Short Paper

Molecular detection of equine piroplasms in donkeys (*Equus asinus*) in North Khorasan province, Iran

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Summary

Equine piroplasmosis is a tickborne disease of equids with worldwide distribution, caused by *Theileria equi* and *Babesia caballi*. The aim of this study was molecular detection of *T. equi* and *B. caballi* in donkeys in northeastern Iran and investigate the association between positivity of piroplasm infection and host-related factors. In the present study, Blood samples were collected from 106 apparently healthy donkeys (*Equus asinus*) in North Khorasan province, Iran. Blood smears were prepared and stained by giemsa method. DNA was extracted from blood and then multiplex-PCR was done for detection of any piroplasms infection. According to the results, four donkeys showed *T. equi* in blood smears but *B. caballi* was not found. Also, fifty four donkeys (50.94%) showed *T. equi* infection using multiplex-PCR. No siginificant difference was observed between the frequency of *T. equi* infection with host-related factors in donkeys. This is the first report on the molecular detection of eqiune piroplamosis in donkeys in Iran. Also, no significant association was found between the rate of *T. equi* infected animals.

Key words: *Babesia caballi*, Donkey, PCR, *Theileria equi*

Introduction

Equine piroplasmosis is an intraerythrocytic parasitic disease of equids that is caused by *Theileria equi* and *Babesia caballi* (Wise *et al*., 2013). Equine piroplasmosis has world wide distribution and is endemic throughout Asia except Siberia and Japan and infection rates in the Middle East are high (Friedhoff *et al*., 1990).

Direct and indirect methods have been used for the diagnosis of equine piroplasmosis. The microscopical examination and molecular methods such as PCR derivatives should be considered as a direct method. The microscopy is suitable in acute cases with clinical signs while PCR is applicable in chronic with low parasitemia. Indirect detection of infection is accomplished by antibody detection via several tests such as complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA) and indircet fluorescent antibody test (IFAT) (Reiter and Weiland, 1989; Bruning, 1996).

Among the ixodid ticks, *Hyalomma*, *Rhipicephalus*, *Haemaphysalis* and *Dermacentor* have been identified as vectors of *T. equi* and *B. caballi* (De waal, 1992). Iatrogenic transmission of the parasites can occur via blood-contaminated instruments and through blood transfusion. *Theileria equi* are transmitted to offspring during pregnancy via placenta. The infected foal may be born healthy but remain a carrier for life (Allsopp *et al*., 2007; Georges *et al*., 2011).

Donkey is an important member of equidae family with a population of more than 40 million. Donkey has been used as working domestic animal in rural areas. Equine piroplasmosis affects health status and work performance of donkeys. The anti-babesial drugs can not completetly eliminate infection in donkey and the infected animal becomes carriers without clinical signs (Kumar *et al*., 2009).

In Iran, equnie piroplasmosis were reported for the first time in 2000 (Aslani, 2000; Seifi *et al*., 2000); however, it seems that there are no other reports about piroplasm infection in donkey in this country. The goal of this study was molecular detection of the *T. equi* and *B. caballi* infection in donkeys and to determine the importance of host-related factors.

Materials and Methods

Field study area

The study was conducted between June and August 2011, in the North Khorasan province. North Khorasan province is level with the southern Caspian sea, and is southern of Turkmenistan, between latitudes 36°37′-38°17′ N and longitudes 55°53′-58°20′ E. The annual rainfall in the province is approximately 250 mm. Also, more than 18000 equids breed in this area (approximately 0.64/km^2).

Blood collection

One hundred and six apparently healthy donkeys from 10 villages were randomly selected. Blood samples were collected from the jugular vein and placed into

EDTA tubes. In addition, blood smears were prepared for each blood sample. Data of each donkey including age, gender, activity, and any grazing in pastures were recorded. The samples were maintained under cool conditions and immediately transferred to the laboratory. The blood EDTA-tubes were stored at -20°C until the time of molecular examination.

Microscopical examination

The smears were fixed in methanol, stained with 10% giemsa solution in phosphate-buffered saline (PBS), pH = 7.2 and examined with an oil immersion lens at a 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). The number of the infected cells was then expressed as a percentage.

