T. equi* and *B. caballi* infections (Rüegg *et al.* 2007; Grandi *et al.* 2011).

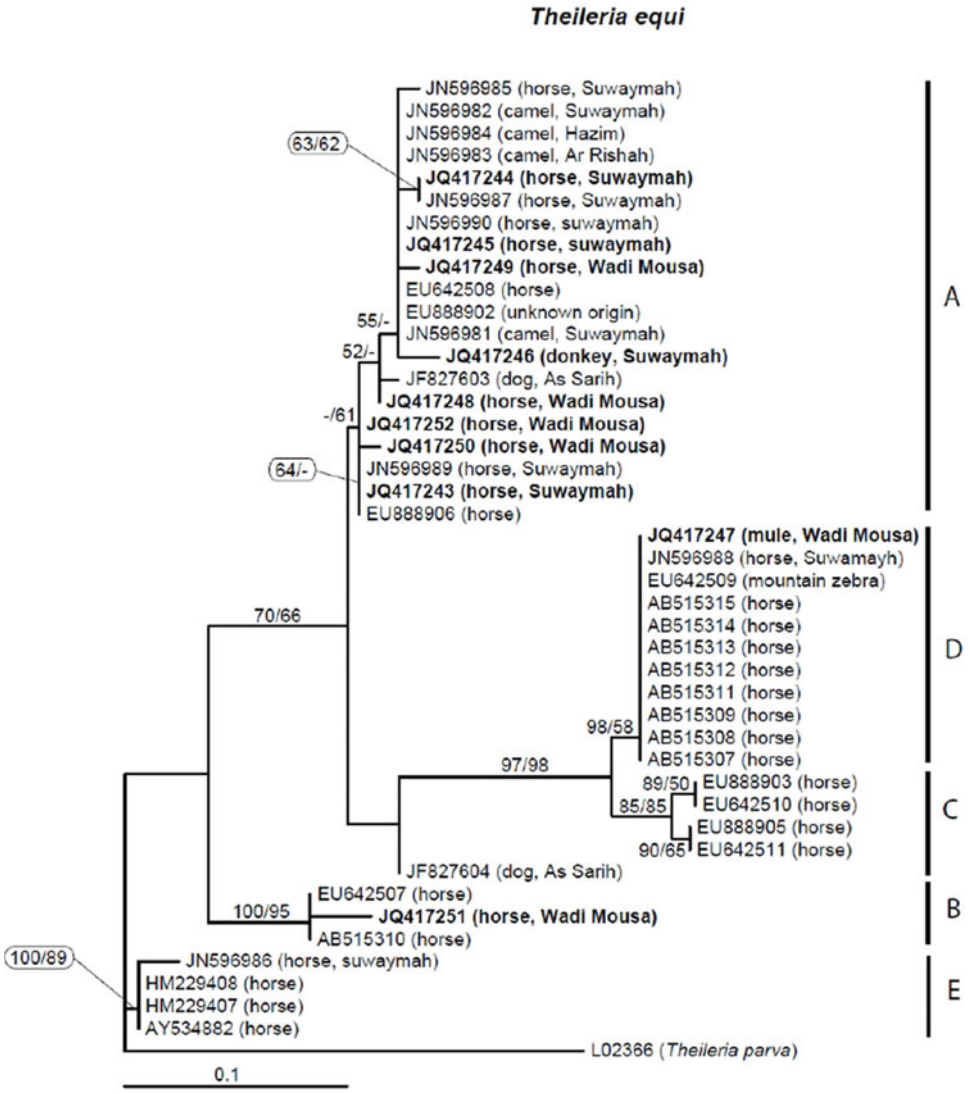

Fig. 2. Phylogenetic tree of *Theileria equi* as inferred from partial sequences of the 18S rRNA gene. The numbers above branches indicate maximum likelihood/maximum parsimony bootstrap supports (1000/1000 replicates). Sequences in bold refer to those obtained in this study. Hosts and localities are in parentheses. A, B, C, D and E denote genotypes.

While Rüegg *et al*. (2007) reported positive and negative correlations between host age and *T. equi* and *B. caballi*, respectively, Grandi *et al*. (2011) found no such correlation. However, we observed a significant effect of age on the prevalence of *B. caballi*, with adults having higher risk of infection. A similar relationship with age was reported for *Babesia bovis*, whereby, under experimental conditions young, animals were more resistant to the parasite (Chauvin *et al*. 2009).

