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Molecular and serological detection of *Theileria equi* and *Babesia caballi* infection in horses and ixodid ticks in Iran

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ABSTRACT

Equine piroplasmosis is a hemoprotozoan tick-borne disease with worldwide distribution that is caused by *Theileria equi* and *Babesia caballi*. However, the geographical distribution of equine piroplasmosis in Iran is unknown. The aim of the current study was to determine the causative agents and vector ticks of equine piroplasmosis in horses in the North Khorasan Province. In the year 2011, 100 horses were randomly selected from 14 villages. Blood samples and ixodid ticks were collected and examined using microscopical, molecular, and serological methods. *Theileria equi* infection was microscopically detected in 5 (5%) of the blood smears with low parasitemia, while serum samples were tested by the indirect immunofluorescent antibody test (IFAT). Antibodies against *T. equi*, *B. caballi*, and a mixed infection were detected in 48 (48%), 2 (2%), and 3 (3%) of the serum samples, respectively. A multiplex PCR was used to detect *T. equi* and *B. caballi* DNA in blood samples. No *B. caballi* infections could be found, but *Theileria equi* DNA was detected in 45 (45%) of the blood samples, and a BLAST analysis of the sequenced samples indicated a 99% similarity with *T. equi* 18S rRNA gene sequences in GenBank. Both molecular and serological results did not identify any significant association between *T. equi* infection and risk factors. A comparision of the results of 3 diagnostic methods demonstrated a poor agreement between microscopical examination with IFAT and PCR and a moderate agreement between IFAT and PCR. Thirty-seven adult ticks (20 females and 17 males) were collected from 15 horses. The most common tick was *Hyalomma marginatum marginatum* ($n=19$), followed by *Hyalomma anatolicum excavatum* ($n=10$), *Rhipicephalus bursa* ($n=4$), *Hyalomma marginatum turanicum* ($n=3$), and *Hyalomma anatolicum anatolicum* ($n=1$). The salivary glands and ovaries were also examined using PCR. The genomic DNA samples of the salivary glands of 3 ticks, *H. a. excavatum* ($n=2$) and *R. bursa* ($n=1$), had a positive reaction for *T. equi*, but no tick contained *B. caballi* DNA. Thus, our results indicate that *T. equi* occurs more frequently than *B. caballi* in the investigated geographical region.

Introduction

Equine piroplasmosis is an important tick-borne disease of equids (horses, donkeys, zebras, and mules) that is caused by *Theileria equi* and *Babesia caballi* (Mehlhorn and Schein, 1998; Rothschild and Knowles, 2007). The disease has a worldwide distribution (De Waal, 1992).

The clinical signs of equine piroplasmosis are categorized as peracute, acute, subacute, and chronic. The peracute form is observed in neonatal foals following infection in utero. The clinical signs for acute piroplasmosis, which occurs frequently, include fever, anemia, anorexia, limb edema, icterus, and hemoglobinuria. The subacute form is characterized by anorexia, intermittent pyrexia, anemia, weight loss, limb edema, lethargy, and poor performance. The clinical signs of the chronic cases are ambiguous and display in appetence, poor body condition, and a poor performance. Anemia may be absent or minimal in the chronic form (Friedhoff and Soule, 1996; Rothschild and Knowles, 2007).

In endemic areas, infected horses are apparently healthy without any clinical signs, although stress and excessive exercise results in subclinical manifestations of the disease in those horses that suffer from a chronic infection (Hailat et al., 1997; Takeet et al., 2009) (Fig. 1).

Several laboratory methods have been used for the diagnosis of infection. Microscopical examination is reasonable only in case of clinical disease because parasitemia is low in the latent phase, and serodiagnosis is suitable for epidemiological studies. Three

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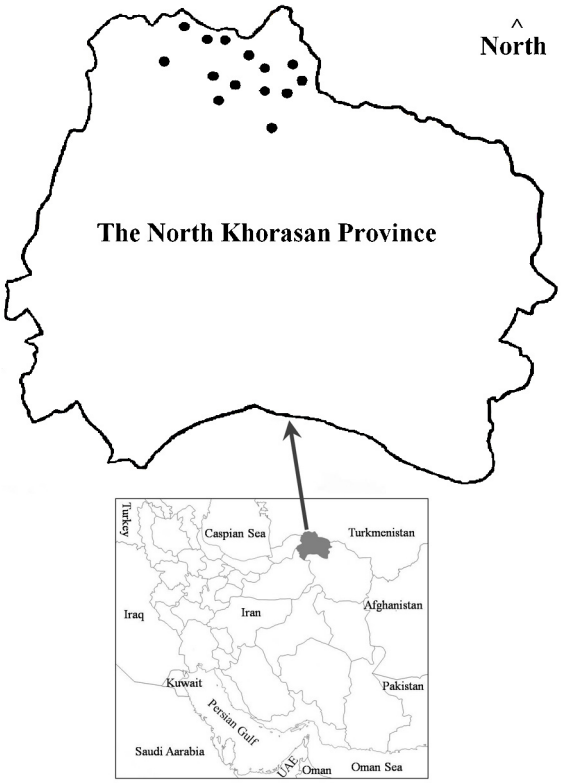