DNA extraction and PCR

DNA was extracted from 100 µL of the blood samples using a commercial kit (Molecular Biological System Transfer (MBST), Tehran, Iran), according to the manual, 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). In brief, 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´-TGC CTT AAA CTT CCT TGC GAT-3´ and for *B. caballi* (CabR: 5´-CTC GTT CAT GAT TTA GAA TTG C-3´), 2 µL of a common forward primer (UFP: 5´-TCG AAG ACG ATC AGA TAC CGT CG-3´), and 16 µL of distilled water were added to a hotstart PCR mastermix (Accupower PCR PreMix kit, Bioneer, Seoul, South Korea) with a final concentration of 250 µM of each dNTP in 10 mM Tris-HCl, pH = 9.0, 30 mM KCl, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase. The reactions were subjected to the following cycling conditions using a thermocycler (BioRad™): 96°C for 10 min, 36 cycles with a denaturing step at 96°C for 1 min, annealing step at 60.5°C for 1 min, and an extension step at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The products were then chilled to 4°C. Five µL of the PCR product was subjected to electrophoresis in a 1.5% agarose gel with the TAE buffer, submerged in an ethidium bromide tank for 15 min, and the distinct bands of *T. equi* (430 bp) and *B. caballi* (540 bp) were visualized by UV illumination. The positive controls were preparted from the blood of infected horses (Seifi *et al*., 2000), and water was employed as a negative control for each PCR amplification.

Sequencing

Three positive products were selected and sequenced in the facilities of Bioneer Inc. (Seoul, South Korea). Sequences were analyzed by using NCBI BLAST, National Institutes of Health, USA (http://www.ncbi.nlm.nih.gov).

Statistical analysis

The relationship between infection rate and risk factors, age and gender was analyzed by the Chi-square test. Significant association were identified when a p-value of less than 0.05 was observed.

Results

Theileria equi infections were microscopically detected in 4 (3.77%) blood smears with low parasitemia (approximately 0.001%-0.003%). In multiplex-PCR, 54 (50.94%) donkeys had predictive band at approximately 430 bp for *T. equi*. No significant relationship was observed between the various risk factors (age and gender) and the rate of *T. equi* infection (Table 1) (P>0.05). Sequenced PCR products were found 99% identical to the *T. equi* 18S rRNA GenBank reference (EU888902.1) by BLAST analysis. The nucleotide sequences were assembled and edited using CLC bio software and deposited in GenBank (NCBI) under access No. of KJ125440-KJ125442.

Table 1: Frequency of *T. equi* PCR positive by host related factors in 106 donkeys

Risk factor	Results of PCR		
	Number	Number of positive	%
Age			
<1	3	2	66.66
1-2	11	1	9.09
3-5	47	23	48.93
5<	45	28	62.22
Gender			
Male	67	33	49.25
Female	39	21	53.84

Discussion

In this study, all donkeys were apparently healthy. The microscopical examination showed *T. equi* infection in 4 (3.77%) of the donkeys with low parasitemia, while 54 (50.94%) of the donkeys were *T. equi*-PCR positive. Our results confirmed low sensitivity of microscopy for identification of carrier animals compared to PCR methods (Bose *et al*., 1995). A few epidemiological studies on equine piroplasmosis have been done using different methods. The frequency of *T. equi* infections was detected from 0.5 to 12% of blood smears in donkeys of Ethiopia (Mekibib *et al*., 2010; Tefera *et al*., 2011; Gizachew *et al*., 2013). DNA of *T. equi* were detected in 31.81% of donkeys in Brazil using PCR method (Machado *et al*., 2011). Seropositivity rates of *T. equi* infection were in 55.7% of donkeys in Ethiopia (Gizachew *et al*., 2012), 4%-13.1% in Turkey (Acici *et al*., 2008; Balkaya *et al*., 2010), 47.2 in Spain (García-Bocanegra *et al*., 2013), 9.6% in China (Chahan *et al*., 2006) and 73.86% in Brazil (Machado *et al*., 2011). These reports indicated that donkeys are important carriers for *T. equi* infection in different countries. In the

present study, *B. caballi* infection was not detected in the blood samples of donkeys. Although *B. caballi* infection has lower frequency and geographical distribution than *T. equi* worldwide (Schein, 1988; Friedhoff *et al*., 1990), but lack of *B. caballi* in blood and mollecular tests in our work was unclear and require further epidemiological studies in Iran.

For analysis of age as a risk factor, animals were divided into four groups and data was analyzed. The association between the rate of *T. equi* infected animals and different age and gender groups of donkeys was not significant. The management breeding system of donkeys is semiconfined and equids of all ages and with different activity can freely access pasture. Grazing in pasture, as a managenent practice, causes more tick infestation and puts animals at greater risk for occurrence of equine piroplasmosis infection (Shkap *et al*., 1998; Moretti *et al*., 2010). To the authors knowledge, this is the first reserach of molecular survey of *Theileria* and *Babesia* species in donkeys from Iran.

Taken together, the results of this study indicated that *T. equi* is prevalent among donkeys in North Khorasan province, Iran. No significant association was found between the rate of *T. equi* infected animals. All the sampled donkeys were asymptomatic, but could act as carriers to facilitate the spread of *T. equi*.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Acici, M; Umur, S; Guvenc, T; Arslan, HH and Kurt, M (2008). Seroprevalence of equine babesiosis in the Black Sea region of Turkey. Parasitol. Int., 57: 198-200.

