

Original Article**Molecular and microscopic detection of *Theileria equi* and *Babesia caballi* in horses in Kurdistan Province, Iran****Kakekhani¹, S., Rahbari^{1,*}, S., Madani^{1,2}, R., Bokaie³, S.**

1. Department of Microbiology, School of Specialized Veterinary Science, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Department of Protomics & Biochemistry, Razi Vaccine & Serum Research Institute, Tehran, Iran, Agricultural Research, Education and Extension Organization, Tehran, Iran
3. Division of Epidemiology and Zoonoses, School of Veterinary Medicine, University of Tehran, Tehran, Iran

Received 08 December 2015; accepted 30 May 2016

Corresponding Author: srahbari@ut.ac.ir

ABSTRACT

Equine piroplasmosis is a tick-borne disease caused by intra-erythrocyte protozoa, *Theileria equi* and *Babesia caballi*. The present study aimed to detect piroplasm infection in horses in Kurdistan Province, Iran, through molecular and microscopic approaches. In this study, 186 blood samples were randomly collected from horses of five regions of Kurdistan Province. The Tbs-S/Tbs-A primer set was used for amplification of *Theileria equi* and *Babesia caballi* DNA through polymerase chain reaction. Blood smears of each case were also examined by Giemsa staining method. During microscopic examination of Giemsa-stained blood smears, 3 out of 186 (1.61%) blood samples were positive for piroplasm infection. The product of only one blood sample yielded 426-430 bp-sized fragments on a 1.5% agarose gel electrophoresis, and BLAST analysis of the sequenced sample indicated a 100% similarity with *T. equi* 18S rRNA gene sequences in GenBank. The results indicated that one out of 186 blood sample was positive (0.54%) for *Theileria equi* and none of them was positive for *Babesia caballi*.

Keywords: *Theileria equi*, *Babesia caballi*, PCR, Horse, Iran

Détection moléculaire et microscopique de *Theileria equi* et *Babesia caballi* chez les chevaux de la province du Kurdistan, Iran

Résumé: La piroplasmose équine est une maladie transmise par les tiques et causée par les protozoaires intra-érythrocytaires *Theileria equi* et *Babesia caballi*. L'objectif de cette étude était de détecter les piroplasmoses affectant les chevaux de la province du Kurdistan iranien par une approche moléculaire et à l'aide d'analyses microscopiques. A cet effet, 180 échantillons sanguins ont été prélevés de façon aléatoire à partir de chevaux originaires de cinq régions de la province du Kurdistan. La série d'amorce Tbs-S/Tbs-A a été utilisée pour l'amplification en chaîne par polymérase de l'ADN de *T. equi* et *B. caballi*. De plus, des frottis de chaque échantillon sanguin ont été analysés après coloration au Giemsa. Parmi les 186 frottis examinés au microscope, 3 étaient atteints d'une piroplasmose (1,61%). Les échantillons positifs ont été ensuite soumis à une analyse électrophorétique sur gel d'agarose (1,5%). Une recherche BLAST sur les séquences des fragments obtenus a révélé 100 % de similarité entre un fragment d'une taille de 426-430 bp issu de l'un des échantillons avec la séquence ARNr 18S de *T. equi* répertoriée dans GenBank. Ces résultats montrent que parmi les 186 échantillons sanguins analysés, un seul était positif au *Theileria equi* alors qu'aucune contamination au *Babesia caballi* n'a été détectée.

