

## Original papers

# Molecular characterization of *Theileria equi* infection in horse populations belonging to West Azerbaijan, Iran: insights into the importance of Equine Merozoite Antigen (EMA)-1 in its diagnosis

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**ABSTRACT.** The major agent of equine piroplasmiasis (EP), *Theileria equi*, contributes to significant losses in the equine industry. This study was designed to evaluate *T. equi* infection among horses from West Azerbaijan by microscopy and molecular approaches. One hundred and twenty six blood samples were collected from the jugular vein and placed in sterile tubes containing EDTA; these tubes were either used immediately for blood smears or stored at –20°C for later examination by PCR. *T. equi* was detected in 3.2% and 27.7% of the animals examined using light microscopy and PCR methods, respectively. The prevalence of *T. equi* was higher in older animals (30.4%) than young equines (24.6%). Also, the females (31%) demonstrated higher *T. equi* infection rates than the males (23.6%). Additionally, while 12 horses housed with other animals were positive for *T. equi*, 23 not housed with other animals were found to be infected. No significant difference was found between infection rate and associated risk factors (age, sex, and housing with other animals). The results confirm a relatively high prevalence of *T. equi* in horses in the study area and also suggest that Equine Merozoite Antigen (EMA)-1 could be a strong candidate to develop diagnostic methods for *T. equi* infection. Due to the importance of EP in the equine industry, and the ability of animals to be lifelong carriers of *T. equi*, accurate and early diagnosis of the disease, based on specific antigens, is critical. Diagnosis would provide basic information about its epidemiology, distribution and prevalence, especially in apparently healthy animals, and effective control and vaccine measures.

**Key words:** *Theileria equi*, Equine Merozoite Antigen (EMA)-1, PCR, West Azerbaijan

## Introduction

The apicomplexan parasite, *Theileria equi*, is believed to be the main cause of equine piroplasmiasis (EP) in domestic and wild equines, including horses, donkeys, mules and zebras. This tick-borne disease is characterized by fever, anaemia, jaundice, haemoglobinuria, pulmonary oedema, colic and occasionally death. *T. equi* infection is responsible for significant economic losses and limitations on the international transport of horses [1]. *T. equi*, depending on the presence of

tick vectors, is prevalent worldwide. Hard-bodied ticks, mainly *Hyalomma*, *Rhipicephalus* and *Dermacentor*, transmit the protozoa in their salivary glands [2]. The animals which overcome acute infection will typically remain lifetime carriers of the infection lifetime with a low grade of parasitemia [3]. Transportation stress, heavy exercise and poor nutrition may result in the recurrence of the infection and the reappearance of clinical theileriosis in equids [4]. It was confirmed that *T. equi* can be transplacentally transmitted from carrier mare to foals, [5] which may result in

abortion or neonatal death.

A decisive diagnosis requires the observation of parasites in blood smears, but parasites are generally present in very low numbers during chronic infection and cannot be detected by microscopic examination of blood smears [6]. It is noteworthy that even in cases of low parasite burden, *T. equi* can still be transmitted iatrogenically or by competent tick vectors [7]. Asymptomatic chronically infected carriers can act as reservoirs of the infection, which poses a serious challenge to controlling the spread of *T. equi* [8]. Diagnosis of subclinical infections is therefore important for the horse-racing industry in which the movement of apparently healthy horses from enzootic districts may result to an outbreak of piroplasmosis due to *T. equi* in previously disease-free areas [9].

Infections can be detected by molecular and serological methods. The polymerase chain reaction (PCR) has been applied for the detection of many *Theileria* and *Babesia* species and has been reported to have higher sensitivity and specificity compared with serological assays [10]. Various genes have been used as targets for the diagnosis of *T. equi*, one of which is Equine Merozoite Antigen (EMA)-1. EMA-1 (34 kDa) is one of the most important immunodominant surface antigens in *T. equi*, belonging to the major piroplasm surface protein (MPSP) family, and is conserved among the genus *Theileria* [11]. EMA-1 plays a significant role in the recognition of, attachment to, and penetration of host erythrocytes by parasites. It has been proven that EMA-1 expressed during the *T. equi* merozoite stage, is an important candidate for the development of effective diagnostic assays [12].

The aim of the present study was to determine the prevalence of *T. equi* using molecular techniques based on EMA-1 among horses in North-West Iran.

## Materials and Methods

**Study area.** This study was conducted in southern areas of West Azerbaijan province, located in the north-west of Iran, on the border between Iran and Iraq. The mountainous nature and desirable pastures of the area make it an excellent place to breed and keep animals, including horses, cattle and sheep. To conduct the study, 126 horses were chosen from all parts of the area and classified into groups based on sex (55 males and 71 females), age (57 were younger than or equal to three years old

and 69 were over three years old) and housing with other domestic animals (41 were housed and 85 were not). Although all the animals were born and reared in Iran, some were used to carry goods across the border because of the impassable mountain routes.