The different susceptibilities of males and females to protozoan infections observed in many species, including humans, has been attributed to levels of sex hormones and activity patterns (Roberts *et al*. 2001). Data in the literature are contradictory in the case of piroplasmoses, as in some studies sex had a significant effect on the infection (Shkap *et al*. 1998; Moretti *et al*. 2010; Grandi *et al*. 2011), while others failed to see such a correlation (Rüegg *et al*. 2007; Karatepe *et al*. 2009; Kouam *et al*. 2010*a*; Dos Santos *et al*. 2011). In our study, some effect of sex was proven for the *B. caballi* infections, as males were more frequently infected.

We used two PCR assays, the 'catch-all' primers that permit the detection of piroplasmid infection in general and multiplex PCR that permits distinction between *T. equi* and *B. caballi* (and mixed infection) in a single reaction with both PCR strategies, showing almost identical sensitivity. Sequencing of amplicons obtained by the former PCR assay permits the identification of piroplasmids other than *B. caballi* and *T. equi*, while multiplex PCR is particularly suitable to detect mixed infections. In contrast to previous studies (Boldbaatar *et al*. 2005; Kouam *et al*. 2010*b*; Sloboda *et al*. 2011), the mixed infections were absent in our samples.

The 18S rRNA gene is widely used for phylogenetic analyses of piroplasmids (Allsopp and

Allsopp, 2006). Two genotypes were originally proposed within both species (Nagore *et al*. 2004), but later studies from South Africa (Bhoora *et al*. 2009), Sudan (Salim *et al*. 2010) and Jordan (Qablan *et al*. 2012*b*), added three additional genotypes of *T*. *equi*. The intraspecific diversity of *T*. *equi* and *B*. *caballi* is likely to further increase with the addition of sequences from so far unexplored geographical regions. Our phylogenetic analyses show that both parasites exhibited an overlapping occurrence of different genotypes within the same population of equids. We identified a novel genotype C of *B*. *caballi*; apparently, this genotype results from the splitting of genotype A as a result of addition of new sequences from horses (this study) and from Jordanian dog (Qablan *et al*. 2012*b*). Although the majority of sequences of *B*. *caballi* in our dataset belong to genotype B, our analysis does not strongly support further division of this genotype into two subgroups as proposed previously (Bhoora *et al*. 2009), probably a consequence of differences in the length of the gene fragment used in both studies.

Analysis of *T*. *equi* 18S rRNA sequences indicates the occurrence, in our dataset, of all previously known genotypes except genotypes E and C, with only one sequence belonging to the newly assigned genotype E (Qablan *et al*. 2012*b*). Interestingly, genotype E corresponds to *T*. *equi*-like parasites previously detected (Nagore *et al*. 2004; Kouam *et al*. 2010*b*) from lethal cases of equine theileriosis in Greece and Spain, respectively. The clustering of *T*. *equi* genotypes and the length of branches may suggest the existence of one or even more new species. However, in order to make such a conclusion, not only an extended set of genetic markers has to be evaluated, but also a better knowledge of the vectors will be necessary.

ACKNOWLEDGEMENTS

We thank Dr Mourad El-Farajat and the staff of Brook Clinic in Petra-Wadi Mousa for help during the field study. We are pleased to acknowledge the support by the National Center for Research and Development/Badia Research Program and the Badia Center for Ecological Education. The help of the Safawi field staff during the field sampling in the Eastern Desert is appreciated. We also appreciate the administrative assistance of the Ministry of Agriculture Amman Jordan represented by Dr Nasser Eddin Al-Hawamdeh.

FINANCIAL SUPPORT

This work was supported by the project 'CEITEC-Central European Institute of Technology' from the European Regional Development Fund (CZ.1·05/1·1·00/02·0068) and the Grant Agency of the Czech Republic (523/09/1972) to P. H., the Institute of Parasitology project (Z60220518) to M. O. and J. L., and the Praemium Academiae award to J. L., who is also a Fellow of the Canadian Institute for Advanced Research.

REFERENCES

Abutarbush, S. M., Alqawasmeh, D. M., Mukbel, R. M. and Al-Majali, A. M. (2011). Equine babesiosis: seroprevalence, risk factors and comparison of different diagnostic methods in Jordan. *Transboundary and Emerging Diseases* **59**, 72–78.

Al-Eisawi, D. M. (1985). Vegetation of Jordan. In *Studies in the History and Archaeology of Jordan. I* (ed. Hadidi, A.), pp. 45–57. Ministry of Archaeology, Amman, Jordan.

Alhassan, A., Pumidonming, W., Okamura, M., Hirata, H., Battsetseg, B., Fujisaki, K., Yokoyama, N. and Igarashi, I. (2005). Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. *Veterinary Parasitology* **129**, 43–49.

