Original Article

Molecular Study of *Babesia caballi* and *Theileria equi* in Horses, in Ardabil Province, Iran

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ABSTRACT

Background: Equine piroplasmosis (EP), caused by *Babesia caballi* and *Theileria equi*, is a prevalent tick-borne hemoprotozoan disease worldwide. In Iran, the disease is endemic and often manifests in subclinical form. To control the disease by detecting carrier horses, real-time polymerase chain reaction (PCR) is a suitable method.

Objectives: We aimed to detect *B. caballi* and *T. equi* in horses in Ardabil Province, Iran, using SYBR Green I real-time PCR.

Methods: This study was conducted from April to August 2015 in 5 cities (Ardabil, Nir, Namin, Sareyn, and Meshginshahr) in Ardabil Province, Iran. Blood samples were randomly collected from 92 healthy horses, noting their age, gender, and sampling location. Genomic DNA was extracted from each sample and assessed, followed by SYBR green I real-time PCR. Data analysis was conducted using Rotor-Gene Q Series software to calculate the target DNA concentration based on the unknown sample's cycle threshold (Ct) value. Positive values were defined as exceeding 1 ng/reaction. The relationship between horse infection rate and different age groups (1-5, 6-10, and >10 years), gender, and sampling locations was investigated using a chi-square test through SPSS statistical software. $P<0.05$ were considered significant.

Results: In this study, the frequency of *B. caballi*-infected horses was 9(9.8%), and that of *T. equi*-infected horses was 4(4.4%). A significant relationship was observed between the *B. caballi* infection and the age group of 5-10 years. However, there was no significant relationship between the *B. caballi* infection and gender or sampling locations. Furthermore, no significant relationship was observed between the *T. equi* infection and the age groups, gender, and sampling locations of the horses.

Conclusion: The infection found in apparently healthy horses suggests endemic EP in this region. Therefore, greater focus is needed on controlling and treating carrier horses.

Keywords: *Babesia caballi*, Piroplasmosis, Real-time polymerase chain reaction (PCR), SYBR green, *Theileria equi*

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Introduction

Equine piroplasmosis (EP) is a hemoprotozoan tick-borne disease with worldwide distribution that results from *Babesia caballi* and *Theileria equi*. In the acute form of equine piroplasmosis, the prominent clinical signs are fever, inappetence, lethargy, edema, petechial hemorrhages on the conjunctiva, icterus, tachycardia, tachypnea, weakness, and hemoglobinuria (Almazán et al., 2022). The chronic form has clinical signs such as weight loss, decreased body condition score, inappetence, depression, and poor performance (Wise et al., 2013; Almazán et al., 2022). However, the covert carrier state of both *B. caballi* and *T. equi* infections has no obvious clinical signs (Tamzali, 2013; Torres et al., 2021).

In endemic areas such as Iran, the infected horses are commonly in the carrier state and look healthy. Identification of the carrier horses is necessary for the prevention of EP in these regions. Microscopic examination with Giemsa staining lacks accuracy in identifying carrier horses. Therefore, using a serologic or a molecular method seems necessary (Kim et al., 2008; Soliman et al., 2021). Among serological methods, competitive enzyme-linked immunosorbent assay (cELISA) is the most sensitive method for the detection of carrier and chronically infected horses (Wise et al., 2014). However, serological methods have some limitations related to the identification of antibodies and cross-reactivity with other pathogens (Kim et al., 2008). Several molecular techniques, including conventional polymerase chain reaction (PCR), have been identified for the genomic detection of the disease (Bashiruddin et al., 1999). Although PCR has high sensitivity and specificity, it depends on end-point measurement, and its results must be exhibited on the agarose gel (Bashiruddin et al., 1999). The real-time PCR technique lacks the abovementioned problem and has more sensitivity and specificity than conventional PCR (Bashiruddin et al., 1999).

