

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

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Running Title: Molecular Study of Equine Piroplasmosis

## **Abstract**

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

**Objectives:** This study 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 five cities (Ardabil, Nir, Namin, Sareyn, and Meshginshahr) in Ardabil province. Blood samples were collected randomly

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, calculating target DNA concentration based on each 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. The values of  $P < 0.05$  was considered significant.

**Results:** In this study, the frequency of *B. caballi*-infected horses was 9 (9.8%), and *T. equi*-infected horses were 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 among the *B. caballi* infection and gender and sampling locations. Furthermore, no significant relationship was observed among 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 equine piroplasmosis in this region. Therefore, greater focus is needed on controlling and treating carrier horses.

**Keywords:** *Babesia caballi*, Piroplasmosis, Real-time PCR, SYBR Green, *Theileria equi*

## Introduction

Equine piroplasmiasis is a hemoprotozoan tick-borne disease with worldwide distribution that is caused by *B. caballi* and *T. equi*. In the acute form of equine piroplasmiasis, the most important 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 inapparent 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 apparently 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, either a serologic or a molecular method is necessary (Kim *et al.*, 2008; Soliman *et al.*, 2021). Among serological methods, competitive enzyme-linked immunosorbent

assay (c-ELISA) 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 methods, 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 does not have the abovementioned problem and has more sensitivity and specificity than conventional PCR (Bashiruddin *et al.*, 1999).

Study on the epidemiology of EP has great importance in the control of it. However, some limited studies exist on the status of EP in Iran (Malekifard *et al.*, 2014; Abedi *et al.*, 2014; Bahrami *et al.*, 2014; Taktaz-Hafshejani and 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 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<sup>2</sup>. 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. Ninety-two apparently 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 they were required for 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 optimal quality of the extracted genomic DNAs.

### **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 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  $\mu$ L distilled water was used to reach the final volume of 10  $\mu$ L. 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 Ct for standard samples. In addition, the melting curve was analyzed to ensure specific amplification. The absolute abundance of 18S rRNA gene for *B. caballi* and Chromosome I gene for *T. equi* was calculated based on standard curves using Rotor-Gene Q Series Software 2.0.2 (Qiagen, Germany),  $R^2 > 0.998$ . The detection limit was  $10^1$  genes/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 21.0, 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* values less than 0.05 were considered significant.

### **Results**



In all extracted genomic DNA samples, the ratio of the absorbance at 260 nm and at 280 nm (A260/A280) was between 1.7-2, which indicated purity and high quality of DNA samples. Moreover, agarose gel electrophoresis revealed that the extracted genomic DNAs have 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 the temperature of 85 °C (Figure 3 and 4).

### **Results for *B. caballi***

The frequency and percentage of *B. caballi* infection and its relationship with age, gender, and sampling location was shown in table 2. Of the 92 samples, nine horses (8.9%) were infected with *B. caballi*. Statistical analysis showed that there is 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 5-10 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, seven males (7.6%) and two 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).

### **Results for *T. equi***

The frequency and percentage of *T. equi* infection and its relationship with age, gender, and sampling location was shown in table 2. There was no significant relationship between *T. equi* infection and the age of the horses ( $P>0.05$ ). Of the 92 horses, four horses (4.4%) were infected with *T. equi*, of which two horses (2.2%) were between 5-10 years old; one horse (1.1%) had less than 5 years old and another one (1.1%) was over 10 years old. In this study, three males (3.3%) and one 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).

### **Discussion**

According to the findings from molecular analysis in the current investigation, the frequency 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 IFAT, the prevalence of *T. equi* (21.5%) was lower than *B. caballi* (34.6%) which is in accordance with the results of our study (Acici *et al.*, 2008). The same result has been reported in several studies (Mujica *et al.*, 2011; Taktaz-Hafshejani and Khamesipour, 2017; Nugraha *et al.*, 2018; Tirosh-Levy *et al.*, 2021). In a study conducted using PCR in 2014 in Urmia city in Iran, the rate of infection with *B. caballi* and *T. equi* in horses was 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%, while 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*, and their results are contrary to our results (Abedi *et al.*, 2014; Kalantari *et al.*, 2022; Aziz and Al-Barwary, 2019; Moretti *et al.*, 2010; Purmehdi-Chelickdani, 2014). 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 where EP is endemic like Iran, 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; Kizilarlan *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 and Long, 2013). However, in some cases, 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 detection of these organisms but the real-time PCR method is faster, more sensitive, and more specific than conventional PCR (Shiple, 2007). It was shown that the sensitivity of the SYBR Green I real time PCR method is hundred times higher than the conventional PCR method (Park *et al.*, 2009). The real-time PCR method is able to determine the subclinical infection and the small amounts of the infection, so it will be helpful in follow-up of the treatment and eradication of the disease (Bell and Ranford-Cartwright, 2002).

