

Original Article

Prevalence and phylogenetic analysis of *Theileria equi* in Iranian dromedaries

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ABSTRACT

Considering the importance of *Theileria equi* infection in horse breeding industry and marketing, in the present study, we aimed to determine the prevalence of *T. equi* among dromedaries in central Iran, where a considerable number of camels and horses are raised and equine theileriosis is quite prevalent. For this purpose, a total of 161 blood samples from camels were examined in terms of *T. equi* infection, using parasitological and molecular methods. For molecular detection of *T. equi*, primers targeting the 18S rRNA gene were selected. Microscopic examination revealed that 0.6% of camels were positive for the intraerythrocytic stage of *Theileria* species, while polymerase chain reaction (PCR) method detected *T. equi* in 7 (4.3%) out of 161 camels. Sequences of 18S rRNAs from all the isolates showed more than 99% homology to each other and *T. equi* isolates in the GenBank. With respect to the single-nucleotide substitution in 18S rRNA gene of the studied camels, three different genotypes were identified and submitted to the GenBank. Considering the homology between 18S rRNA sequences of *T. equi* in the studied samples and those available in the GenBank, the phylogenetic tree formed three distinct, but highly-related clusters. In this study, age, gender, and locality were not determined as risk factors for *T. equi* infection in camels. In conclusion, this study demonstrated that *T. equi* is present among Iranian camels.

Keywords: *Theileria equi*, Camel, Polymerase chain reaction, Phylogenetic analysis, Yazd

Prévalence et analyse phylogénétique de *Theileria equi* infectant les dromadaires iraniens

Résumé: Etant donnée l'importance des infections causées par *Theileria equi* dans l'industrie et le marché des chevaux d'élevages, l'objectif de cette étude était de déterminer la prévalence de *T. equi* chez les dromadaires du centre de l'Iran. Cette région, où les élevages de camélidés et de chevaux sont abondants, est aujourd'hui particulièrement touchée par la theilériose équine. Dans cette étude, la présence d'infection au *T. equi* a été examinée sur un total de 161 prélèvements sanguins de dromadaires un utilisant des méthodes de parasitologie et moléculaires. Pour la détection moléculaire de *T. equi*, des amorces visant la séquence du gène 18S de l'ARNr ont été sélectionnées. Alors que nos observations microscopiques indiquaient une contamination au *Theileria spp.* en phase intraérythrocytaire pour 0.6% des dromadaires, nos analyses moléculaires par PCR nous ont permis de détecter 7 cas (4,3%) d'infection à *T. equi* sur les 161 échantillons testés. Les séquences du gène 18S de l'ARNr de tous les isolats détectés et celles des isolats de *T. equi* répertoriées dans GenBank étaient homologues à plus de 99%. Trois génotypes différents ont été identifiés et annotés dans GenBank selon leurs substitutions nucléotidiques uniques sur la séquence 18S de l'ARNr. Etant donnée la forte homologie entre les séquences 18S de l'ARNr des isolats de *T. equi* identifiés dans nos échantillons et celles disponibles dans GenBank, ces génotypes constituent trois groupes distincts, mais étroitement liés dans l'arbre phylogénétique. De plus, cette étude montre que l'âge, le sexe et la localité des dromadaires n'influent pas sur le risque d'infection au *T. equi*. En conclusion, nos analyses ont révélé la présence d'infections au *T. equi* chez les dromadaires iraniens.

Mots clés: *Theileria equi*, Dromadaire, Réaction en chaîne de la polymérase, Analyse phylogénétique, Yazd