Understanding the epidemiology of EP is crucial for effective control. However, limited studies exist on the status of EP in Iran (Malekifard et al., 2014; Abedi et al., 2014; Bahrami et al., 2014; Taktaz-Hafshejani & Khamesipour, 2017; Kakekhani et al., 2017; Kalantari et al., 2022). In these studies, the blood smear microscopic examination, indirect fluorescent antibody technique (IFAT), and molecular methods based on conventional PCR have been used to evaluate the status of EP. The purpose of our study was to investigate the status of EP in horses of Ardabil province in Iran by detecting *B. caballi* and *T. equi* using the SYBR green I real-time PCR technique.

Materials and Methods

Field study area

This study was conducted from April to August 2015 in Ardabil Province, Northwest Iran. The total area of Ardabil Province is 18634 km^2. The latitude and longitude for Ardabil Province are 38°29'7.18"N and 47°53'28.04"E, respectively. This province has a cold climate with an average annual rainfall of 256 mm and a mean precipitation of 21.4 mm per year. The winters are severely cold, with temperatures falling to -25 °C.

Blood sampling

The present study was performed in the villages of five cities (Ardabil, Nir, Namin, Sareyn, and Meshginshahr) in Ardabil Province, Iran. Ninety-two healthy horses (20 females and 72 males) were randomly selected for blood sampling. The age, gender, and sampling location of each horse were recorded. Blood samples were collected from the jugular vein and placed into EDTA tubes (Hebei Xinle Sci&Tech Co, Ltd). The samples were maintained under cool conditions and immediately transferred to the laboratory. Subsequently, the EDTA tubes were stored at -20 °C until the time of molecular examination.

DNA extraction

The genomic DNA from the blood samples was extracted using the YTA Genomic DNA Extraction mini kit (Yekta Tajhiz Azam, Tehran, Iran) following the manufacturer's guidelines. Subsequently, the concentration of the extracted DNA was determined using a photometer (Biophotometer, Eppendorf, Germany) at 260 nm and 280 nm optical density. In cases where the genomic DNA concentration was high, appropriate dilutions were performed. The isolated DNA samples were then stored at -20 °C until further analysis.

Agarose gel electrophoresis

Following the genomic DNA extraction, 1.5% agarose gel electrophoresis was carried out to confirm the accuracy of the procedure and to assess the integrity and purity of the extracted genomic DNAs.

Table 1. Primer sequences used for detecting *B. caballi* and *T. equi*

Parasite	Gene	Primer Name	Sequence (5'–3')	Product (bp)
B. caballi	*18S rRNA*	BABcab-F	TTCCCTTCGCTTTTTGTTTT	260
		BABcab-R	CCCCTAACTTTCGTTCTTGA	
T. equi	*Chromosome I*	BABequ-F	CAGTTAATGTGGTGGCGAAG	238
		BABequ-R	CCAAGTCTCACACCCTATTT	

Primer sequence

The primers were used to amplify the *18S rRNA* gene of *B. caballi* and the *Chromosome I* gene of *T. equi* (Table 1).

SYBR green I real-time PCR assays

All real-time PCR reactions were accomplished in the Rotor-Gene Q real-time PCR instrument (Qiagen Company, Germany). In each reaction, 1 µL of template DNA, 0.5 µL of 0.25 µMol of each primer, 5 µL of SYBR green I real-time PCR master mix (AccuPower® 2X GreenStar™ master mix, Bioneer Co., South Korea), and 3 µL distilled water were used to reach the final volume of 10 µL. Thermal cycling conditions were adopted as follows: The initial cycle of heating at 95 ˚C for 10 min, 40 cycles of 95 ˚C for 10 s, 58 ˚C for 15 s, and 72 ˚C for 20 s. In the dissociation stage, the temperature was ramped up from 72 to 95 ˚C (at the rate of 1 ˚C/min). The fluorescent products were detected at the last step of each cycle. A fluorescent intensity for *B. caballi* and *T. equi* was produced based on the cycle threshold (Ct) for standard samples. In addition, the melting curve was analyzed to ensure specific amplification. The absolute abundance of the *18S rRNA* gene for *B. caballi* and the *Chromosome I* gene for *T. equi* was calculated based on standard curves using Rotor-Gene Q Series Software, version 2.0.2 (Qiagen, Germany) (R^2>0.998). The detection limit was 10^1 gene copies per reaction. The standard curve and Ct for *T. equi* and *B. caballi* were drawn. The DNA concentration in each unknown sample and the Ct value for *B. caballi* and *T. equi* are determined.