The results of the present study indicated that there is 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 ten years old are similar to this study, but in the age group above ten years old, a decrease in 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 different geographical conditions and 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 (Kizilarlan *et al.*, 2015; Moretti *et al.*, 2010; Farkas *et al.*, 2013). In addition, no significant relationship was found between equine piroplasmosis infection and gender, which is

similar to the results of other studies (Malekifard *et al.*, 2014; Abedi *et al.*, 2014; Aziz and Al-Barwary, 2019).

The result of the present study indicated that there is no difference between the infection with *T. equi* and *B. caballi* and different sampling areas, and this revealed that the distribution of infection in the different cities under study in Ardabil province follows a similar pattern.

### **Conclusion**

In conclusion, the presence of infection in apparently healthy horses indicated that EP is endemic in this region and more attention should be paid for the control and treatment of carrier horses and real-time PCR can be used to detect subclinical infection and effectively control the disease.

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### **Conflict of Interest**

The authors declared no conflict of interest.

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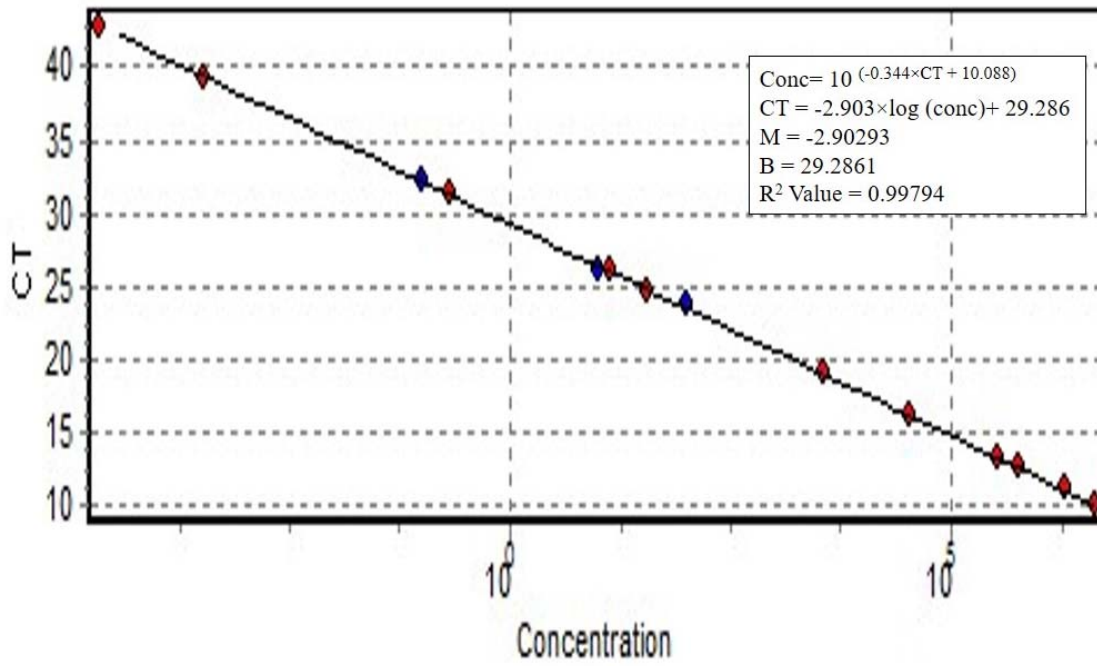
**Table 1.** Primer sequences used for the detection of *B. caballi* and *T. equi*

