Diagnosis of subclinical equine theileriosis in center of Iran using parasitological and molecular methods

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Abstract. A total of 105 blood samples from healthy horses from different stables in Yazd province, center of Iran, were examined for the presence of *Theileria equi* infection using parasitological and molecular methods. Out of the 105 samples, the parasitological method detected *T. equi* infection in 5 (4.76%) cases while the PCR method gave 24 (22.86%) positive results. Age, gender and breed were not determined as risk factors for *T. equi* infection in this study. Since blood samples were taken from healthy animals, this implies that 22.86% of horses had subclinical theileriosis in the current study. In conclusion, this study demonstrated that *T. equi* is present in horses in the center of Iran. Despite the healthy appearance of horses, these carrier animals can transmit the parasites to ticks and are a potential continuous source for maintaining and disseminating the organisms to the horse population. We concluded that it is important to make further studies on definitive host and vectors in the respective areas.

INTRODUCTION

Equine theileriosis (ET) is a febrile, tick-borne disease caused by *Theileria equi* (*T. equi*). The etiological agent of ET is specific for solipeds. The disease poses a serious threat to the horse raising industry and international movement of horses (Avarzed et al., 1997). Equine theileriosis has a worldwide distribution and is endemic in most tropical and subtropical areas as well as in some temperate regions (de Waal, 1992). This distribution is closely related to areas of higher population of vector ticks (Friedhoff, 1988). Clinical signs of the disease in equids are intermittent fever, listlessness, depression, anorexia, watering of the eyes, swelling of the eyelids, intravascular hemolysis, anemia, jaundice, hemoglobinuria and edema (de Wall, 1992). In acute cases death may occur from one to four weeks after the onset of clinical signs and such cases are therefore of considerable veterinary economic importance (Kuttler 1988; Schein, 1988). In chronic and subclinical cases, despite the healthy appearance, infected horses can carry the piroplasms in their blood for many years and carrier animals are thought to be responsible for the maintenance of infection (Schein, 1988).

The agent can be naturally transmitted by the ticks of the family Ixodidae. Ten tick species from three distinct genera (*Dermacentor*, *Rhipicephalus* and *Hyalomma*) have been identified in *T. equi* transmission. Transplacental transmission may occur which becomes a serious economic problem for horse farmers, and the infected mares may be likely carriers of *T. equi* for their lifetime. In addition to this biological route, *T. equi* has the potential to
be transmitted iatrogenically. This hemoparasite can be mechanically transmitted through contaminated needles and surgical instruments (De Wall, 1992).

There is currently no available vaccine for this disease and treatment options are not generally effective. A variety of drugs like imidocarb, tetracyclin, diminazene and buparvaquone are used for the treatment of *T. equi* infection. Most of the drugs improve the clinical signs but are unable to completely eliminate the infection from the body (de Waal & van Heerden, 1994). Since animals that survive the acute phase of the infection may continue to carry the parasite for long periods of time, current control measures include euthanasia or lifetime quarantine for a positive horse. In addition, to avoid spread of disease, it is important to eliminate contact with ticks to prevent the transfer of blood from one equid to another.

While the identification of parasites in blood smears constitutes the definitive diagnosis of equine infection, it bears certain limitations, particularly during apparent or chronic infection due to low parasitemia (Krause *et al*., 1996). Thus, several serological assays, *in vitro* culture methods and molecular tools that are often more sensitive and specific have been developed to advance diagnosis of equine piroplasmosis (Xuan *et al*., 2001). Detecting serum antibodies against merozoite antigens using serological tests is a useful technique but major shortfall of these tests is the existence of false positive and negative results due to cross reactivity and weak specific immune response of the host (Ikadai *et al*., 2002). Moreover, antibody detection using specific and sensitive ELISA cannot differentiate between current and previous infections due to the persistence of antibodies. Therefore, several molecular techniques have been developed with high sensitivity and specificity for the detection and quantification of piroplasm infection (Alhassan *et al*., 2005).

