

## Detection of *Theileria equi* and *Babesia caballi* using microscopic and molecular methods in horses in suburb of Urmia, Iran

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Article Info	Abstract
<b>Article history:</b> Received: 26 June 2013 Accepted: 02 December 2013 Available online: 15 June 2014	Equine piroplasmiasis is a severe disease of horses caused by the intra-erythrocyte protozoan, <i>Theileria equi</i> and <i>Babesia caballi</i> . The aim of this study was to identify equine piroplasmiasis based on molecular and morphometrical features in horses in suburb of Urmia, West Azerbaijan province, Iran. From April to September 2011, a total number of 240 blood samples were collected randomly from horses of 25 villages. The specimens were transferred to the laboratory and the blood smears stained with Geimsa, and the morphological and biometrical data of parasite in any infected erythrocyte were considered. Extracted DNA from each blood sample was used in multiplex PCR in order to confirm the presence of <i>B. caballi</i> and <i>T. equi</i> . Microscopic observation on 240 blood smears determined that 15 (6.25%) and 5 (2.80%) samples were infected by <i>T. equi</i> and <i>B. caballi</i> , respectively. The mixed infections occurred in 2 (0.83%) samples. The results of the PCR assays showed 26 (10.83%), 14 (5.83%) and 4 (1.66%) were distinguished as <i>T. equi</i> , <i>B. caballi</i> and mixed infection, respectively. Differences in infection rates were statistically nonsignificant between male and female horses and among different age groups. Our findings indicated that <i>T. equi</i> and <i>B. caballi</i> were prevalent in horse population.
<b>Key words:</b> <i>Babesia caballi</i> Horse Iran Multiplex PCR <i>Theileria equi</i>	
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### تشخیص تیلریا اکویی و بابزیا کابالی بوسیله روش های مولکولی و میکروسکوپی در اسبهای اطراف شهرستان ارومیه، ایران

#### چکیده

پیروپلاسموزیس یک بیماری در اسبها بوده که توسط تک یاخته های داخل سلولی تیلریا اکویی و بابزیا کابالی ایجاد می شود. هدف از این مطالعه تشخیص پیروپلاسموزیس به روش مولکولی و مشخصات مورفولوژیکی در اسبهای شهرستان ارومیه واقع در استان آذربایجان غربی، ایران بود. از آوریل تا سپتامبر ۲۰۱۱ در مجموع ۲۴۰ نمونه خون اسب به صورت تصادفی از ۲۵ روستا جمع آوری شد. نمونه ها به آزمایشگاه منتقل و گسترش های خونی با گیمسا رنگ آمیزی شده و شاخص های مورفولوژیکی و بیومتریکی آنکل در هر گلبول قرمز آلوده مورد بررسی قرار گرفت. دی ان ای استخراج شده از هر نمونه خونی در واکنش زنجیره ای پلی مراز چندگانه به منظور تشخیص تیلریا اکویی و بابزیا کابالی مورد استفاده قرار گرفت. در بررسی میکروسکوپی ۱۵ نمونه آلوده به تیلریا اکویی (۶/۲۵ درصد) و ۵ نمونه آلوده به بابزیا کابالی (۲/۸۰ درصد) بودند. آلودگی همزمان در ۲ نمونه (۰/۸۳ درصد) مشاهده شد. نتایج حاصل از واکنش زنجیره ای پلی مراز نشان داد که ۲۶ نمونه آلوده به تیلریا اکویی (۱۰/۸۳ درصد)، ۱۴ نمونه آلوده به بابزیا کابالی (۵/۸۳ درصد) و ۴ نمونه آلوده به هر دو آنکل (۱/۶۶ درصد) به ترتیب آلوده بودند. تفاوت معنی داری ما بین آلودگی در اسبهای نر و ماده و گروه های سنی مختلف دیده نشد. یافته های ما شیوع تیلریا اکویی و بابزیا کابالی در میان جمعیت اسب را نشان داد.