Statistical analysis

Statistical analysis was performed using SPSS software, version 21 (SPSS Inc.). The relationship between *T. equi* and *B. caballi* infection with age, gender, and sampling location was investigated using a chi-square test. The P<0.05 were considered significant.

Results

In all extracted genomic DNA samples, the ratio of the absorbance at 260 nm and 280 nm (A260/A280) was between 1.7 and 2, which indicated the purity and high quality of the DNA samples. Moreover, agarose gel electrophoresis revealed that the extracted genomic DNA was of good quality.

The Ct is the cycle number at which the fluorescence generated within a reaction crosses the threshold line. Ct values are logarithmic and are used directly or indirectly for quantitative analyses. After running real-time PCR, the accuracy of the results can be measured. The data are reliable if identical replicate samples have a Ct standard deviation <0.3 and or a standard curve has a correlation coefficient (R^2 value) >0.99. Ct values and standard curve of the present study for *B. caballi* (Figure 1) and *T. equi* (Figure 2) showed the correctness of the PCR results.

The analysis of the melting curve revealed that the maximum concentration of DNA during the polymerase chain reaction occurred at a temperature of 85 ˚C (Figures 3 and 4).

Results for *B. caballi*

The frequency and percentage of *B. caballi* infection, along with its relationship with age, gender, and sampling location, are presented in Table 2. Of 92 samples, 9 horses (8.9%) were infected with *B. caballi*. Statistical analysis showed a significant relationship between infection with *B. caballi* and the age of the horses (P<0.05). Seven horses (7.6%) infected with *B. caballi* were between 5 and 10 years old, and two horses (2.2%) were over 10 years old. In the horses less than 5 years old, there was no infection with *B. caballi*. In this study, 7 males (7.6%) and 2 females (2.2%) were infected with *B. caballi*. No significant difference was observed between *B. caballi* infection and gender (P>0.05). In addition, there was no significant difference between *B. caballi* infection and different sampling locations (P>0.05) (Table 2).

Table 2. Infection rate of *B. caballi* and *T. equi* and its relationship with age, gender, and sampling location

Variables		No. (%)						P		Chi-square	
		Positive Samples		Negative Samples		Total					
		B. caballi	*T. equi*	*B. caballi*	*T. equi*	*B. caballi*	*T. equi*	*B. caballi*	*T. equi*	*B. caballi*	*T .equi*
Age (y)	1-5	0(0)	1(1.1)	27(29.3)	26(28.2)	27(29.3)	27(29.3)	P<0.05	P>0.05	6.31	0.13
	5-10	7(7.6)	2(2.2)	31(33.7)	36(39.1)	38(41.4)	38(41.4)				
	>10	2(2.2)	1(1.1)	25(27.2)	26(28.3)	27(29.3)	27(29.3)				
	Total	9(9.8)	4(4.4)	83(90.2)	88(95.6)	92(100)	92(100)				
Gender	Female	2(2.2)	1(1.1)	18(19.6)	19(20.6)	20(21.7)	20(21.7)	P>0.05	P>0.05	0.001	0.001
	Male	7(7.6)	3(3.3)	65(70.7)	69(75)	72(78.3)	72(78.3)				
	Total	9(9.8)	4(4.4)	83(90.2)	88(95.6)	92(100)	92(100)				
Sampling location	Ardabil	3(3.3)	1(1.1)	30(32.6)	32(34.8)	33(35.8)	33(35.8)	P>0.05	P>0.05	0.263	1.41
	Nir	3(3.3)	1(1.1)	25(27.2)	27(29.3)	28(30.4)	28(30.4)				
	Namin	1(1.1)	1(1.1)	11(12)	11(12)	12(13.1)	12(13.1)				
	Sareyn	1(1.1)	0(0)	6(6.5)	7(7.6)	7(7.6)	7(7.6)				
	Meshgin-shahr	1(1.1)	1(1.1)	11(12)	11(12)	12(13.1)	12(13.1)				
	Total	9(9.8)	4(4.4)	83(90.2)	88(95.6)	92(100)	92(100)				

