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Short communication

A survey for piroplasmids in horses and Bactrian camels in North-Eastern Mongolia

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ABSTRACT

Equine piroplasmosis caused by *Babesia caballi* and *Theileria equi* is widespread in Asia. The presence of these haemozoans in Mongolia was previously confirmed in domestic as well as in reintroduced Przewalski horses in which they cause significant pathology. The data on occurrence of piroplasms from Bactrian camels in Asia is lacking. A total of 192 horses, 70 Bactrian camels, and additional 16 shepherd dogs from the Hentiy province were included in our study. No clinical signs typical for piroplasmid infection were observed during the field survey. Microscopic examination revealed the presence of *T. equi* in blood smears from 67% of examined horses, with camels and dogs being negative. A two step PCR approach was used to detect piroplasms in peripheral blood. In the first "catch all" PCR reaction, amplification of the 496 bp-long fragment of the SSU rRNA gene enabled the detection of *Babesia* and *Theileria* spp. Second round multiplex PCR reaction used for species discrimination allowed the amplification of *T. equi*- and *B. caballi*-specific 340 bp and 650 bp-long regions of the SSU rRNA, respectively. This assay detected *T. equi* in 92.7% of horses, while the infections with *B. caballi* and dual infections were rare. In both PCR setups, camels and dogs were negative indicating that in the studied region, these hosts do not share piroplasms with horses.

1. Introduction

Piroplasmids are worldwide distributed apicomplexan parasites of domestic animals and wildlife transmitted by ticks (Homer et al., 2000). Over 30 species belonging to the genera *Babesia* and *Theileria* have been described to date (Criado-Fornelio et al., 2004; Uilenberg, 2006). As such, they were traditionally referred to as host-specific protists. However, several recent studies revealed the occurrence of atypical piroplasmid species in various hosts opening the question of the host specificity and its barriers in these parasites (Criado-Fornelio et al., 2003; Buling et al., 2007; Gimenez et al., 2009; Fritz, 2010). Undoubtedly, the tick species shared by different species of hosts represent a bridge responsible for the transmission.

In our study, we have chosen the piroplasms infecting equines as a model of possible co-occurrence of piroplasmids in host communities consisting of several species living in close contact. The studied species, namely *Babesia caballi* and *Theileria equi*, cause in horses the disease termed equine piroplasmosis (EP) endemic in most

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tropical and subtropical areas (Friedhoff et al., 1990). Besides horses, donkeys and zebras, molecular techniques including sequencing of the resulting products enabled the detection of equine priplasmids also in dogs (Criado-Fornelio et al., 2003; Criado et al., 2006; Beck et al., 2009; Fritz, 2010). In contrast to horses and dogs, piroplasmid infections were only rarely recorded in camelids (Boid et al., 1985; Rao et al., 1988; Nassar, 1992; Egbe-Nwiyi, 1994), and the available data remains rather limited.

Mongolia is a country that chiefly depends on the animal production, with an estimated number of horses reaching two millions. Previous studies detected piroplasms in Mongolian horses by means of several detection methods (Avarzed et al., 1997; Xuan et al., 1998; Ikadai et al., 2000; Boldbaatar et al., 2005), and proved high prevalences and even fatal consequences for Mongolian equids (Ruegg et al., 2007). In contrast, data about these parasites in Bactrian camels and dogs in Mongolia is totally lacking.

The major goal of our study was to (i) define the prevalence of piroplasmids in Mongolian horses in Hentyi province and (ii) to search for these species in camels and dogs to prove and/or exclude their role as infection reservoirs in those places, where they are kept in close contact with horses. Moreover, we aimed to validate some of the available PCR-based diagnostic assays.