**Blood samples.** Blood samples were taken from 126 randomly-selected apparently healthy horses by jugular venipuncture and placed in sterile tubes containing EDTA; these tubes were either used immediately for blood smears or stored at  $-20^{\circ}\text{C}$  for later use in PCR. When the samples were collected, no clinical disease signs were seen or reported by owners and the animals had not been treated for *T. equi*.

**Parasitological examination.** To check for blood parasites, blood smears were prepared the same day, fixed in methanol and stained with 10% Giemsa solution in phosphate-buffered saline (PBS) at pH 7.2. Then, the slides were inspected using light microscopy (magnification 1000 $\times$ ). Smears with no parasites in 100 fields were considered negative.

**DNA extraction and PCR amplifications.** DNA was extracted from 200  $\mu\text{l}$  of each EDTA-coated tube using a DNA purification kit (Cinna Gen, Iran) according to the manufacturer's protocol. Purified DNA was stored at  $-20^{\circ}\text{C}$  until used as a template for subsequent PCR amplifications. For detection of *T. equi*, a 750 bp region of the EMA-1 gene was amplified using a forward primer (EMA-1 F) with the sequence (5'-ATAAGCTTATTATG GAGGAGA-3') and a reverse primer (EMA-1 R) with the sequence (5'-AGGGATCCTCAAATAGA GTAG-3') that was chosen based on the EMA-1 sequence (GenBank<sup>TM</sup> accession number U97169). PCR reactions were performed in a total volume of 20  $\mu\text{l}$ , containing 10  $\mu\text{l}$  of commercial Master Mix (TopBio, Slovakia), 0.5  $\mu\text{l}$  of each primer and 3  $\mu\text{l}$  of template DNA. Distilled water was used as negative control in each PCR reaction. The positive control was prepared from the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. PCR cycling comprised an initial denaturation step at  $94^{\circ}\text{C}$  for five min, followed by 34 cycles of denaturation at  $94^{\circ}\text{C}$  for one min, annealing at  $58^{\circ}\text{C}$  for 45s, extension at  $72^{\circ}\text{C}$  for one min. This was followed by a final extension step at  $72^{\circ}\text{C}$  for five min. The amplified PCR products were visualized by 1.5% agarose gel electrophoresis in TAE-buffer stained with DNA Safe stain under UV light.

**Statistical analysis.** All obtained data was

Table 1. Prevalence, confidence interval (95%), odds ratio and associated of risk factors of *T. equi* infection among horses from West Azerbaijan, Iran

Risk factors	No. of examined	Positive	Prevalence (95% CI) <sup>1</sup>	Odds Ratio	P-value
Sex					
Female	22	71	31% (23.2–38.6)	1.4	0.17
Male	55	13	23.6% (15.4–31.8)		
Age					
> 3	69	21	30.4% (22.4–38.1)	1.3	0.25
≤ 3	57	14	24.6% (16.5–32.3)		
Contact with other animals					
Yes	41	12	29.2% (21.3–37.4)	1.1	0.17
No	85	23	27.1% (19.7–34.9)		
Total	126	35	27.7%		

<sup>1</sup> Confidence Interval

analyzed using SPSS and Microsoft Excel® software (Version 20). The *Chi-square* test was applied to determine any association between risk factors and the prevalence of *T. equi*: *P*-values less than 0.05 were considered significant. The odds ratio (OR) along with 95% CI were calculated for different potential determinants of the infection.

## Results

Out of 126 examined horses, the specific *T. equi* merozoite 750 bp gene fragment (EMA-1) was detected in 35 (27.7%) horses (Fig. 1), whereas only four (3.1%) blood smears were found to be positive for *T. equi* with low parasitemia (about 0.008–0.01%) by microscopy. The prevalence of *T. equi* was higher in older animals (30.4%) than the young ones (24.6%). Also, the females (31%) showed higher infection with *T. equi* than the males (23.6%). Twelve horses housed with other animals and 23 not housed with other animals were positive for *T. equi*. No statistically significant differences were found between the presence of infection and associated risk factors: age, sex and housing with other domestic animals. All information about the prevalence of *T. equi*, confidence interval (95%), odds ratio and associated of risk factors are summarized in Table 1.

## Discussion

For years, the diagnosis of EP was a challenge for researchers and veterinarians as Giemsa-stained blood smears are ineffective at detecting low parasite burden. Several serological assays have been developed for the detection of *T. equi*, including the enzyme-linked immunosorbent assay (ELISA), the indirect fluorescent antibody test (IFAT), the complement fixation test (CFT), the Latex agglutination test (LAT) and the immunochromatographic test (ICT) [13]. However, these assays are restricted due to their antibody-detected limitation and/or potential cross-reactivity to other pathogens [14]. Molecular diagnostic tests such as PCR are more sensitive than serological tests and can detect parasite DNA from 2.5 µl blood sample with a parasitemia of 0.000001% [15]. The result obtained by microscopic examination of the blood smears in the present study revealed a low prevalence of *T. equi*, which was in agreement with data reported from Iran [16] and Brazil [3]. In spite of its high specificity, this method has very low sensitivity especially in cases of chronic and subclinical infections [17]. Diagnostic methods based on DNA amplification have been suggested as reliable tools for the detection of EP both in the acute phase of infection and in chronically infected animals [9].