Results for *T. equi*

The frequency and percentage of *T. equi* infection, along with its relationship with age, gender, and sampling location, are presented in Table 2. There was no significant relationship between *T. equi* infection and the age of the horses (P>0.05). Of 92 horses, 4(4.4%) were infected with *T. equi*, of which 2(2.2%) were between 5 and 10 years old; one horse (1.1%) was less than 5 years old, and another one (1.1%) was over 10 years old. In this study, 3 males (3.3%) and 1 female horse (1.1%) were infected with *T. equi*. Statistical analysis revealed that there is no significant relationship between *T. equi* infection and gender (P>0.05). In addition, no significant difference was observed between *T. equi* infection and different sampling locations (P>0.05) (Table 2).

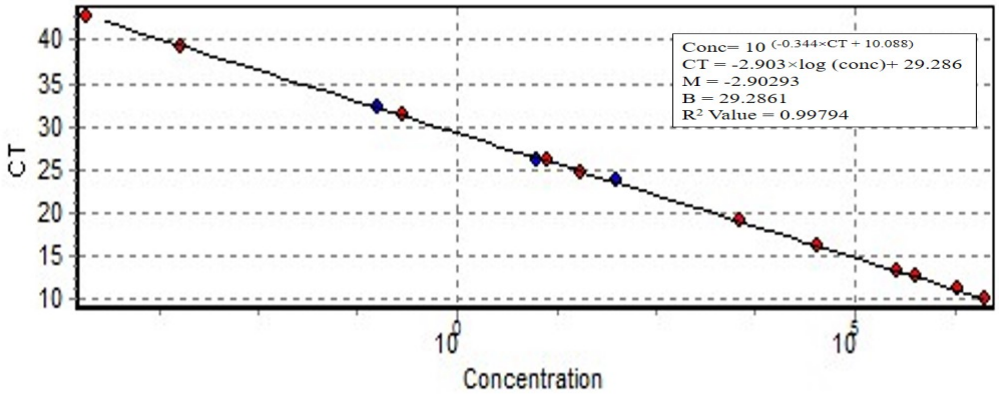

Figure 1. The standard curve based on Ct for *B. caballi*

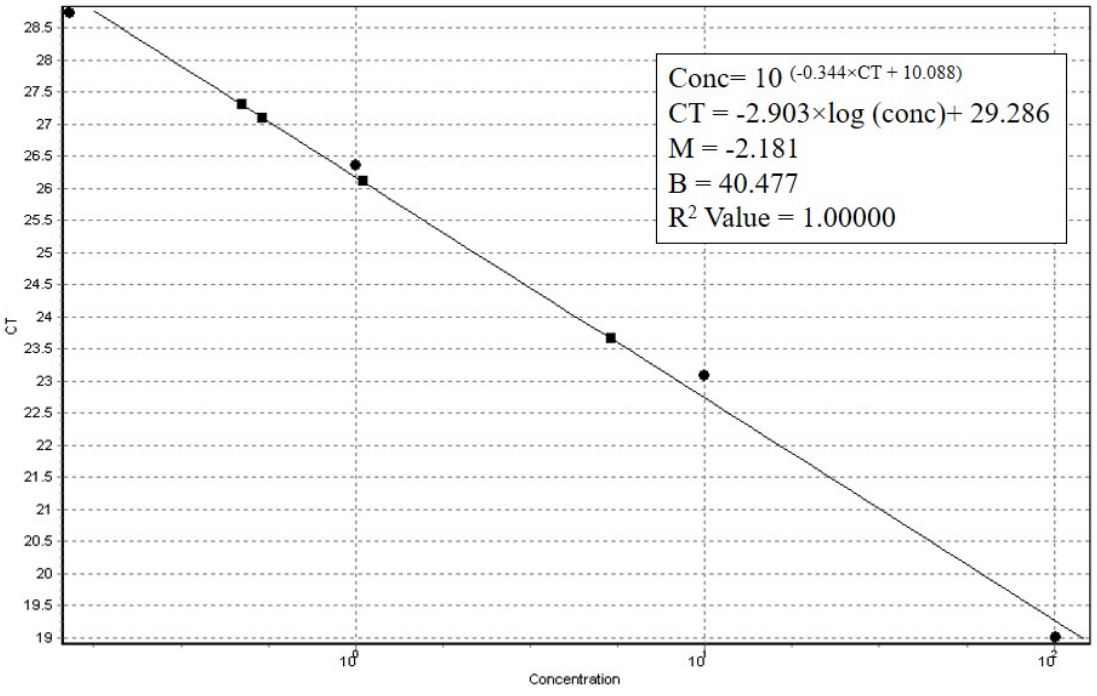