Mots clés: *Theileria equi*, *Babesia caballi*, PCR, Cheval, Iran

INTRODUCTION

Equine piroplasmiasis, caused by *Theileria equi* (recently re-classified from *Babesia equi* (Mehlhorn and Schein, 1998) and *Babesia caballi*, is known as an important protozoan infection from the veterinary and economic viewpoints (Schein, 1988). Equine piroplasmiasis, which occurs throughout the tropical and subtropical areas of the world, imposes great economic losses to endemic countries (Friedhoff et al., 1990; de Waal, 1992). Tick species of the genera *Boophilus*, *Dermacentor*, *Hyalomma*, and *Rhipicephalus* are the biological vectors of equine piroplasmiasis (Battsetseg et al., 2001). Equine piroplasmiasis is characterized by fever, anemia, dyspnea, icterus, hepato- and hemorrhages in mucosal surfaces, hemoglobinuria, and even death (Schein, 1988; Uilenberg, 2006). Infection caused by *T. equi* may be suppressed by anti-theilerial drugs, but only some of them can eliminate infection. After recovery from theileriosis, horses may remain carriers for years or for life without further clinical diseases; these animals can act as a source of infection to tick vectors and naive equids (de Waal, 1992; Brüning, 1996). The Giemsa staining of the blood smears is the common method for the identification of piroplasm infection, but this method has low sensitivity and provides some false positive or false negative results (Shayan and Rahbari, 2005). In addition, serological procedures are usually employed in determining subclinical infections. False positive and false negative results are also commonly observed in serological tests due to cross-reactions or sometimes inadequate antibody level in carriers due to long-term infection (Gubbels et al., 2000). The previous studies performed in Iran on equine piroplasmiasis are mostly in the form of case reports (Nouri and Lotpholahzadeh, 1993; Aslani, 2000; Seifi et al., 2000; Sakha, 2007; Erfei Akhole et al., 2013). Recently, serological and molecular methods have been applied for diagnosis of equine piroplasmiasis (Seifi et al., 2000; Malekifard et al., 2011; Abedi et al., 2013; Bahrami et al., 2014). The polymerase chain reaction (PCR) method has more sensitivity and specificity in

comparison with the serological tests. The PCR method can detect parasitic infections with or without clinical signs (D'oliveira et al., 1995; Almeria et al., 2001; Dumanli et al., 2005). Herein, we aimed to estimate the prevalence of equine piroplasmiasis caused by *Theileria equi* and *Babesia caballi* in Kurdistan Province, Iran.

MATERIALS AND METHODS

Study area and sampling. Kurdistan Province, a mountainous region with a temperate climate, is located in the west of Iran. The study was conducted during May-September (the tick activity season) 2012 and 2013, in five areas (north, south, east, west, and center) of Kurdistan Province. Regardless of age and gender, a total of 186 horses were randomly selected from each area. Blood samples were collected from the jugular vein of each horse into sterile vacuum tubes with anticoagulant agent (EDTA). Additionally, two thin blood smears were prepared from each sample, and immediately 500 µl of each collected blood sample was mixed with 1 ml ethanol in 1.5 ml sterile eppendorf tube; the specimens were transferred to the Laboratory of Parasitological of the School of Veterinary Medicine, Azad University of Sanandaj, Iran. The blood samples were stored at -20 °C for further analysis. Data of the sampled animals (e.g., gender, age, and area) were recorded during sample collection.

Microscopic examination. The fixed blood smears were stained with Geimsa method in order to determine the presence of haemoprotozoal parasites. The stained smears were microscopically examined to identify haemoprotozoal parasites.

DNA extraction and PCR. For detection of *Theileria* spp. and *Babesia* spp, primers derived from 18S rRNA were used (Shayan, P. and S. Rahbari 2005). Tbs-S/Tbs-A primer set was employed for PCR amplification of 18S rRNA of *Theileria* spp. and *Babesia* spp. Weights of the amplified sequences by this primer set for *Theileria* spp. and *Babesia* spp. were 426-430 bp and 389-402 bp, respectively (Shayan and Rahbari, 2005). Distilled water was used as negative control in each PCR reaction. The positive control was

prepared from the School of Veterinary of Tehran University and Ferdowsi University of Mashhad. The positive sample was sent to Bioneer Laboratory in South Korea for sequencing (3-49, Munpeong-Dong, Daedok-Gu, Daejeon 220-306, Korea), and the result was assessed by BLAST analysis in National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

Out of 186 examined horses, 62 were female and 124 male; all the horses were aged more than 1 year. During microscopic examination of Giemsa-stained blood smears, 3 out of 186 (1.61%) blood samples were positive for *Theileria equi*. The genomic DNA from blood samples was amplified with Tbs-S/Tbs-A primer. The product of only one blood sample yielded 426-430 bp-sized fragments on a 1.5% agarose gel electrophoresis (Figure1), and BLAST analysis of the sequenced sample indicated a 100% similarity with *T. equi* 18S rRNA gene sequences in GenBank (*T. equi* isolate Sample2, KJ573370.1, Brazil; *T. equi* genotype A, KC465785.1, Tunisia). The sequenced sample was in alignment with *T. equi* 18S rRNA gene sequences in PubMed including numbers 23542456 (Tunisia), 23399005 (US), 19019541 (South Africa), and 12719133 (Southern Europe). Therefore, the result indicated that one out of 186 blood sample was positive (0.54%) for *Theileria equi* and none of them was positive for *Babesia caballi*. The previous studies on equine piroplasmiasis conducted in Iran are mostly case reports (Nouri and Lotpholazadeh, 1993; Aslani, 2000; Seifi et al., 2000; Sakha, 2007; Erfei Akhole et al., 2013). In this study, the result of microscopical observation showed an infection rate of 1.61%, while the obtained result by PCR was 0.54%, and BLAST analysis of the sequenced sample indicated a 100% similarity with *T. equi* 18S rRNA gene sequences in GenBank. Recently, serological and molecular methods have been applied to diagnose equine piroplasmiasis in Iran. Abedi et al. (2014) reported that 45% of horses from North Khorasan Province are infected with *T. equi*. On the other hand,