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

Variable	Positive samples (%)		Negative samples (%)		Total (%)		P- value		Chi-square		
	<i>B. caballi</i>	<i>T. equi</i>	<i>B. caballi</i>	<i>T. equi</i>	<i>B. caballi</i>	<i>T. equi</i>	<i>B. caballi</i>	<i>T. equi</i>	<i>B. caballi</i>	<i>T. equi</i>	
Age	1-5 years	0 (0%)	1 (1.1%)	27 (29.3%)	26 (28.2%)	27 (29.3%)	27 (29.3%)	<i>P</i> < 0.05	<i>P</i> > 0.05	6.31	0.13
	5-10 years	7 (7.6%)	2 (2.2%)	31 (33.7%)	36 (39.1%)	38 (41.4%)	38 (41.4%)				
	> 10 years	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%)	<i>P</i> > 0.05	<i>P</i> > 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%)				

	Ardabil	3 (3.3%)	1 (1.1%)	30 (32.6%)	32 (34.8%)	33 (35.8%)	33 (35.8%)		
	Nir	3 (3.3%)	1 (1.1%)	25 (27.2%)	27 (29.3%)	28 (30.4%)	28 (30.4%)		
Sampling	Namin	1 (1.1%)	1 (1.1%)	11 (12%)	11 (12%)	12 (13.1%)	12 (13.1%)		
location	Sareyn	1 (1.1%)	0 (0%)	6 (6.5%)	7 (7.6%)	7 (7.6%)	7 (7.6%)	$P > 0.05$	$P > 0.05$
	Meshginshahr	1 (1.1%)	1 (1.1%)	11 (12%)	11 (12%)	12 (13.1%)	12 (13.1%)	0.263	1.41
	Total	9 (9.8%)	4 (4.4%)	83 (90.2%)	88 (95.6%)	92 (100%)	92 (100%)		

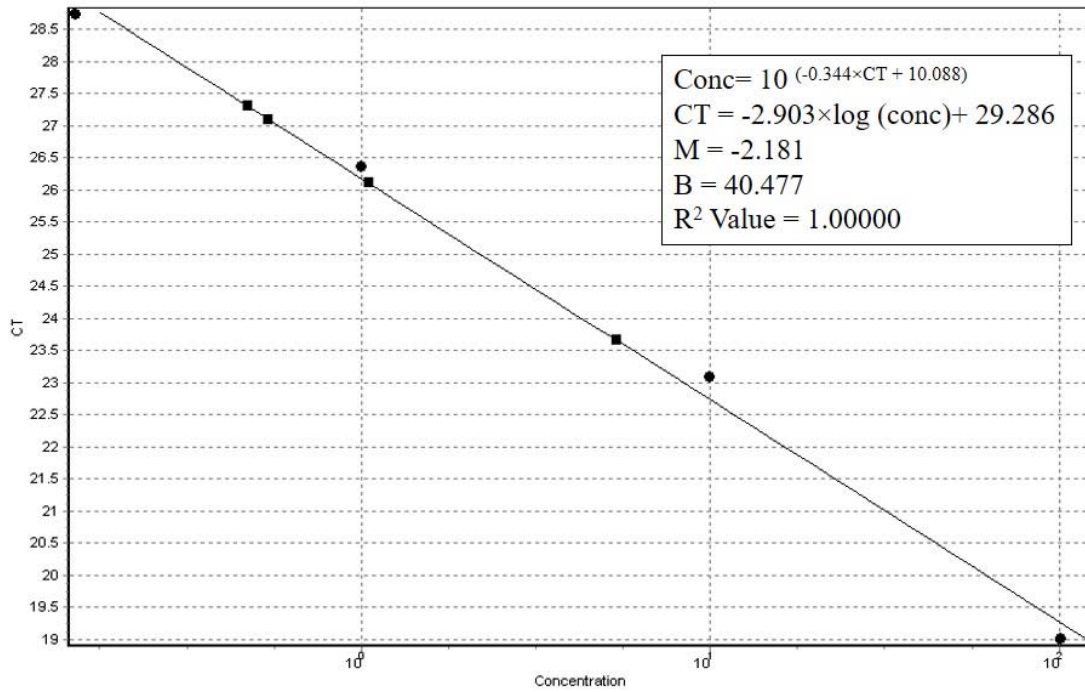
**Table 2:** Infection rate of *B. caballi* and *T. equi* and its relationship with age, gender, and sampling location