INTRODUCTION

Tick-borne diseases, as an important group of diseases among domestic animals, commonly occur in Iran and have economic consequences for the country (Bahrami et al., 2014a; Bahrami et al., 2014b). Equine theileriosis, caused by *Theileria equi*, is a tick-borne protozoan disease of horses, mules, donkeys, and zebras. *Theileria* parasites have a unique life cycle, involving asexual and sexual developmental stages in the erythrocytes of vertebrates and tissues of ticks (de Waal, 1992). Although equine theileriosis is mainly endemic to tropical and subtropical areas, regions with a moderate climate may be affected, as well. This disease poses a serious threat to horse breeding industry and marketing (Avarzed et al., 1997). Equine theileriosis may be asymptomatic or manifest with severe signs such as fever, hemolytic anemia, jaundice, hemoglobinuria, and death in acute cases (Heim et al., 2007; Ruegg et al., 2007). The causative agent of equine theileriosis can be naturally transmitted by the ticks of the family *Ixodidae*. Overall, 10 tick species from three distinct genera (i.e., *Dermacentor*, *Rhipicephalus*, and *Hyalomma*) have been identified in *T. equi* transmission. Transplacental transmission can be a serious economic burden on horse breeders, and the infected mares are likely to be carriers of *T. equi* for a lifetime. In addition to this biological route, *T. equi* has the potential to be iatrogenically transmitted. In fact, this hemoparasite can be mechanically transmitted through contaminated needles and surgical instruments (de Waal, 1992). Since carrier animals, as the major source of infection, play the most important role in altering the piroplasm life cycle between horses and ticks, accurate diagnosis of carriers is necessary for disease prevention. Piroplasmids are generally considered as parasites with high host specificity (Uilenberg, 2006). However, the level of specificity in the intermediate host is probably lower than expected for non-typical species detected in several vertebrates, such as canine *Babesia canis*, bovine *B. bovis*, equine *B. bigemina*, and canine *T. equi* or *B. caballi* (Criado-Fornelio et al., 2003; Beck et al., 2009). Also, (Qablan

et al., 2012b) detected *T. equi* in the blood samples of Jordanian camels; therefore, camels could be also carriers of equine piroplasmidosis. Camel (*Camelus dromedarius*) is an important and popular multi-purpose local animal in central Iran. Yazd province in Iran is home to a considerable population of camels. However, despite the general endurance and resilience of camels, they are vulnerable to various infections (Walker et al., 2005) and parasitic agents (Bukachi et al., 2003). In Iran, similar to other countries, parasitic diseases among camels, particularly those caused by protozoans, have been neglected. Therefore, the aim of the present study was to investigate the prevalence of *T. equi* among camels and perform phylogenetic analyses in Iran, where equine theileriosis has become endemic.

MATERIALS AND METHODS

Study setting and animals. The province of Yazd is located in a plateau in central Iran at an altitude of 29.52 to 33.27 and longitude of 52.55 to 56.37. The annual rainfall in this region ranges between 50 and 100 mm. Yazd province has hot summers, mild springs, and cold winters, and changes in temperature are too dramatic in winter and summer; the temperature may even vary from +45°C during the day to -20°C at night. In the present study, a total of 161 camels in direct or indirect contact with horses were randomly selected. The samples were chosen from three regions of Yazd province, where a major population of camel herds is raised. These regions were as follows: Ardekan in the north of Yazd province (n=61), Saddough in the northeast of Yazd province (n=60), and Mehriz in the south of Yazd province (n=40). Farms cooperating with this study were randomly selected. Anamnestic data (i.e., age, sex, prior health problems, and contact with other animals) were gathered for each inspected animal, using bilingual questionnaires. Overall, coughing, locomotion problems, and skin lesions were the most commonly reported issues by the questioned owners. Although adult ticks were observed in the majority of camels, clinical signs which are typical of equine

piroplasmiasis (e.g., hemoglobinuria and jaundice) were not recorded.

Sample collection. The blood samples were collected from the jugular vein, transferred into sterile vacuum tubes, containing ethylenediaminetetraacetic acid (EDTA), and stored at -20°C. Thin blood smears were prepared and fixed with absolute methanol (1 min). Afterwards, they were stained with 10% Giemsa solution (30 min) and examined via oil immersion microscopy to observe intraerythrocytic forms of *T. equi*. Approximately 20,000 red blood cells were carefully searched per slide.