Figure 2. The standard curve based on Ct for *T. equi*

Discussion

According to the findings from molecular analysis in the current investigation, the frequency rates of *B. caballi* and *T. equi* infections in horses from Ardabil Province were 8.9% and 4.4%, respectively. These results reveal a higher occurrence of *B. caballi* infection compared to *T. equi* in the studied population. Moreover, simultaneous infection with both agents was not found. In a study on 153 horses in Turkey using immunofluorescent antibody test (IFAT) the prevalence of *T. equi* (21.5%) was lower than that of *B. caballi* (34.6%), which follows the results of our study (Acici et al., 2008). The same result has been reported in several studies (Mujica et al., 2011;

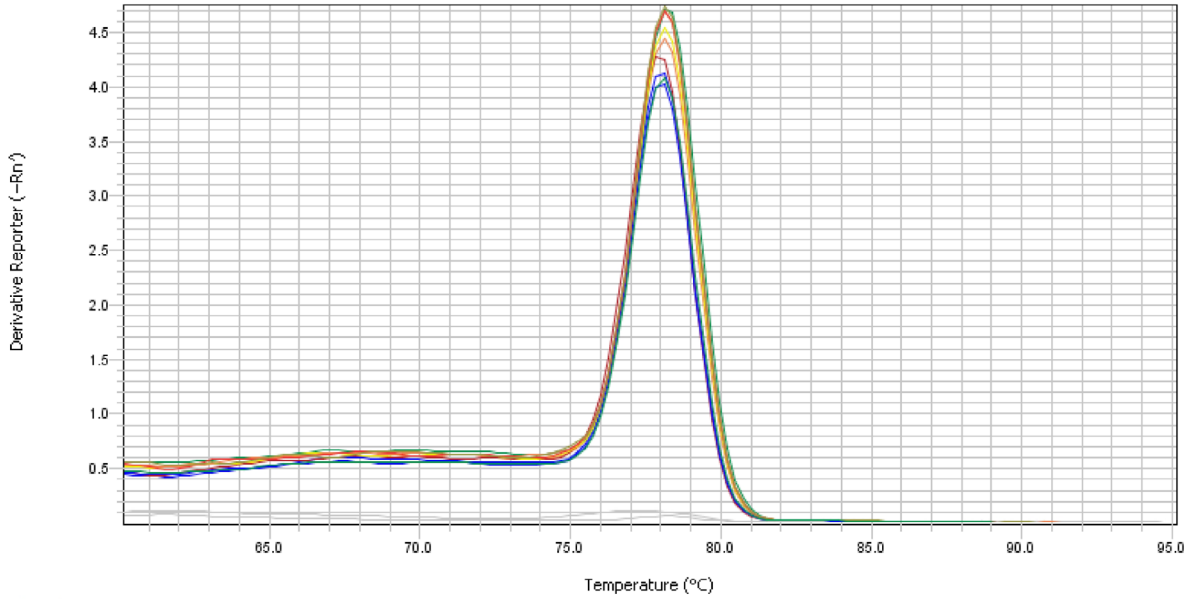

Figure 3. Melting curve analysis of real-time PCR products for *B. Caballi*

Note: Gray lines are the non-template control and negative control. Colorful lines include positive samples and control.