Malekifard et al. (2011) reported that 10.83% of horses are infected with *T. equi* in the suburbs of Urmia in the northwest of Iran. Similar studies conducted in Turkey (neighboring northwest of Iran) described the high prevalence of equine piroplasmiasis with *T. equi* (Akkana et al., 2003; Oncel et al., 2007; Acici et al., 2008).

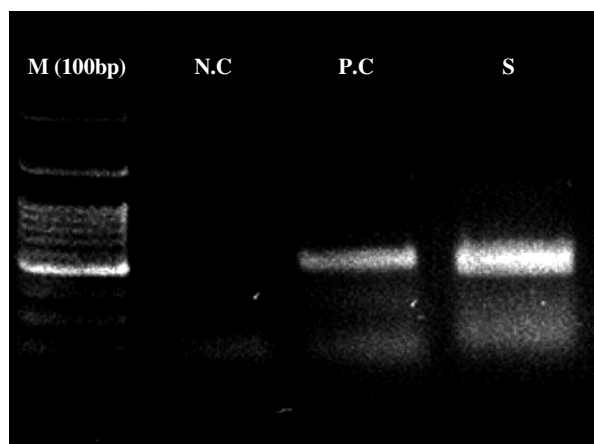


Figure 1. Agarose gel electrophoresis: analysis of polymerase chain reaction amplification for detection of *Theileria sp.* by using Tbs-S/Tbs-A primer set derived from the 18SrRNA encoding gene M:100 bp DNA marker; .NC: Negative control; PC: Positive control for *T. equi* (426-430 bp) S: Sample 116

These differences between Urmia and Kurdistan might be associated with the restriction that has been implemented to control the animals' movement between the border of Iraq and Iran. It seems that the entry of horses across the border of Turkey and Iran can play a major role in causing higher babesial infection in those regions. In addition, Kurdistan is located in a mountainous area, where climatic factors may influence tick population; accordingly, the lower prevalence rate of infection compared to previous result can be acceptable. Bahrami et al. (2014) examined 105 blood samples from healthy horses from different stables in Yazd Province, Iran. They demonstrated that the prevalence of *T. equi* infection by Giemsa staining method was 4.76%, while by PCR assay this rate was 22.86% (Bahrami et al., 2014). Malekifard et al. (2011) reported that the positive infection rates with *T. equi*

and *B. caballi* by Giemsa staining method were 6.25% and 2.80%, respectively, while 10.83% and 5.83% of cases were infected with *T. equi* and *B. caballi*, respectively, by PCR (Malekifard et al., 2011). Abedi et al. (2014) examined 100 blood samples of horses from Turkoman breed only by microscopy and indirect immunofluorescent antibody test. They reported that the prevalence rate of *T. equi* infection by Giemsa staining method was 5%, while by indirect immunofluorescent antibody, this rate was 48% (Abedi et al., 2013; Abedi et al., 2014) and by PCR was 45% (Abedi et al., 2014). In conclusion, this study showed that the prevalence of *Theileria equi* is low in the horses of Kurdistan Province and there was no infection with *Babesia caballi*.

Ethics

We 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.

Acknowledgment

We would like to thank Dr. Parviz Shaian for his comments on primer design and Dr Nikbakht, Dr Shaikhi, and Mrs Hashemi for their technical assistance.