DNA extraction and polymerase chain reaction (PCR). DNA was extracted from the blood samples, using a genomic DNA purification kit (SinaClon Bioscience Co., Iran). For the detection of *T. equi*, primers targeting the 18S rRNA gene were selected from the literature (Alhassan et al., 2005). Primers (Bioneer Inc., South Korea) used in the reaction consisted of BEC-UF2 as the forward primer (sequence: 5'- TCGAAGACGATCAGATACCGTGC-3') and Equi-R as the reverse primer (sequence: 5'- TGCCTTAAACTTCCTTGCGAT-3'), yielding a 435 bp product. A negative control, consisting of the reaction mixture and 2 µl of DNase/RNase-free water (instead of DNA), was included in PCRs. Also, a positive control, including a DNA sample extracted from the blood of a horse with clinical theileriosis, was used. All PCRs were performed in a 25 µl reaction mixture, containing 12.5 µl of Taq DNA Polymerase Master Mix Red (Amplicon, Denmark), 1 µM primers, and 50 ng of DNA templates. PCR cycling included an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45 sec, annealing at 60 °C for 50 sec, extension at 72 °C for 60 sec, and a final extension at 7 °C for 5 min. The PCR products were electrophoresed on 1.5% agarose gel (SinaClon Bioscience Co., Iran) in tris-acetate-EDTA (TAE) buffer and were stained with Safe stain (SinaClon Bioscience Co., Iran); then, they were visualized under ultraviolet light (positive samples showed 435 bp bands).

DNA sequencing and phylogenetic analysis.

Amplified fragments (corresponding to the predicted size of *T. equi*) from camels, as well as two samples from horses, were purified, using a PCR purification kit (Fermentas, Lithuania). The PCR fragments were sequenced, using specific primers and Big Dye Terminator V.3.1 Cycle Sequencing Kit in an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). DNA sequences were analyzed, using Vector NTI[®] software (Life Technology, USA). Multiple-sequence alignment of sequences from the studied camels and those in the GenBank (available on <http://ncbi.nlm.nih.gov>) was analyzed, using BLAST (available on <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and ClustalW2 software. A phylogenetic tree was drawn, using the neighbor-joining method and MEGA 4.0 software. The topological stability of the tree was evaluated by 1000 bootstrap replications.

Statistical analysis. Fisher's exact test and Chi-square were performed to compare infection rates among different age and sex groups of animals. P-value less than 0.05 was considered statistically significant.

RESULTS

Microscopic examination revealed that 1 (0.6%) out of 161 examined blood smears from camels was positive for the intraerythrocytic stage of *Theileria* species. In 7 (4.3%) samples, PCR was positive, and a band of approximately 435 bp was seen on the agarose gel, suggesting an infection with *T. equi* (Figure 1). In order to confirm the specificity of PCR analysis, 435 bp amplicons obtained from seven camels and two horses were sequenced. The PCR products were confirmed as *T. equi*, thus establishing the specificity of PCRs. Sequences of 18S rRNAs from all the isolates showed more than 99% homology to each other and *T. equi* isolates from horses (genotype A1_RBEQ178, GenBank accession No: EU642508 and Wadi Musa isolate, GenBank accession No: JQ417248) and camels in the GeneBank (Suwaymah isolate, GenBank accession No: JN596981 and Wadi Araba isolate, GenBank accession No: JN596983). Based on the

single-nucleotide substitution in 18S rRNA gene from the studied camels, three different genotypes were identified and submitted to the GenBank with the following characteristics: Saddough isolate with GenBank accession No.: KM047412, Ardakan isolate with GenBank accession No.: KM047413, and Mehriz isolate with GenBank accession No.: KM047414. Considering the homology between the submitted GenBank sequences and 18S rRNA sequences of *T. equi* in the studied samples, the phylogenetic tree formed three distinct, but highly related clusters. Three different *T. equi* genotypes, isolated from camels and horses in Yazd province, were clustered in one group.

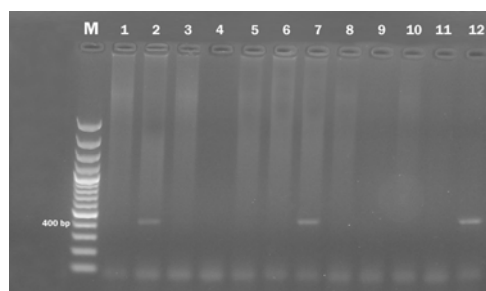


Figure 1. Amplification of *T. equi* DNA. Lane M is a 100 bp ladder, lane 12 is a positive control DNA, lane 11 is a negative control DNA, lanes 2 and 7 represent positive field samples, and lanes 1, 3-6, and 8-10 represent negative field samples.