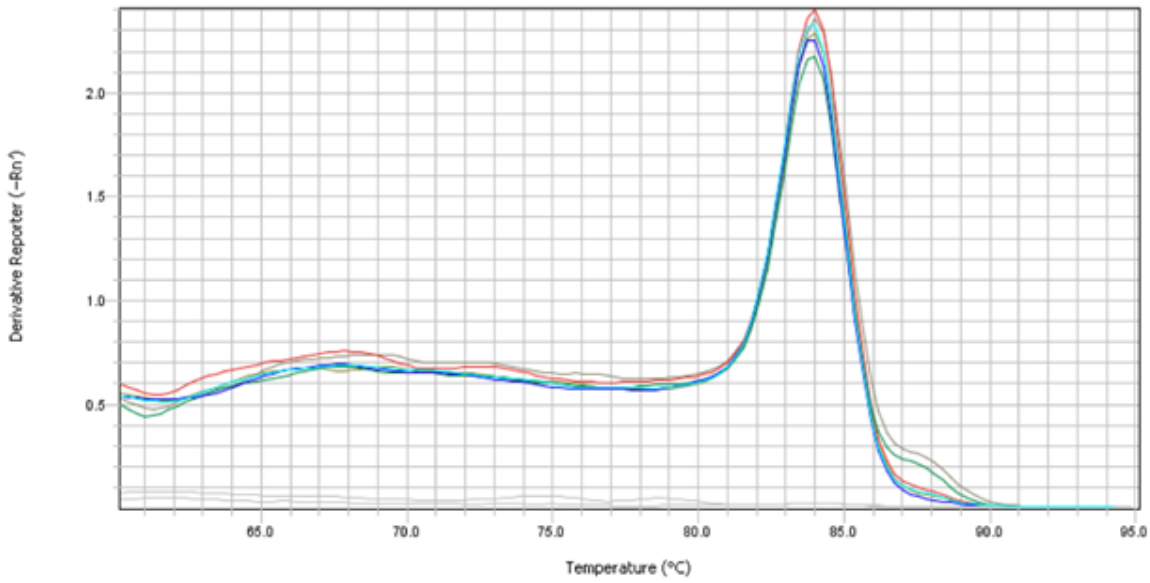

Figure 4. Melting curve analysis of real-time PCR products for *T. equi*

Note: Gray lines are the non-template control and negative control. Colorful lines include positive samples and control.

Taktaz-Hafshejani & Khamesipour, 2017; Nugraha et al., 2018; Tirosh-Levy et al., 2021). In a survey conducted using PCR in 2014 in Urmia City in Iran, the rates of infection with *B. caballi* and *T. equi* in horses were reported as 5.83% and 10.83%, respectively (Malekifard et al., 2014). Furthermore, in a molecular investigation carried out in the Kurdistan Province of Iran, involving 186 horses, the prevalence of *T. equi* infection was found to be 0.54%. In contrast, no cases of *B. caballi* infection were detected (Kakekhani et al., 2017). In several other studies, it has been shown that the prevalence of infection with *T. equi* is higher than that of *B. caballi*. These findings contrast with those of our study (Abedi et al., 2014; Kalantari et al., 2022; Aziz & Al-Barwary, 2019; Moretti et al., 2010). This discrepancy in the prevalence of *B. caballi* and *T. equi* in different studies may be related to the difference in sample size, diagnostic methods, regions, and tick vectors (Moretti et al., 2010; Rocafort-Ferrer et al., 2022).