Fig. 1. Locations in the North Khorasan Province, Iran, where horses were sampled in the present study.

serological tests such as complement fixation test (CFT), indirect fluorescence antibody test (IFAT), and enzyme-linked immunosorbent assay (ELISA) have been used in epidemiological studies of equine piroplasmosis. The CFT is the primary test used for horses traveling between countries. Because the CFT may not identify all infected animals, especially not those that have been treated, and because of anti-complementary reactions produced by some sera, the IFAT is used as a supplementary test. A strongly positive IFAT reaction is simply recognized, but detecting weak and negative reactions requires considerable experience in interpretation (Taylor et al., 2007). Molecular methods are more sensitive than other diagnostic techniques to detect carrier animals (Böse et al., 1995). Several PCR methods based on ribosomal 18S RNA sequence, *EMA-1* gene, Rhoptry-associated protein 1, and 16S-rRNA have been developed to detect piroplasms in horses (Bahiruddin et al., 1999; Nicolaiewsky et al., 2001; Battsetseg et al., 2001; Rampersad et al., 2003). Recently, a multiplex PCR and multiplex real-time PCR were used for the *B. caballi* and *T. equi* detection (Alhassan et al., 2005; Heim et al., 2007a,b).

Equine piroplasmosis is endemic throughout Asia except Siberia and Japan, and the infection rates in the Middle East are high (Friedhoff et al., 1990; Ruegg et al., 2007).

In Mediterranean countries, which are endemic for piroplasmosis, symptomatic infections can occur all the year round with specific or nonspecific signs (Zobba et al., 2008). Although Iran is located in the endemic areas and *B. caballi* and *T. equi* infections have been reported in horses (Aslani, 2000; Seifi et al., 2000), there is no epidemiological information about equine piroplasmosis available for Iran.

The aim of our current study was to determine the relative frequency of *T. equi* and *B. caballi* infections in horses in the North Khorasan Province of Iran. In addition, tick infestation of horses and piroplasm infection in ticks were investigated.

Materials and methods

Field study area

The study was conducted in the North Khorasan Province from June to August 2011. The North Khorasan Province, which covers an area of more than 28,400 km^2, is located next to the north-eastern border of Iran (36°37′–38°17′N and 55°53′–58°20′E). The average annual rainfall in the province is approximately 250 mm. The province is a breeding center for Turkoman horses, an oriental breed from the steppes of Central Asia.

Sampling

One hundred horses from 14 villages were randomly selected. First, the data of each horse, including age, sex, activity, and any grazing in pastures, were recorded. Blood samples were collected from the jugular vein and placed into serum and EDTA tubes. In addition, blood smears were prepared from each blood sample. Simultaneously, the whole skin of the horses was carefully searched for ticks, which were then removed and preserved in 70% ethanol. The samples were maintained under cool conditions and immediately transferred to the laboratory. The serum tubes were centrifuged, and sera were separated. The sera and blood EDTA tubes were stored at −20 °C until the time of serological and molecular examination.

Microscopical examination

The smears were fixed in methanol and stained in 10% Giemsa solution in phosphate buffered saline (PBS), pH 7.2. The slides were examined with an oil immersion lens at a final magnification of 1000×. To identify *Theileria* and *Babesia* species, the full length of the intraerythrocytic mature piroplasm organism was measured using a graded ocular microscope. Parasitemia was assessed by counting the number of infected red blood cells on examination of 50 microscopic fields (approximately 50,000 cells).

Tick identification

The ticks collected from infected horses were counted and speciation was done using the identification keys by Hoogstraal (1956), Walker et al. (2003), and Estrada-Peña et al. (2004). The collected ticks were grouped according to species and level of engorgement, and then the salivary glands and the uterus of each tick were dissected in 0.85% saline solution under a stereomicroscope. Subsequently, the tick samples were maintained at −20 °C until use.

Indirect fluorescent antibody test (IFAT)

IgG antibodies against *T. equi* and *B. caballi* infections were detected by IFAT according to the manufacturer's instructions for *T. equi* (Fuller Laboratories, Fullerton, California, USA) and *B. caballi* antibodies (MegaScreen, Horbranz, Austria). Sera with a strong fluorescence at a dilution of 1:80 were considered to be positive.

DNA extraction and PCR

DNA was extracted from 100 μl of blood and from tick samples using a commercial kit [Molecular Biological System Transfer (MBST), Tehran, Iran], and kept at −20 °C until use. A multiplex PCR was conducted for the detection of *T. equi* and *B. caballi* using the method of Alhassan et al. (2005). Briefly, 20 μl of a mixture containing 1 μl of template DNA, 1 μl (10 pmol) of each of the reverse primers for *T. equi* (EquiR: 5′-TGCCTTAAACTTCCTTGCGAT-3′ and for