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

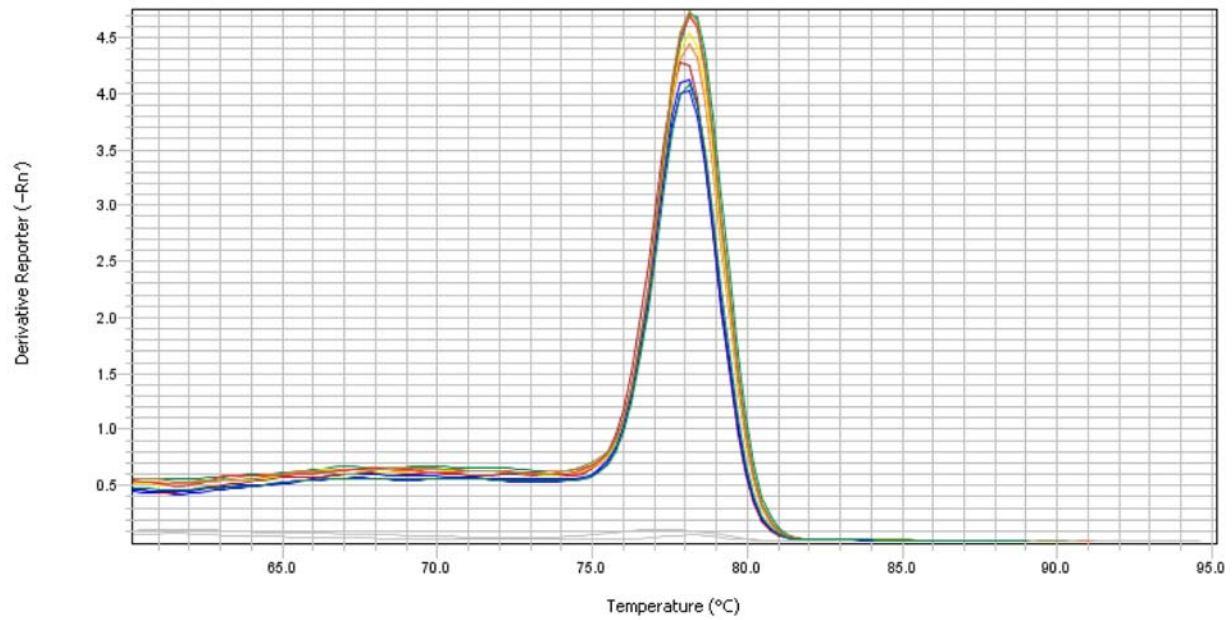
Uncorrected





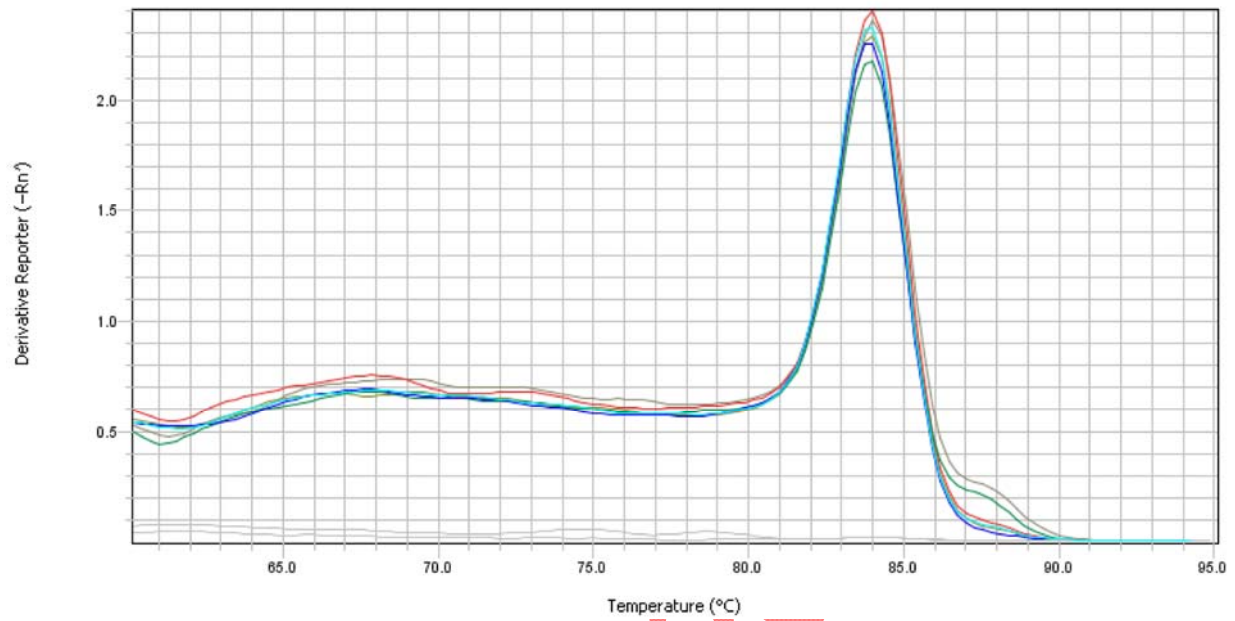
**Figure 2:** The standard curve based on CT for *T. equi*