**واژه های کلیدی:** اسب، ایران، بابزیا کابالی، تیلریا اکویی، واکنش زنجیره ای پلیمرز چندگانه

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## Introduction

Equine piroplasmosis is a tick-borne disease caused by intra erythrocyte protozoa, *Babesia equi* (recently re-classified as *Theileria equi*)<sup>1</sup> and *Babesia caballi*.<sup>2</sup> It is characterized by fever, anemia, icterus, hepatomegaly, edema, intra-vascular hemolysis, hemoglobinuria and even death.<sup>3,4</sup> These parasites affect horse industry worldwide, causing economic loss and impacting the international movement of horses.<sup>5</sup> This disease is distributed in Asia, Europe, Africa and South America. Prevalence of disease is related to distribution of vector ticks.<sup>6,7</sup> Tick species of the genera *Boophilus*, *Dermacentor*, *Hyalomma* and *Rhipicephalus* are the biological vectors of equine piroplasmosis.<sup>8</sup> The clinical picture of piroplasmosis is variable and often nonspecific.<sup>9</sup> It is not possible to distinguish between *T. equi* and *B. caballi* infections based on clinical signs alone. Several studies have documented mixed infections of *T. equi* and *B. caballi*.<sup>10,11</sup> Recovered horses from acute phase of infection serve as reservoirs for both *Babesia* species.<sup>6</sup>

*Theileria equi* is a small piroplasm whereas *B. caballi* is a larger form. The shape of *T. equi* parasite in the infected erythrocyte varies from spherical, ovoid or Maltese cross shape. The organism may be found either singly, in pairs, or in tetrads. *Babesia caballi* organisms are pyriform round or oval in shape and commonly seen singly or in pairs.<sup>3,12</sup>

Equine *Babesia* species detection was performed traditionally using Giemsa staining of thin blood smears and their morphology in infected erythrocytes. This method may have been accompanied with some technical problems.<sup>13</sup> Recently, several studies have been conducted to describe biometrical and genetical characterization of babesiosis in Iran.<sup>13,14</sup>

Several sero-epidemiological studies concerning equine babesiosis have been conducted among horses of many parts of the world. The lack of the specificity due to cross reactivity with other species of *Babesia* has been observed in serological investigations.<sup>15</sup> Molecular techniques have been considered as perfect methods for detection of many species of *Babesia* and *Theileria*. Reportedly, PCR assays have higher sensitivity and specificity compared with serological assays.<sup>16-18</sup>

The presence of equine piroplasmosis has previously been reported from different parts of Iran. These studies have been performed by microscopic examination and serological methods.<sup>19-23</sup> The aim of this study was to identify equine piroplasmosis based on molecular techniques and morphometrical indices in horses in suburb of Urmia, West Azerbaijan province, Iran.

## Materials and Methods

**Study area.** The study was conducted during the tick activity seasons (spring and summer) in 25 villages of Urmia suburb, capital of West Azerbaijan province. Urmia

is semi-humid, with mean precipitation of 350 mm, maximum monthly temperature of 28.3 °C in August and minimum monthly temperature of -5 °C in January. This area has borders with Turkey and Iraq and some residents of the area usually travel and carry goods by working horses across the borders due to the arduous mountain routes.<sup>24</sup>

**Sampling and morphometric procedures.** From April to September 2011, a total number of 240 blood samples were collected randomly from horses in the mountainous, mountainside, and plain areas of Urmia suburb. Blood samples were aseptically obtained from the jugular vein of each horse. The age and sex were recorded for each animal. The blood samples were collected in the presence of the EDTA anticoagulant and used immediately for blood smears stained with Giemsa. The samples were transferred to the laboratory of Parasitology, Faculty of Veterinary Medicine, Urmia University, Urmia, for further analysis.

**Giemsa staining.** The Giemsa stained blood smears were examined to determine the presence of hemoprotozoal parasites. The morphological and biometrical parameters including the shape, site location and size of parasite in any infected erythrocyte have been considered.<sup>14</sup> In microscopic examination, *B. caballi* was identified as large paired pyriform parasites, while the small *T. equi* parasites were identified as paired pyriform, rounded and tetrad or Maltese cross arrangement of merozoites.<sup>12,25</sup>

**DNA extraction.** Genomic DNA was extracted according to Alhassan *et al.* with some modifications.<sup>11</sup> Briefly, 50 µL of each horse blood samples were washed three times with cold phosphate buffered saline by centrifuging at 1000 *g* for 5 min at 4 °C and re-suspending in 100 µL of DNA extraction buffer (0.1 mM Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM EDTA, and 100 µg mL<sup>-1</sup> proteinase K) and incubating at 55 °C for 2 hr. The parasitic DNA was extracted with phenol-chloroform and precipitated with ethanol. The purified DNA pellets were dissolved in 20 µL of double-distilled water for subsequent PCR reactions.<sup>11</sup>