In countries such as Iran, where EP is endemic, the incidence of clinical cases is low. However, clinical cases are sometimes reported (Seifi et al., 2000; Aslani, 2000; Sakha, 2007). In addition, the disease is endemic in most countries of the Middle East, including Jordan, Saudi Arabia, Pakistan, and Turkey in the neighborhood of Iran (Alanazi et al., 2012; Qablan et al., 2013; Hussain et al., 2014; Kizilarslan et al., 2015). In such countries, it is essential to identify carrier horses. Several methods have been described to identify carriers of *B. caballi* and *T. equi* in horses (Wise et al., 2013; Salinas-Estrella et al., 2022). Giemsa staining cannot help to identify carrier cases, so molecular and serological methods have been used for this purpose (Kim et al., 2008; Abedi et al., 2014; Alanazi et al., 2012; Vieira et al., 2013; Onyiche et al., 2019). The most sensitive serologic method for the diagnosis of chronic and subclinical cases of infection with *T. equi* is cELISA (Sellon & Long, 2013). However, in some instances, serological tests have problems in identifying subclinical cases due to the cross-reaction between antibodies (Kim et al., 2008). Furthermore, the conventional PCR can be used for the detection of these organisms, but the real-time PCR method is faster, more sensitive, and more specific than conventional PCR (Shipley, 2007). It was shown that the sensitivity of the SYBR green I real-time PCR method is 100 times higher than the conventional PCR method (Park et al., 2009). The real-time PCR method can determine subclinical infections and small amounts of infection, so that it will be helpful in the follow-up of treatment and eradication of the disease (Bell & Ranford-Cartwright, 2002).

The results of the present study indicated no infection with *B. caballi* in horses less than 5 years old, and infection with *B. caballi* in the age group of 5–10 years old is significantly higher than in other age groups. In Mongolia, the prevalence of both infections increases with rising age (Rüegg et al., 2007). The results of our study in age groups less than 10 years old are similar to this study, but in the age group above 10, a decrease in the prevalence was observed. It is difficult to explain this relationship with age, and our results are inconsistent with the results

of many other studies (Faraj et al., 2019; Malekifard et al., 2014; Moretti et al., 2010), which may be due to the small sample size in our study. However, another study showed a decrease in the prevalence of infection at the age of 10-16 years old (Avarzed et al., 1997), which is consistent with our study, although this study also had a small sample size. In addition, some studies have found no relationship between age and the prevalence of infection with *B. caballi* (Faraj et al., 2019; Malekifard et al., 2014; Moretti et al., 2010). These different results may be due to various geographical conditions and the distribution of vectors.

The results obtained in the present study indicated that the infection with *T. equi* has no significant relationship with the age of the horses, and these results are similar to the results of other studies (Kizilarslan et al., 2015; Moretti et al., 2010; Farkas et al., 2013). In addition, no significant relationship was found between EP infection and gender, which is similar to the results of other studies (Malekifard et al., 2014; Abedi et al., 2014; Aziz & Al-Barwary, 2019).

The results of the present study indicate no difference in infection rates between *T. equi* and *B. caballi*, nor between different sampling areas. This finding suggests that the distribution of infection in the various cities under study in Ardabil Province follows a similar pattern.

Conclusion

In conclusion, the presence of infection in apparently healthy horses indicates that EP is endemic in this region. Therefore, more attention should be paid to controlling and treating carrier horses. Real-time PCR can be used to detect subclinical infections and effectively control the disease.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. The patient's owners were informed of the purpose of the research.

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The paper was extracted from the doctor of veterinary medicine thesis of the Farnoosh Farsijani, approved by the Faculty of Veterinary Medicine, Tabriz University, Tabriz, Iran.

Authors' contributions

Conceptualization and supervision: Hamid Akbari and Abbas Imani Baran; Methodology: Farnoosh Farsijani, Raziallah Jafari Jozani and Hamid Akbari; Data collection: Farnoosh Farsijani, Hamid Akbari and Abbas Imani Baran; Data analysis: Hamid Akbari, Arman Shokri and Raziallah Jafari Jozani; Investigation and writing: All authors.

Conflict of interest

The authors declared no conflict of interest.

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