Uncorrected



**Figure 3:** Melting curve analysis of real-time PCR products for *B. Caballi*. Gray lines are non-template control and negative control. Colorful lines include positive samples and control.

Uncorrected



**Figure 4:** Melting curve analysis of real-time PCR products for *T. equi*. Gray lines are non-template control and negative control. Colorful lines include positive samples and control.

## بررسی مولکولی بابزیا کابالی و تیلریا اکوئی در اسب‌های استان اردبیل، ایران

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چکیده:

**پیش زمینه مطالعه:** پیروپلاسموزیس اسبان یک بیماری تک یاخته‌ای خونی قابل انتقال از کنه با گسترش جهانی است که بوسیله

تیلیریا اکوئی و بابزیا کابالی ایجاد می‌شود. این بیماری در ایران بومی است و اغلب به شکل تحت بالینی ظاهر می‌شود. برای کنترل این بیماری، با شناسایی اسب‌های حامل، واکنش زنجیره ای پلیمرز زمان واقعی به عنوان یک روش مناسب مطرح شده است.

**هدف:** SYBR Green I مطالعه حاضر، شناسایی بابزیا کابالی و تیلیریا اکوئی در اسب‌های استان اردبیل، ایران با استفاده از real-time PCR است.

**مواد و روش کار:** این مطالعه از فروردین تا مرداد 1394 در پنج شهر (اردبیل، نیر، نمین، سرعین و مشگین شهر) در استان اردبیل انجام شد. نمونه‌های خون به صورت تصادفی از ورید وداچ 92 اسب به ظاهر سالم با ذکر سن، جنس و شهر محل نمونه انجام گرفت. SYBR Green I real-time PCR ژنومی استخراج و ارزیابی شد و سپس DNA گیری گرفته شد. در هر نمونه، در هر نمونه Ct هدف بر اساس مقادیر DNA آنالیز شد. غلظت Rotor-Gene Q series اطلاعات با استفاده از نرم افزار نامشخص محاسبه شد. مقادیر وقتی که بیش از 1 نانوگرم به ازای هر واکنش بودند، مثبت تلقی میشدند. برای ارزیابی ارتباط بین میزان آلودگی اسب ها و گروه‌های سنی مختلف (5-1، 10-6 و بیش از 10 سال)، جنس و محل اخذ نمونه، اطلاعات با استفاده از معنی‌دار در نظر گرفته شد.  $P < 0/05$  آنالیز شدند. مقادیر SPSS 0/05 آزمون مربع کای در نرم افزار

نتایج: در این مطالعه فراوانی اسب های آلوده به *بازیا کابالی* 9 (9/8) و *تیلریا اکوئی* 4 (4/4) بود. ارتباط معنی داری بین عفونت با *بازیا کابالی* و گروه های سنی 5-10 سال مشاهده شد. با این حال، ارتباط معنی داری بین عفونت با *بازیا کابالی* و جنس و محل اخذ نمونه وجود نداشت. به علاوه، ارتباط معنی داری بین عفونت با *تیلریا اکوئی* و گروه های سنی، جنس و محل اخذ نمونه وجود نداشت.

نتیجه گیری: وجود عفونت در اسب های به ظاهر سالم نشان دهنده بومی بودن *پیروپلاسموزیس* اسب ها در این منطقه است. بنابراین، توجه بیش تری برای درمان و کنترل اسب های حامل مورد نیاز است.

کلمات کلیدی: *بازیا کابالی*، *پیروپلاسموزیس*، واکنش زنجیره ای پلیمرز زمان واقعی، سایبرگرین، *تیلریا اکوئی*