References

- Abedi, V., Razmi, G., Seifi, H., Naghibi, A., 2014. Molecular and serological detection of *Theileria equi* and *Babesia caballi* infection in horses and ixodid ticks in Iran. *Ticks Tick Borne Dis* 5, 239-244.
- Abedi, V., Razmi, G.H., Seifi, H., Naghibi, A., 2013. Seroprevalence of *Theileria equi* and *Babesia caballi* infection in Turkoman breed horses in Iran. *Iran J Vet Sci Technol* 5 45-52.
- Acici, M., Umur, S., Guvenc, T., Arslan, H.H., Kurt, M., 2008. Seroprevalence of equine babesiosis in the Black Sea region of Turkey. *Parasitol Int* 57, 198-200.
- Akkana, H.A., Karacaa, M., Tutuncua, M., Degerb, S., Kelesa, I., Agaoglu, Z., 2003. Serologic and microscopic studies on Babesiosis in horses in the Eastern border of Turkey. *J Equine Vet Sci* 23, 181-183.
- Almeria, S., Castellà, J., Ferrer, D., Ortuño, A., Estrada-Peña, A., Gutiérrez, J.F., 2001. Bovine piroplasms in Minorca (Balearic Islands, Spain): a comparison of PCR-based and light microscopy detection. *Vet Parasitol* 99, 249-259.
- Aslani, M.R., 2000. A Case Report of *Babesia caballi* Infection in a Foal. *J Appl Anim Res* 17, 253-256.
- Bahrami, S., Ghadrán, A.R., Pourmahdi Borujeni, M., Vafayi Salarpur, M., 2014. Epidemiology of *Theileria equi* in Persian Arab horses from Iran. *Vet Med* 59 409-414.
- Battsetseg, B., Xuan, X., Ikadai, H., Bautista, J.L.R., Byambaa, B., Boldbaatar, D., et al., 2001. Detection of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. *Int J Parasitol* 31, 384-386.
- Brüning, A., 1996. Equine piroplasmosis an update on diagnosis, treatment and prevention. *British Vet J* 152, 139-151.
- D'oliveira, C., Van der Weide, M., Habela, M.A., Jacquiet, P., Jongejan, F., 1995. Detection of *Theileria annulata* in Blood Samples of Carrier Cattle by PCR. *J Clean Microbiol* 33, 2665-2669.
- de Waal, D.T., 1992. Equine piroplasmosis: A review. *British Vet J* 148, 6-14.
- Dumanli, N., Aktas, M., Cetinkaya, B., Cakmak, A., Koroglu, E., Saki, C.E., et al., 2005. Prevalence and distribution of tropical theileriosis in eastern Turkey. *Vet Parasitol* 127, 9-15.
- Erfei Akhole, A., Rasooli, A., Razi Jalali, M., Hamidi Nejat, H., Rohizade, A., Raki, A., 2013. Equine Theileriosis in two pure arab mares in Ahwaz city. *Iran Vet J* 9, 103-108
- Friedhoff, K.T., Tenter, A.M., Muller, I., 1990. Haemoparasites of equines: impact on international trade of horses. *Rev Sci Tech* 9, 1187-1194.
- Gubbels, M.-J., Katzer, F., Hide, G., Jongejan, F., Shiels, B.R., 2000. Generation of a mosaic pattern of diversity in the major merozoite-piroplasm surface antigen of *Theileria annulata*. *Molecul Biochem Parasitol* 110, 23-32.
- Malekifard, F., Tavassoli, M., Yakhchali, M., Darvishzadeh, R., 2011. Detection of *Theileria equi* and *Babesia caballi* using microscopic and molecular methods in horses in suburb of Urmia, Iran. *Vet Res Forum* 5 129 - 133.
- Mehlhorn, H., Schein, E., 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. *Parasitol Res* 84, 467-475.
- Nouri, M., Lotpholahzadeh, S., 1993. A case report of babesiosis in the Horse in Ahvaz. *J Faculty Vet Med* 48 49-54.

- Oncel, T., Vural, G., Gıcık, Y., Arslan, M.O., 2007. Detection of Babesia (Theileria) equi (Laveran, 1901) in horses in the Kars province of Turkey. *Turkiye Parazitolojisi Dergisi* 31, 170-172.
- Sakha, M., 2007. Successful treatment of babesiosis in a horse. *J Vet Res* 62, 155-157.
- Schein, E., 1988. *Equine babesiosis*, CRC Press, Boca Raton, FL.
- Seifi, H.A., Mohri, M., Sardari, K., 2000. A mixed infection of Babesia equi and Babesia caballi in a racing colt: A report from Iran. *J Equine Vet Sci* 20, 858-860.
- Shayan, P., Rahbari, S., 2005. Simultaneous differentiation between Theileria spp. and Babesia spp. on stained blood smear using PCR. *Parasitol Res* 97, 281-286.
- Uilenberg, G., 2006. Babesia--a historical overview. *Vet Parasitol* 138, 3-10.