Table 1. Prevalence of *T. equi* infection among camels in Yazd province, central Iran and the related factors

Factors	Number of examined camels	Positive	%
Prevalence	161	7	4.3
Gender			
Male	100	3	3
Female	61	4	6.5
Age			
≤ 5 years	52	1	1.9
> 5 years	109	6	5.5
Locality			
Ardekan	61	3	4.9
Saddough	60	1	1.6
Mehriz	40	3	7.5

Cluster two contained different *T. equi* genotypes from horses in the GenBank database, while *T. equi* genotypes isolated from the camels were clustered in another branch (Figure 2). Based on the molecular

analysis, 3 (3%) out of 100 males and 4 (6.5%) out of 61 females were found to be positive for *T. equi*. Therefore, in terms of gender, there was no significant difference in the prevalence of infection among males and females ($P > 0.05$). The prevalence of infection among camels below five years of age was 1.9%, while a prevalence rate of 5.5% was reported among camels above five years. Based on the findings, age was identified as a risk factor for *T. equi* infection in camels. The highest prevalence of *T. equi* infection was observed in Mehriz (7.5%), while the prevalence rates in Saddough and Ardekan were 1.6% and 4.9%, respectively (Table 1). In this study, there was no significant geographical variation in the prevalence of *T. equi* infection among camels ($P > 0.05$).

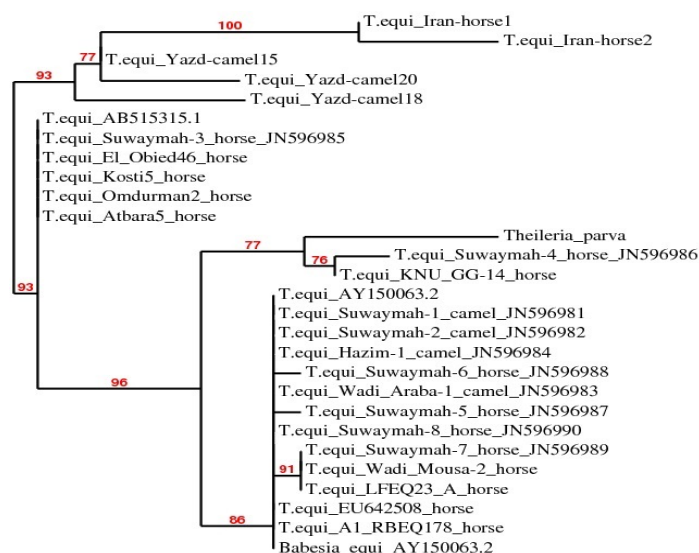


Figure 2. Phylogenetic tree of *T. equi*, inferred from the partial sequence of 18S rDNA gene. The numbers above the branches indicate maximum likelihood/maximum parsimony bootstrap support (500/1000 replicates). *T. equi*-Iran-horse 1, *T. equi*-Iran-horse 2, *T. equi*-Yazd-camel 15, *T. equi*-Yazd-camel 20, and *T. equi*-Yazd-camel 18 represent the sequences obtained in our study.

DISCUSSION

Carrier animals are the major source of spreading infection and play the most important role in altering the parasite life cycle between horses and ticks. Therefore, accurate diagnosis of carriers is necessary for disease prevention and effective control measures. However, diagnosis of piroplasmiasis in the carrier by

means of blood smear examination is difficult and subjective. Direct detection of piroplasms in erythrocytes of stained blood smears is possible in the acute phase of infection. However, during the latent phase, parasitemia becomes too low to detect positive cases via microscopic examinations. Serological methods are frequently employed to determine subclinical infections. However, false positive and negative results are commonly reported in serological tests due to cross-reactions, weakened specific immune responses, and unspecified antibodies in the carriers (due to long-term infection) (Leemans et al., 1999). In the early 1990's, molecular methods were introduced for *T. equi* DNA detection in blood samples (Posnett and Ambrosio, 1991; Posnett et al., 1991). The comparison of *in vitro* cultivation and molecular methods, such as loop-mediated isothermal amplification and PCR, showed that the latter are more sensitive (Alhassan et al., 2007). Since a considerable number of camels are in direct or indirect contact with horses in central Iran, the aim of this study was to investigate the presence and prevalence of *T. equi* infection in camels. In our previous studies, the overall molecular prevalence of *T. equi* was estimated at 22.86% among horses in this region (Bahrami et al., 2014a). According to the microscopic examination in the present study, the piroplasmic form of *Theileria* species was reported in 1 (0.6%) out of 161 prepared blood smears from camels. PCRs were positive in seven samples, and 435 bp bands were seen on the agarose gel, demonstrating *T. equi* infection. Based on the findings, no significant difference was found between the studied age groups ($P>0.05$), which could be attributed to the enzootic stability status of the studied area for theileriosis. The age difference of horses in the present study ranged between 11 months and 28 years, suggesting the possible exposure of all the camels to infections caused by this piroplasm. Moreover, in terms of sex, no significant difference was observed in the frequency of *T. equi* infection among the studied camels. These results were in agreement with previous