2. Materials and methods

2.1. Study site, sampling

The study was conducted in July 2009 at localities in the following districts of the Hentyi province in North-Eastern Mongolia—Batnorov, Bayan Ovoo, Bayankhutag, Dadal, Galshar, and Norovlin. Anamnestic data (age, sex, and utilization, health problems in the past, tick burden, and contact with other animals) was obtained for each inspected animal using bilingual questionnaires. Horses and camels were blood-sampled by puncture of the jugular vein using plastic containers (Hemos H-01, Gama Group) equipped with 18 G needles (BD Microlance). Blood samples of dogs were obtained by puncture of the cephalic vein using the containers mentioned above equipped with thinner 21 G needles (BD Microlance). Two thin blood smears made from each specimen were fixed with methanol later the same day. Blood aliquotes were stored in 3 ml EDTA containers (Terumo) for further DNA analysis.

2.2. Blood smears, DNA extraction, PCR and sequencing

Blood smears were stained with Giemsa (Merck) and examined using light microscopy (Olympus AX 70) for 30 min. Total DNA was extracted from 100 μl of EDTA-fixed blood using DNeasy® Blood and Tissue kit (Qiagen). To amplify a diagnostic fragment of the piroplasmid SSU rRNA gene, we have designed the forward primer TB-F (5′-CTTCAGCACCTTGAGAGAAAT-3′) and the reverse primer TB-R (5′-TCDATCCCCRWCACGATGCRBAC-3′), which universally amplified the target gene from both *Theileria* and *Babesia* species. The 25 μl PCR reaction consisted of 2 μl of DNA sample, 12.5 μl of Combi PPP Master Mix (Top-Bio), 1 μl of each of the primers and 8.5 μl of PCR water; amplification conditions were: 5 min at 94 °C, 39 cycles each of 94 °C for 45 s, 62 °C for 30 s, and 72 °C for 45 s, with the addition of a final extension period of 10 min at 72 °C. To distinguish between *T. equi* and *B. caballi*, initially the primers and PCR protocol of Alhassan et al. (2005) were used. However, due to technical issues (see below), following PCR protocol has been developed. Primers were designed by visual inspection of the alignment of the SSU rRNA genes from 8 *Theileria* spp., 7 *Babesia* spp., as well as the same gene from their hosts (horse, camel and dog), so that only the piroplasmid gene is amplified. The possibility of self/pair dimer or hairpin formations was checked using the software package PrimerSelect (DNAstar Inc.). In a single PCR reaction, the forward primer TBM (5′-CTTCAGCACCTTGAGAGAAATC-3′) (this study), and the reverse primers Equi-R (5′-TGCCTTAAACTTCCTTGCGAT-3′) (Alhassan et al., 2005) and BC-R (5′-GATTCGTCGGTTTTGCCTTGG-3′) (this study) were used. The amplification program consisted of 5 min denaturation at 94 °C followed by 40 cycles of 94 °C for 1 min, 63 °C for 1 min, and 72 °C for 1 min, and a final extension period of 10 min at 72 °C.

In both universal and species-specific PCR reactions, DNA isolated from the blood of a piroplasmid-free horse served as a negative control. DNA isolated from the blood of a donkey co-infected with *B. caballi* and *T. equi* obtained from Italy was used as a positive control. Products of all PCR reactions were subjected to ethidium-bromide stained 1.5% agarose gel electrophoresis, and documented using Vilber Lourmat system. Selected PCR products were extracted from agarose gel using QIAquick gel extraction kit (Qiagen) and sequenced in their entirety.

3. Results

In total, 192 horses (99 females, 89 males), 70 camels (31 females, 37 males), and 16 dogs (4 females, 12 males) were sampled; data on sex for 4 horses and the same numbers of camels was lost. The age of horses and camels ranged from 2 to 20 years; dogs were 6 months to 10 years old. Clinical examination revealed various health disorders, general poor condition was observed in 30 horses (21%), 9 camels (13%), and 3 dogs (19%). Most commonly coughing, locomotion problems, and skin lesions and/or erosions were mentioned by the questioned owners. However, clinical signs typical for piroplasmosis (hemoglobinuria, jaundice) were not recorded. It is worth mentioning that the owners of horses were frequently reporting infestation by ticks in the past months. Occasionally, adult ticks were observed on camels from the Bayan Ovoo district. Microscopic examination revealed that 59 out of 88 examined blood smears (67%) from horses were positive for intraerythrocytic stages of *Theileria* sp. Rarely, merogonial stages within the lymphocytes were also observed (data not shown). Developmental stages of *Babesia* sp. were not found in any of the examined horses and finally, blood smears of all the camels and dogs were negative for the presence of piroplasms.