Allsopp, M. T. E. P. and Allsopp, B. A. (2006). Molecular sequence evidence for the reclassification of some *Babesia* species. *Annals of the New York Academy of Sciences* **1081**, 509–517.

Balkaya, I., Utuki, A. E. and Piskin, F. C. (2010). Prevalence of *Theileria equi* and *B. caballi* in donkeys from eastern Turkey in winter season. *Pakistan Veterinary Journal* **30**, 245–246.

Bashiruddin, J. B., Cammà, C. and Rebêlo, E. (1999). Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16s rRNA gene. *Veterinary Parasitology* **84**, 75–83.

Battsetseg, B., Lucero, S., Xuan, X., Claveria, F. G., Inoue, N., Alhassan, A., Kanno, T., Igarashi, I., Nagasawa, H., Mikami, T. and Fujisaki, K. (2002). Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. *Veterinary Parasitology* **107**, 351–357.

Bhoora, R., Franssen, L., Oosthuizen, M. C., Guthrie, A. J., Zweygarth, E., Penzhorn, B. L., Jongejan, F. and Collins, N. E. (2009). Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. *Veterinary Parasitology* **159**, 112–120.

Boldbaatar, D., Xuan, X., Battsetseg, B., Igarashi, I., Battur, B., Batsukh, Z., Bayambaa, B., and Fujisaki, K. (2005). Epidemiological study of equine piroplasmosis in Mongolia. *Veterinary Parasitology* **127**, 29–32.

Brüning, A. (1996). Equine piroplasmosis, an update on diagnosis, treatment and prevention. *British Veterinary Journal* **152**, 139–151.

Cacciò, S., Cammà, C., Onuma, M. and Severini, C. (2000). The *β*-tubulin gene of *Babesia* and *Theileria* parasites is an informative marker for species discrimination. *International Journal for Parasitology* **30**, 1181–1185.

Chauvin, A., Moreau, E., Bonnet, S., Plantard, O. and Malandrin, L. (2009). *Babesia* and its host: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Veterinary Research* **40**, 37.

Crawley, M. J. (2007). *The R Book*. John Wiley & Sons, Chichester, UK.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A. and Barba-Carretero, J. C. (2003). Molecular studies on *Babesia, Theileria* and *Hepatozoon* in southern Europe: Part II. Phylogenetic analysis and evolutionary history. *Veterinary Parasitology* **114**, 173–194.

De Waal, D. T. (1992). Equine piroplasmosis: a review. *British Veterinary Journal* **148**, 6–14.

Dos Santos, T. M., Roier, E. C., Santos, H. A., Pires, M. S., Vilela, J. A., Moraes, L. M., Almeida, F. Q., Baldani, C. D., Machado, R. Z. and Massard, C. L. (2011). Factors associated to *Theileria equi* in equids of two microregions from Rio de Janeiro, Brazil. *Brazilian Journal of Veterinary Parasitology* **20**, 235–241.

Grandi, G., Molinari, G., Tittarelli, M., Sassera, D. and Kramer, L. H. (2011). Prevalence of *Theileria equi* and *Babesia caballi* infection in horses from northern Italy. *Vector-Borne Zoonotic Diseases* **11**, 955–956.

Guindon, S. and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696–704.

Hailat, N. Q., Lafi, S. Q., Al-Darraji, A. M. and Al-Ani, F. K. (1997). Equine babesiosis associated with strenuous exercise: clinical and pathological studies in Jordan. *Veterinary Parasitology* **69**, 1–8.

Hall, T. A. (1999). BioEdit. A user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Hunfeld, K. P., Hildebrandt, A. and Gray, J. S. (2008). Babesiosis: recent insights into an ancient disease. *International Journal for Parasitology* **38**, 1219–1237.

Karatepe, B., Karatepe, M., Cakmak, A., Karaer, Z. and Ergun, G. (2009). Investigation of seroprevalence of *Theileria equi* and *Babesia caballi* in horses in Nigde province, Turkey. *Tropical Animal Health and Production* **41**, 109–113.

Kouam, M. K., Kantzoura, V., Gajadhar, A. A., Theis, J. H., Papadopoulos, E. and Theodoropoulos, G. G. (2010*a*). Seroprevalence of equine piroplasms and host-related factors associated with infection in Greece. *Veterinary Parasitology* **169**, 273–278.

Kouam, M. K., Kantzoura, V., Masouka, P. M., Gajadhar, A. A. and Theodoropoulos, G. (2010*b*). Genetic diversity of equine piroplasms in Greece with a note on speciation within *Theileria* genotypes (*T. equi* and *T. equi*-like). *Infection, Genetic and Evolution* **10**, 963–968.