Alhassan, A; Pumidonming, W; Okamura, M; Hirata, H; Battsetseg, B; Fujisaki, K; Yokoyama, N and Igarashi, I (2005). Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. Vet. Parasitol., 129: 43-49.

Allsopp, MTEP; Lewis, BD and Penzhorn, BL (2007). Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. Vet. Parasitol., 148: 130-136.

Aslani, MR (2000). A case report of *Babesia caballi* infection in a foal. J. Appl. Anim. Res., 17: 253-256.

Balkaya, I; Utuk, AE and Piskin, FC (2010). Prevalance of *Theileria equi* and *Babesia caballi* in donkeys from Eastern Turkey in winter season, Pak. Vet. J., 30: 245-246.

Bose, R; Jorgensen, WK; Dalgliesh, RJ; Friedhoff, KT and De Vos, AJ (1995). Current state and future trends in the diagnosis of babesiosis. Vet. Parasitol., 57: 61-74.

Brüning, A (1996). Equine piroplasmosis an update on diagnosis, treatment and prevention. Bri. Vet. J., 152: 139-151.

Chahan, B; Zhang, S; Seo, J; Nakanura, C; Zhang, G; Bannai, H; Jian, Z; Inokuma, H; Tuchiya, K; Sato, Y; Kabeya, H; Maruyama, S; Mikami, T and Xuan, X (2006). Seroepidemiological evidence for the possible presence of *Babesia* (*Theileria*) *equi* and *Babesia caballi* infections in donkeys in Western Xinjiang, China. J. Vet. Med. Sci., 68: 753-755.

De waal, DT (1992). Equine piroplasmosis: a review. Bri. Vet. J., 148: 6-14

Friedhoff, KT; Tenter, AM and Muller, I (1990). Haemoparasites of equines: impact on international trade of horses. Rev. Sci. Tech. Off. Int. Epiz., 9: 1187-1194.

García-Bocanegra, I; Arenas-Montes, A; Hernández, E; Adaszek, L; Carbonero, A; Almería, S; Jaén-Téllez, JA; Gutiérrez-Palomino, P and Arenas, A (2013). Seroprevalence and risk factors associated with *Babesia caballi* and *Theileria equi* infection in equids. Vet. J., 195: 172-178.

Georges, KC; Ezeokoli, CD; Sparagano, O; Pargass, I; Campbell, C; Abadie, RD and Yabsley, MJ (2011). A case of transplacental transmission of *Theileria equi* in a foal in Trinidad. Vet. Parasitol., 175: 363-366.

Gizachew, A; Schuster, RK; Joseph, S; Wernery, R; Georgy, NA; Elizabeth, SK; Asfaw, Y; Regassa, F and Wernery, U (2012). Piroplasmosis in donkey—A hematological and serological study in Central Ethiopia. J. Equine Vet. Sci., 33: 1-5.

Kumar, S; Kumar, R and Sugimoto, C (2009). A perspective on *Theileria equi* infections in donkeys. Jpn. J. Vet. Res., 54: 171-180.

Machado, RZ; Toledo, CZP; Teixeira, MCA; André, MR; Freschi, CR and Sampaio, PH (2012). Molecular and serological detection of *Theileria equi* and *Babesia caballi* in donkeys (*Equus asinus*) in Brazil. Vet. Parasitol., 186: 461-465.

Mekibib, B; Manegerew, M and Tadesse, A (2010). Prevalence of haemoparasite and associated risk factors in working donkeys in Adiguden and Kwiha districts of Tigray region, Northern Ethiopia. J. Anim. Vet. Adv., 9: 2249-2255.

Moretti, A; Mangili, V; Salvatori, R; Maresca, C; Scoccia, E; Torina, A; Moretta, I; Gabrielli, S; Tampieri, MP and Pietrobelli, M (2010). Prevalence and diagnosis of *Babesia* and *Theileria* infections in horses in Italy: a preliminary study. Vet. J., 184: 346-350.

Reiter, I and Weiland, G (1989). Recently developed methods for the detection of babesial infections T. Roy. Soc. Trop. Med. Hyg., 83: 21-23.

Seifi, HA; Mohri, M and 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.

Shkap, V; Cohen, I; Leibovitz, B; Savitsky Pipano, E; Avni, G; Shofer, S; Giger, U; Kappmeyer, L and Knowles, D (1998). Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. Vet. Parasitol., 76: 251-259.

Tefera, M; Worku, A and Tolosa, M (2011). Prevalence and risk factors for donkey babesiosis in and around Debre Zeit, Central Ethiopia. Vet. Res., 4: 56-60.

Wise, LN; Kappmeyer, LS; Mealey, RH and Knowles, DP (2013). Review of equine piroplasmosis. J. Vet. Intern. Med., 27: 1334-1346.