**Multiplex PCR.** In order to specify the morphological findings and simultaneous differentiation of *B. caballi* and *T. equi*, multiplex PCR based on the 18S ribosomal RNA genes was performed. A set of primers, Bec-UF2 5'-TCGAAGACGATCAGATACCGTCG-3', Cab-R 5'-CTCGTT CATGATTTAGAATTGCT-3' and Equi-R 5'-TGCCTTAAAC TTCCTTGCGAT-3', were used to amplify DNA fragments of 540 and 392 bp from *B. caballi* and *T. equi*, respectively. The primer's specificity and sensitivity and also the PCR condition had been described previously by Alhassan *et al.*<sup>11</sup>

The PCR reaction was performed in 50 µL of a mixture (10 mM Tris-HCl [pH 8.3], 50 mM KCl, and 1.5 mM MgCl<sub>2</sub>) containing 3 µL of the template DNA, 2.5 pmol of each of the primers, 0.2 mM dNTP mixture and 2.5 U of *Taq* DNA polymerase (Fermentas, Schwerte, Germany). Cycling condition was 96 °C for 10 min, followed

by 40 cycles at 96 °C for 1 min, 60.5 °C for 1 min, and 72 °C for 1 min with a final extension step of 72 °C for 10 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis, followed by ethidium bromide staining and photography.<sup>11</sup> Positive controls were consisted of DNA from blood samples known to be infected by *B. caballi* and *T. equi* through microscopic examination of blood smears. Distilled water was used as negative control in PCR amplification.

**Statistical analysis.** Data were analyzed using SPSS (Version 17; SPSS Inc., Chicago, USA). A value of  $p < 0.05$  was considered as statistically significant.

**Results**

Out of 240 examined horses, 129 were females and 111 males. The number of infected horses based on age and sex was summarized in Table 1. Prevalence of *B. caballi* and *T. equi* in all age groups and between male and female horse were not statistically significant ( $p > 0.05$ ).

**Table 1.** The frequency (positive/examined) of *Babesia* spp. infection in horses based on age and sex.

Methods	Age (%)		Sex (%)	
	< 3	≥ 3	Female	Male
Microscopy	9/106 (8.49)	13/134 (9.70)	12/129 (9.30)	10/111 (9.00)
	19/106 (17.92)	25/134 (18.65)	23/129 (17.82)	21/111 (18.91)

**Morphological and morphometric findings.**

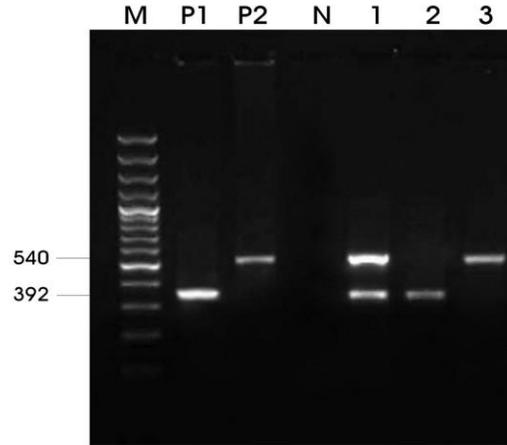
Microscopic observation on 240 blood smears determined that 15 (6.25%) and 5 (2.80%) samples were infected by *T. equi* and *B. caballi*, respectively. The mixed infections were occurred in 2 samples (0.83%). The parasites shapes were distinguished based on single round, double round, single pyriform and double pyriform with obtuse or acute angle. The size of *T. equi* and *B. caballi* typical paired pyriforms and round forms are summarized in Table 2.

**Table 2.** The morphological features of *T. equi* and *B. caballi*. The data are presented as mean ± standard deviation.

Parasite	Morphological feature	Size (µm)
<i>T. equi</i>	Double pyriform -acute angle	1.14 ± 0.15 × 1.40 ± 0.11
	Double pyriform -obtuse angle	1.60 ± 0.07 × 1.88 ± 0.13
	Round	1.5 ± 0.19
<i>B. caballi</i>	Double pyriform -acute angle	2.60 ± 0.08 × 2.88 ± 0.11
	Double pyriform -obtuse angle	3.53 ± 0.14 × 3.91 ± 0.07
	Round	2.53 ± 0.28

**Molecular findings.**

The results of the PCR assays showed, 26 (10.83%) and 14 (5.83%) were infected with *T. equi* and *B. caballi*, respectively. The PCR product of *T. equi* and *B. caballi* were 392 bp and 540 bp, respectively. A mixed infection of *B. caballi* and *T. equi* was found just in 4 horses (1.66%), (Fig.1).