Kumar, S., Kumar, R., Gupta, A. K. and Dwivedi, S. K. (2008). Passive transfer of *Theileria equi* antibodies to neonate foals of immune tolerant mares. *Veterinary Parasitology* **151**, 80–85.

Lack, J. B., Reichard, M. V. and Van Den Bussche, R. A. (2012). Phylogeny and evolution of the piroplasmida as inferred from 18S rRNA sequences. *International Journal for Parasitology* **42**, 353–363.

Lassmann, T. and Sonnhammer, E. L. (2005). Kalign – an accurate and fast multiple sequence alignment algorithm. *BMC Bioinformatics* **6**, 298.

Moretti, A., Mangili, V., Salvatori, R., Maresca, C., Scoccia, E., Alessandra, T., Moretta, I., Gabrielli, S., Tampieri, M. P. and Pietrobelli, M. (2010). Prevalence and diagnosis of *Babesia* and *Theileria* infections in horses in Italy: a preliminary study. *Veterinary Journal* **184**, 346–350.

Nagore, D., García-Sanmartín, J., García-Pérez, A. L., Juste, R. A. and Hurtado, A. (2004). Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. *Veterinary Parasitology* **123**, 41–45.

Pearson, R. A., Nengomasha, E. and Krecek, R. (2000). The challenge in using donkeys for work in Africa. In *Donkeys, People and Development, a Resource Book of the Animal Traction Network for Eastern and Southern Africa (ATNESA)*. (ed. Starkey, P. and Fielding, D.), pp. 194–195. ACP-EUT Technical Center for Agricultural and Rural Cooperation (CTA), Wageningen, the Netherlands.

Qablan, M. A., Kubelová, M., Široký, P., Modrý, M. and Amr, Z. S. (2012*a*). Stray dogs of northern Jordan as reservoirs of ticks and tick-borne haemopathogens. *Parasitology Research* **111**, 301–307.

Qablan, M. A., Sloboda, M., Jirků, M., Oborník, M., Dwairi, S., Amr, Z. S., Hořín, P., Lukeš, J. and Modrý, M. (2012*b*). Quest for the piroplasms in camels: identification of *Theileria equi* and *Babesia caballi* in Jordanian dromedaries by PCR. *Veterinary Parasitology* **186**, 456–460.

Roberts, C. W., Walker, W. and Alexander, J. (2001). Sex-associated hormones and immunity to protozoan parasites. *Clinical Microbiology Reviews* **14**, 476–488.

Rüegg, S. R., Torgerson, P., Deplazes, P. and Mathis, A. (2007). 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. *Parasitology* **134**, 939–947.

Salim, B., Bakheit, M. A., Kamau, J., Nakamura, I. and Sugimoto, C. (2010). Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene *Theileria equi* from horses in Sudan. *Parasitology Research* **106**, 493–498.

Shkap, V., Cohen, I., Leibovitz, B., Savitsky, Pipano, E., Avni, G., Shofer, S., Giger, U., Kappmeyer, L. and Knowles, D. (1998). Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. *Veterinary Parasitology* **76**, 251–259.

Schnittger, L., Rodriguez, A. E., Florin-Christensen, M. and Morrison, D. A. (2012). *Babesia*: a world emerging. *Infection, Genetic and Evolution* **12**, 1788–1809.

Sloboda, M., Jirků, M., Lukešová, D., Qablan, M. A., Batsukh, Z., Fiala, I., Hořín, P., Modrý, M. and Lukeš, J. (2011). A survey of piroplasmids in horses and Bactrian camels in North-Eastern Mongolia. *Veterinary Parasitology* **179**, 246–249.

Starkey, P. and Starkey, M. (2000). Regional and world trends in donkey populations. In *Donkeys, People and Development, a Resource Book of the Animal Traction Network for Eastern and Southern Africa (ATNESA)*. (ed. Starkey, P. and Fielding, D.), pp. 10–12. ACP-EUT Technical Center for Agricultural and Rural Cooperation (CTA), Wageningen, the Netherlands.

Swofford, D. L. (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10 (Alvitec)*. Sinauer Associates, Sunderland, MA, USA.

Zobba, R., Ardu, M., Niccolini, S., Chessa, B., Manna, L., Cocco, R. and Parpaglia, M. L. P. (2008). Clinical and laboratory findings in equine piroplasmosis. *Journal of Equine Veterinary Science* **28**, 301–308.