The molecular diagnosis was initiated by subjecting DNA samples isolated from the blood of examined animals to PCR reaction using primers TB-F and TB-R that univer-

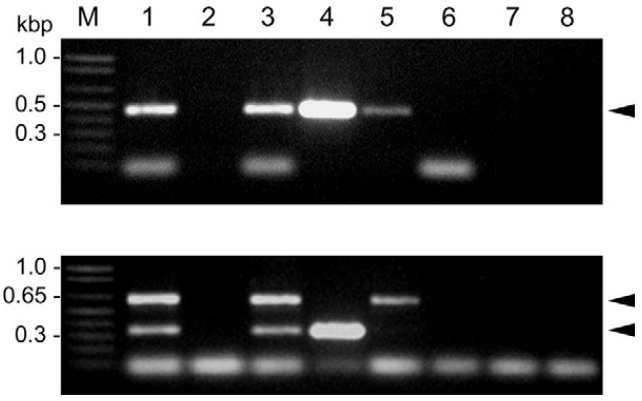

Fig. 1. (A) PCR-based detection of piroplasmids using "catch all" primer pair TB-F and TB-R that amplifies a 496 bp-long fragment of the SSU rRNA gene (arrowhead). (B) Multiplex PCR reaction containing primers TBM, Equi-R and BC-R was used in the second step and allowed discrimination between *Theileria equi* (360 bp) and *Babesia caballi* (650 bp) (arrowheads). M: DNA marker; lane 1: positive control; lane 2: negative control; lane 3: horse with mix infection of *T. equi* and *B. caballi*; lane 4: *T. equi*-positive horse; lane 5: *B. caballi*-positive horse; lane 6: negative horse; lane 7: negative dog; lane 8: negative camel (identical samples were loaded in both panels).

sally amplify a 496 bp-long part of the SSU rRNA gene of *Theileria* and *Babesia*. This "catch all" approach revealed that from 192 examined horses, 92.7% were positive, while all the camels and dogs were found negative for the presence of piroplasmid DNA in peripheral blood (Fig. 1). Next, we wanted to establish to which genus the parasites responsible for such a high prevalence belong, and we have employed the PCR assay of Alhassan et al. (2005) for that purpose. However, despite we have obtained amplicons of expected size for *B. caballi* (540 bp), sequencing revealed that the used primer pair amplified not only the parasite SSU rRNA gene, but also the orthologous horse or camel host gene (data not shown), making the assay unreliable due to false positivity. Therefore, we aligned available piroplasmid SSU rRNA genes along with host SSU rRNA sequences. We were then able to identify short *Babesia* or *Theileria*-specific regions of the gene, into which novel primers TBM and BC-R were placed. Combination of this primer pair with the Equi-R primer in a single multiplex reaction allowed instant discrimination between *Babesia* and *Theileria* thanks to the species-specific amplicon size. Control reactions showed that the primer pair TBM and BC-R produced a 650 bp-long *B. caballi*-specific amplicon, while the other primer pair (TBM and Equi-R) ensured specific amplification of a 360 bp-long fragment of the target gene from *T. equi* (Fig. 1). Sequencing of the PCR products confirmed their predicted homologies with the given parasite species (data not shown). Application of this multiplex PCR approach revealed the presence of *T. equi* in all animals positive in the initial "catch all" PCR round (Fig. 1). A mixed infection of *B. caballi* and *T. equi* was found in just two horses (1.2%) originating from the Batnorov district; a single animal from the same district harbored infection with *B. caballi* only (Fig. 1). All the examined camels and dogs were confirmed to be negative (data not shown).