**Fig. 1.** PCR detection of *B. caballi* and *T. equi* with a set of primer combinations (Bec-UF2, Cab-R, and Equi-R). M = 100 bp DNA marker; P1= Positive control for *T. equi*; P2 = Positive control for *B. caballi*; N= Negative control; Lane 1 = Mixed of *T. equi* and *B. caballi*; Lane 2 = *T. equi*; Lane 3 = *B. caballi*.

**Discussion**

Visual detection of piroplasms by microscopic examination as the simplest and most accessible diagnostic test, confirms the clinical diagnosis of the babesiosis.<sup>26</sup> Considering the possible falsely diagnosed cases of babesiosis, the combination of microscopy and PCR based diagnostics is recommended.<sup>27</sup>

Equine piroplasms can be recognized based on biometrical and morphometric data. Soulsby described *T. equi* as small *Babesia* being 2 µm in length (< 2.5 µm), pyriform or comparatively rare round or amoeboid and *B. caballi* as a large *Babesia* measuring 2.5 to 4 µm (> 2.5 µm) with acute angle in pyriform. The morphological characteristics observed in *T. equi* and *B. caballi* in current study was in agreement with the findings of Soulsby.<sup>28</sup>

Previous research focused on diagnosis using microscopic examinations of Giemsa-stained blood smears has been reported infection rates of *T. equi* varying 3.50 to 7.00% in Iran.<sup>19,21</sup> In the present study, 6.25, 2.80 and 0.83% samples were infected by *T. equi*, *B. caballi* and mixed infections, respectively. Microscopic examination of Geimsa stained blood smears is the common method for diagnosis of these piroplasms in Iran. The low sensitivity of this method does not permit its use in epidemiological investigations.<sup>14</sup> The results of this study confirmed findings of Bashiruddin *et al.* in that PCR is more sensitive in diagnosing piroplasmosis than microscopy.<sup>29</sup>

In our study no differences were observed between the *T. equi* and *B. caballi* prevalence in all age and sex groups of the horse examined. It may be due to high number of ticks in this area and continuous exposure of young and old horses to infected ticks.<sup>30</sup>

The results of molecular and microscopic examinations confirmed the simultaneous infection of horses in the study

region with both equine *Babesia* species, which was consistent with findings of Seifi *et al.* and Abedi *et al.* They reported mixed infection of *T. equi* and *B. caballi* in horses of Turkmen region in Iran.<sup>19,22</sup>

*Theileria equi* is more common and pathogenic than *B. caballi* in endemic countries.<sup>31-33</sup> The results of the present study demonstrated that *T. equi* was more prevalent than *B. caballi*. Our findings were in agreement with the previous study in Iran.<sup>19</sup> A possible reason for the low prevalence of *B. caballi* could be associated with the earlier removal of the parasite after a short term of infection.<sup>34</sup>

Several investigations on the prevalence of equine piroplasmiasis in Turkey, which shares a border with the study area, have been published.<sup>35-38</sup> These reports demonstrated that both *T. equi* and *B. caballi* infections in horses have been widespread in Turkey, with the prevalence rate of 7.00% and 3.00%, respectively.<sup>38</sup>

Because of certain geographical specifications of studied area, the sampled horses had close communication with horses of neighboring countries and probably, they had been contentiously exposed to ticks and protozoa. This situation emphasizes the importance of border control and quarantine.<sup>35</sup> Probably, an increase in the number of imported horses from neighboring countries and the distribution of vector ticks are factors which increase the occurrence of infection in this region.

Based on our results, it is concluded that *T. equi* and *B. caballi* were prevalent among horses in West Azerbaijan province, Iran. Moreover, this report suggests the possibility of an endemic nature of equine piroplasmiasis in this area. These data are essential to establish adequate control measures in this area. The tick vectors for equine piroplasmiasis in studied region are still unknown to date, therefore, there is a need to investigate the potential tick vectors involved in the transmission of both *T. equi* and *B. caballi* in horses in this region.

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