studies on horses in Iran (Abedi et al., 2014; Bahrami et al., 2014b), Italy (Moretti et al., 2010), Switzerland (Sigg et al., 2010), Venezuela (Mujica et al., 2011), and Turkey (Acici et al., 2008; Karatepe et al., 2009). However, the present findings were not in consistence with the results of some previous studies, which proposed a relationship between the frequency of *T. equi* infection and sex of horses in Turkey (Sevinc et al., 2008), Mongolia (Ruegg et al., 2007), and Spain (Garcia-Bocanegra et al., 2013). In some studies, marked differences in the prevalence of *T. equi* infection between geographical areas could be attributed to variations in the management of animals (e.g., nutrition and tick control) (Salim et al., 2008), host activity (Kouam et al., 2010), and climatic conditions (Moretti et al., 2010). Since the management of animals was homogenous in the present study and the climate was not significantly different in the studied regions, no significant geographical variation was observed in the prevalence of *T. equi* infection among camels. In the present study, evaluation of the sequence alignment of *T. equi* 18S rRNA gene in the studied camels and other isolates in the GenBank revealed more than 99% homology. The data presenting the significant taxonomical relationship between *T. equi* from camels and horses in Iran and other geographical regions provide molecular evidence to confirm the interspecies transmission of piroplasms. In consistence with the present findings, Qablan et al. (2012b) detected *T. equi* in the blood samples of four clinically healthy camels from three districts of Jordan, using a specific PCR assay. Historically, the taxonomy of piroplasms in dromedaries is rather uncertain. Two species, namely *T. camelensis* and *T. dromedarii*, have been described so far (Yakimoff et al., 1917; Rao et al., 1988), although the validity of both taxa is questionable. Furthermore, the description of *T. camelensis* does not provide any information on the developmental stages (Boid et al., 1985), and no proper taxonomic description of *T. dromedarii* is yet available (Rao et al., 1988). Similarly, in a previous

study, the *Babesia*-like infection in camels (Egbe-Nwiyi, 1994) failed to explain the life cycle of parasites. It should be mentioned that in 1926, Wenyon suggested the similarity of *T. camelensis* to *Nuttallia* (today known as *T. equi*). Besides horses and camels, *T. equi* of genotype A has been detected in dogs in Croatia (Beck et al., 2009), France (Fritz, 2010), and recently in Jordan (Qablan et al., 2012a). These findings indicate that dogs may contribute to the circulation of infection and suggest the potential of this genotype to infect a wider spectrum of hosts. Overall, we should determine which tick species are responsible for the transmission of diseases to and/or between camels. It is well established that several tick species (e.g., *Hyalomma anatolicum*, *Boophilus annulatus*, and *Rhipicephalus sanguineus*) infest both camels and horses (El-Rabie et al., 1990; Walker et al., 2005) and play a possible role in the transmission of piroplasmida infections. In addition, *Hyalomma dromedarii*, the most prevalent tick on camels in Iran, is known to infest domestic animals other than camelids (Apanaskevich et al., 2008), even though this species has not been reported among equids in Iran. In conclusion, this study demonstrated that *T. equi* infection is present among Iranian camels; also, the camels shared piroplasms with horses in the studied regions. Although it is too early to conclude, the possibility of natural *T. equi* infection among camels may complicate the epidemiology of equine theileriosis. Therefore, further studies are required to decide if tick vectors are able to transfer *T. equi* from infected camels and cause infection in horses.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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