There is a considerable population of horses in Yazd province, central region of Iran (>1800). The variety of horses within Yazd includes Turkeman, Darreh Shuri, Throughbred and Arabian horses. Also, Yazd has been hosting races for several years and every year the number of participant is increasing. Based on our knowledge, there is no previous study regarding the prevalence of *T. equi* infections in Iran. In this study, we aimed to determine the presence of parasite DNA in the equine population in Yazd province, center of Iran.

**MATERIALS AND METHODS**

**Blood samples:**
More than half of the horse population in Yazd province is located in Yazd city. The province of Yazd is located in the central part of Iranian plateau at 29.52 to 33.27 latitude and 52.55 to 56.37 longitudes. The annual rainfall is between 50 to 100 mm. The province has hot summers, mild spring and cold winters. Changes of temperature are too much in winter and summer, even day and night, between +45 to -20°C. In this study, blood samples were taken from 105 horses based at stables. Samples had been collected between February and May of 2012. The farms participating in this study had been selected randomly. On average, 20% of the total numbers of animals in the farms were sampled. The farms participated in the present study had acceptable management (animals reared in stalls, fed with forage and concentrate rations, systematic control of ticks with frequent veterinary care). There were 66 (62.85%) females and 39 (37.15%) males. Blood samples were obtained from a total of 105 horses which consisted of 34 horses equal and less than three years old and 71 horses more than three years old. The breed composition comprised of 65 (61.9%) Arabian horses and Arabian horse crosses and 40 (38.1%) non-Arabian horses consisted of 3 Darreh Shuri (7.5%), 5 Turkeman (12.5%), 7 Throughbred (17.5%) and 25 local breeds (25%). The blood samples were collected from the jugular vein into sterile vacuum tubes containing EDTA and kept in -20°C until analyzed. For every animal, information had been recorded including age, sex, breed and management. All horses were healthy at the time of blood collection.
Parasitological examination
Thin blood smears were prepared, fixed with absolute methanol (1 min), stained with 10% Giemsa solution (30 min) and examined under oil immersion lens to observe intraerythrocytic forms of *T. equi*. More than 30 microscopic fields of blood films at a magnification of ×1000 were examined. Approximately 20,000 red blood cells (RBCs) were carefully searched per slide.

DNA extraction and polymerase chain reaction (PCR) amplification
DNA was extracted using a genomic DNA purification kit (Cinna Gen, Iran). For detection of *T. equi*, primers targeting the 18S rRNA gene were selected from the literature (Alhassan et al., 2005). Primers used in the reaction were the forward primer BEC-UF2 with the sequence 5'-TCGAAGACGATCAGATACCGTCG-3' and the reverse primer Equi-R with the sequence 5'-TGCCTTAAACTTCCTTGCGAT-3', yielding a 392 bp product. PCR reactions included a negative control, consisting of the reaction mix and 2 µl of DNase/RNase-free water and a positive control that consisted of DNA sample from the blood of a horse with clinical theileriosis. All PCR reactions were performed in 20 µl of a mixture consisting of 10µl Taq master mix, 1 µM primers and 5 µl DNA template. PCR cycling included an initial denaturation step at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45s, annealing at 60°C for 50s, extension at 72°C for 60s. This was followed by a final extension step at 72°C for 5 min. PCR products were electrophoresed in 1.5% agarose in Tris–acetate–EDTA (TAE) buffer, and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet light. Positive samples showed a band of approximately 400 bp.

Statistical analysis
Fisher Exact and Chi-square tests were used to compare infection rates among different age, sex and breed groups. *P* values of <0.05 were considered statistically significant.

RESULTS
In 5 out of 105 prepared blood smears, piroplasmic forms of *T. equi* (4.76%) were demonstrated. In 24 samples of these 105 examined samples, the PCR was positive and a band of approximately 400bp was seen on the agarose gel which considered as infection with *T. equi* (22.86%) (Fig. 1).