4. Discussion

Among the three groups of domestic animals included in our study, piroplasms were previously found only in Mongolian equines. Avarzed et al. (1997) reported a combined seroprevalence exceeding 80% in central Mongolia, while Boldbaatar et al. (2005) detected antibodies against *T. equi* and *B. caballi* in 39–96%, and 17–63% horses, respectively. Based on these results, both research groups considered the causative agents of EP to be widespread in Mongolia. However, it is known that in hosts infected with more than a single piroplasmid species the results of serological examinations may be influenced by cross reactions (Papadopoulos et al., 1996) and, more importantly, they may not reflect the actual status of the examined animals. Despite the relatively high prevalence rates of *B. caballi* in various localities in Mongolia reported previously (Avarzed et al., 1997; Xuan et al., 1998; Ikadai et al., 2000; Boldbaatar et al., 2005), we have found only three horses positive for this piroplasmid, whereas the rate of *T. equi* infections was very high as confirmed also by microscopy. Prevalence rates of *T. equi* are generally higher compared to *B. caballi* (Criado-Fornelio et al., 2003; Fritz, 2010; Ruegg et al., 2007) and our results correlate also with PCR-based survey by Ruegg et al. (2007), who detected high prevalence of *T. equi* and a significantly lower occurrence of *B. caballi* in the south-western Mongolia. The latter species was frequently encountered in horses originating from the southern, semi-desert parts of Mongolia, likely a consequence of high density of ticks in this area (Boldbaatar et al., 2005). A very low prevalence of *B. caballi* in our samples might be correlated with the studied area that belongs to the forested zone located close to the southern part of Siberia, where both *T. equi* and *B. caballi* were until recently supposed to be absent (Friedhoff and Soule, 1996). Differences in prevalence rates of both species indicate that the tick vector(s) of *T. equi* are different from those transmitting *B. caballi*, which would thus be virtually absent in the areas sampled by us. Since we did not find any ticks on the sampled horses, no data is currently available to support this notion. Interestingly, several old horses remained negative despite the high prevalence of *T. equi* in the study area, and they will be used for further investigations on possible existence of host resistance genes as identified in other species of domestic animals (Jensen et al., 2008).

Piroplasmids are generally considered as parasites with high host specificity (Uilenberg, 2006). However, the level of the specificity in intermediate host is probably lower than expected based on recent findings of "non-typical" species in several vertebrates, such as the detections of the canine *B. canis* and bovine *B. bovis* and *B. bigemina* in horses, or equine *T. equi* and *B. caballi* in dogs (Criado-Fornelio et al., 2003; Criado et al., 2006; Beck et al., 2009). Based on our results, we might assume that in the conditions of North-Eastern Mongolia, equine piroplasmids are probably not transmitted to camels and dogs, although the infection pressure is high. However, a more extensive study including higher number of animals would be of a great value, despite the obstacles in sampling associated with field studies conducted in similar countries.

Regarding increasing number of reports on the cross-transmissions of various piroplasms, species determination becomes imperative, and this can be achieved by either sequencing the PCR products, or by applying protocols that allow distinguishing the parasite species by the amplicon size. Here we have introduced a PCR assay that allows such an easy distinction between *T. equi* and *B. caballi* of horses. Moreover, it is worth mentioning that some of the published PCR protocols for the detection of equine piroplasms (Bashiruddin et al., 1999; Battsetseg et al., 2001; Ueti et al., 2003; Alhassan et al., 2005) either failed when used on the material from Mongolian equines (Ruegg et al., 2007) or resulted in false positivity (this work). In most PCR surveys, primers specific for a piroplasmid species so far associated with the examined particular host species are used. However, such an approach may lead to the loss of important data as infections with "unexpected" piroplasms would be missed. The two-step PCR approach combined with sequencing of the amplicons used in this study to large extent alleviates this problem.

Conflicts of interest

The authors declare no conflicts of interest.

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