![Figure 1. Amplification of *T. equi* DNA. Lane M is a 100-bp ladder. Lane P is a positive control DNA lane. Lane N is a negative control DNA lane. Lanes 1,5,7,8 represent positive field samples. Lanes 2-4, 6 represent negative field samples](image-url)
Based on molecular results, 10 out of 39 males (25.64%) and 14 out of 66 females (21.21%) were found to be positive. Therefore, in terms of gender, there was no significant difference between the prevalence rate of infection in males and females. The prevalence of infection in horses under three years old was 20.59%, while a prevalence rate of 23.94% was detected in horses more than three years old. 16.92% of Arabian horses and 32.50% of non-Arabian horses were infected with the parasite. Prevalence of infection in non-Arabian horses was higher but the difference was not significant \( (p > 0.05) \). Age and breeds have not been identified as risk factors for T. equi infection in this study (Table 1).

**DISCUSSION**

The Middle East, because of its geographical situation, is extremely prone to the introduction of diseases and the spread of endemic infections. This part of the world is a crossroad between different continents. Equine theileriosis or circulation of the parasite has been reported from different countries in the region, such as Turkey, United Arab Emirates, Israel and Sudan (Salim et al., 2008; Karatepe et al., 2009; Jaffer et al., 2010; Steinman et al., 2012). To the best of our knowledge there is no published data on the prevalence of equine theileriosis in Iran. Equine theileriosis is of great importance because of the international movement of horses in connection with equine sport competitions, and some countries restrict the entrance of horses that are serologically positive for piroplasma species. For this reason, specific and sensitive tests to detect piroplasma infections are needed. The clinical picture of piroplasmosis is variable and often non-specific and gives only a hint for further investigation. Fever, jaundice, anorexia and depression are the most frequent symptoms and low erythrocyte counts, low hemoglobin, low platelets and high bilirubin levels are the main laboratory findings in infected horses (Zobba et al., 2008). A direct detection of the piroplasms in the erythrocytes in a stained blood smear is possible during the acute phase of the infection. Later, during the latent phase, the parasitemia becomes too low to detect positive cases by microscopy. The carrier status of horses can be proven by *in vitro* cultivation (in HL-1 medium supplemented with 20% fetal bovine serum) (Holman et al., 1997; Zweygarth et al., 2002). Already in the early 1990s, molecular methods to detect DNA of T. equi and B. caballi in horse blood (Posnett & Ambrosio, 1991; Posnett et al., 1991) were introduced.

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**Table 1. Prevalence of T. equi in equids in Yazd province, center of Iran by PCR and equid related factors**

<table>
<thead>
<tr>
<th>Factor</th>
<th>No. examined</th>
<th>Positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence</td>
<td>105</td>
<td>24</td>
<td>22.86</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39</td>
<td>10</td>
<td>25.64</td>
</tr>
<tr>
<td>Female</td>
<td>66</td>
<td>14</td>
<td>21.21</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3 years old</td>
<td>34</td>
<td>7</td>
<td>20.59</td>
</tr>
<tr>
<td>&gt;3 years old</td>
<td>71</td>
<td>17</td>
<td>23.94</td>
</tr>
<tr>
<td>Breed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabian</td>
<td>65</td>
<td>11</td>
<td>16.92</td>
</tr>
<tr>
<td>Non-Arabian</td>
<td>40</td>
<td>13</td>
<td>32.50</td>
</tr>
</tbody>
</table>
A comparison of *in vitro* cultivation and molecular methods like loop-mediated isothermal amplification and PCR showed that the latter methods are more sensitive (Alhassan *et al*., 2007). Frequently, serological methods are employed in determining subclinical infections. False positive and negative results are commonly observed in serological tests due to cross reaction, weakening in specific immune response as well as lack of determination of antibodies in carriers because of long-term infection (Leemans *et al*., 1999). The objective of this study was to estimate the prevalence of equine theileriosis in the center of Iran using parasitological and molecular techniques. In the present study, only 5 horses out of 105 (4.76%), showed positive blood smears with *T. equi* with a low parasitemia. Methods based on species-specific polymerase chain reaction assays which mainly target the 18S rRNA gene (Caccio *et al*., 2000; Birkenheuer *et al*., 2003; Criado-Fornelio *et al*., 2003; Rampersad *et al*., 2003) have been found sensitive enough to detect parasite DNA from 2.5 µl blood samples with parasitemias as low as 0.000001% (Alhassan *et al*., 2007). In the present study, the overall prevalence rate of *T. equi* infection by PCR was found to be 22.86%. Since we sampled healthy horses, this implies that subclinical infection is common. Although asymptomatic horses have a low parasitemia, transmission of *T. equi* can still occur either iatrogenically, or when competent tick vectors feed on these horses (Ueti *et al*., 2005; Ueti *et al*., 2008). Thus, asymptomatic persistently infected carriers can serve as reservoirs of infection which is one of the challenges for controlling the spread of this parasite. In endemic countries, horses are known to adapt to certain infections, but stress and other factors that cause severe immunosuppression may result in subclinical infections becoming overt and detectable.

For randomly selected 125 horses in different districts of Nigde province of Turkey, *T. equi* seropositivity rates were estimated to be 12.8% (Karatepe *et al*., 2009). Jaffer *et al.* (2010) showed that of 105 horses examined in Dubai, the TaqMan real-time PCR detected DNA of piroplasms in 31.4% samples, while serological methods found antibodies in 36.2% horses. In a study by Steinman *et al.* (2012) the overall molecular prevalence of *T. equi* was 26.4%. In the present study, the factor sex of the experimental horses showed no significant differences among the infected and non-infected animals. This result is similar to previous reports of Olivera & Garcia (2001), Asgarali *et al.* (2006), and Karatepe *et al.* (2009); consequently, it appears that both male and female horses showed the same susceptibility of infection to equine theileriosis. However, several other studies, did find a correlation between gender and positivity (Ruegg *et al*., 2007; Sevnic *et al*., 2008; Moretti *et al*., 2010), although the results were often inconsistent. Significant differences (p>0.05) were also not found between age groups which could be due to the condition of enzootic stability of equine theileriosis in the studied area. The age intervals of the horses in the present study ranged between 5 months and 30 years old which suggests that all these horses could have been previously exposed to challenge infections with this piroplasm. This finding is similar to some reports (Souza *et al*., 2000; Moretti *et al*., 2010; Santos *et al*., 2011; Grandi *et al*., 2011), but different to the data published by Ruegg *et al.* (2007) and Kouam *et al.* (2010). Olivera & Garcia (2001) concluded that horses with ages between 2 and 4 years old showed higher prevalences of equine piroplasmosis. They could be explained in part by different age categorization. In the present study, the prevalence of infection was not significant between Arab and non-Arab horses. According to the Moretti *et al.* (2010) survey differences in prevalences between breeds are difficult to interpret since in many instances the breed influences the management of horse, its exposure to tick insemination and to parasite control program and even its geographical areas and exposure to pasture. In a study by Steinman *et al.* (2012) horse breed has been identified as a risk factor for *T. equi* infection, but they believed
that more research is needed in order to determine whether there is a true predisposition among certain breeds. To our knowledge, this is the first report on prevalence of equine theileriosis using molecular techniques in Iran. In the present study it is noticeable that despite good condition of farms (animals reared in stalls, fed with forage and concentrate rations, systematic control of ticks with frequent veterinary care), subclinical theileriosis was detected. Therefore, the concerns about spreading *T. equi* should be considered (especially in exhibitions and sports) and a mandatory screening process for international and national movement of horses should be implemented. Accurate diagnosis of equine theileriosis is essential for providing baseline information about its epidemiology, distribution, and prevalence in the affected equine population and for effective control measures. The carrier horses are the major sources for spreading the infection and have the most important role in alteration of the parasite life cycle between horses and ticks. The accurate diagnosis of carriers is necessary for determining the immunological conditions of animals and prevention of the disease.

In conclusion, this study demonstrated that *T. equi* is present in the horses of Iran. For randomly selected 105 horses in Yazd province, *T. equi* prevalence was shown to be 21.49%. Despite their healthy appearance, the horses can transmit the parasites to ticks and are a potential continuous source for maintaining and disseminating the organisms to the horse population. Diagnosis of subclinical infections is important to prevent spread of equine piroplasmosis. We concluded that it is important to make further studies on definitive host and vectors in the areas.

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REFERENCES