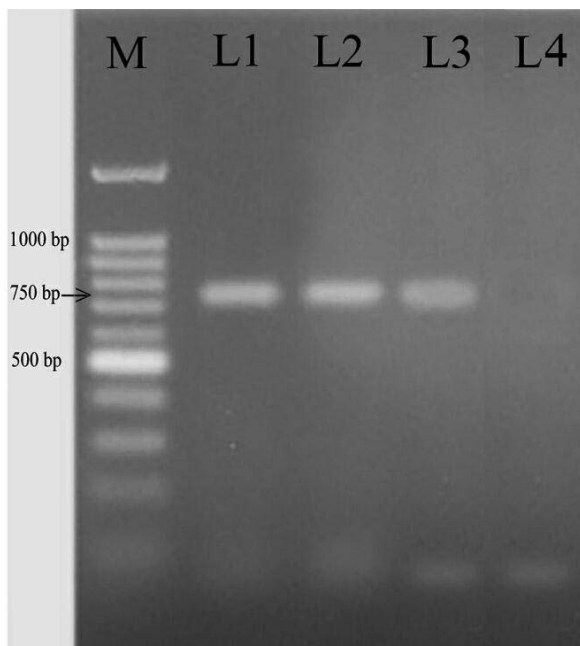


Fig. 1. PCR analysis of EMA-1 fragment with 750 bp on 1% electrophoresis gel, to detect *T. equi* infection in horses rose in West Azerbaijan, Iran  
M: 100 bp DNA Ladder; lane 1: positive control; lane 2 and 3: positive samples; lane 4: negative control

In the current study, the EMA-1 based PCR examination identified a medium prevalence of *T. equi* in horses from northwest Iran. Previous studies conducted in several Iranian states including Yazd [16], Northern Khorasan [18], West Azerbaijan [19], Khuzestan [20] and Kurdistan [21] revealed prevalence rates of 22.8%, 45%, 12.5%, 28.5% and 96.7%, respectively. Some countries neighbouring Iran have reported the prevalence of *T. equi* to range from 10% to 77% [22–25]. Studies carried out in France [26] and Egypt [27] showed prevalences of 80% and 36.4% for *T. equi*, respectively. The difference in prevalence of *T. equi* in various parts of the world, and even in different climatic zones within a country, may be ascribed to variations in sensitivity of the diagnostic tests, difference in the number and prevalence of vectors, activity of equids, management of the horses, and the presence and effectiveness of control programs [28]. In addition, the prepatent period for *T. equi* infections is 12–14 days; therefore sampling time can play a remarkable role in the detection of parasites [6].

Although, no significant relationship was identified between the prevalence of *T. equi* and the age of the equines in the present study, older equines had higher levels of *T. equi* infection than younger ones. Similar findings have previously been recorded from Iran [29], Pakistan [30] and

Turkey [31], where no significant correlation was identified between the infection and age of host. It has been reported that once infected with *T. equi*, equines remain lifelong carriers [32] and this could be a possible explanation for the higher prevalence of the infection in older equines [30]. Older animals are more susceptible to infection with *T. equi* due to increased stress and immune suppression [33].

In the present study, despite the higher prevalence of infection observed in males compared to females, no significant association ( $p > 0.05$ ) was observed between the sex of equines and *T. equi* infection. This finding is concordant with data reported from Switzerland [34]; however, some studies have found females to have a higher prevalence than males [35]. This difference might be due to the differences in the level of care, grooming, the purpose of keeping the animal, and sample size of the study. Male horses are mainly used for working, riding and other sports-related activities; consequently, they are more prone to stress and immune suppression [36]. Differences in the susceptibility of males and females to protozoan infections have been ascribed to levels of sex hormones and activity patterns. However, there is no public consensus regarding the role of host sex in EP, as some studies show the sex of the host to have a significant effect on the infection rates [37] while others do not report any correlation [27]. In the current study, although it was not statistically significant, the horses housed with other domestic animals, had a greater prevalence of *T. equi* infection than those living alone. Similarly, previous studies reported that contact with other domestic animals could be an important factor for *T. equi* infection via introduction of tick vectors [30,38,39].

Despite lacking obvious clinical signs, infected horses can serve as parasite carriers that can be a potential source for maintaining *T. equi* in the population. This can cause transmission of the parasite to different parts of the country and facilitate the spread of EP. As parasitemia generally decreases below 1% for the subclinical forms or asymptomatic carriers [8], it cannot be detected by typical light microscopy methods. Hence, accurate and early diagnosis of *T. equi* is critical for providing basic information about its prevalence, especially in apparently healthy animals, as well as its epidemiology and distribution, and for effective control and vaccine measures.

Equine Merozoite Antigens (EMA)-1 and 2, are

immunodominant proteins which play pivotal roles in the pathogenicity and the survival of equine piroplasms [40]. These proteins are targeted by the host immune system during the host-parasite interaction, indicating their importance for use as a diagnostic reagent and vaccine target. A highly conserved surface protein, EMA-1, expressed by merozoites of *T. equi* has been used as a diagnostic antigens. This protein is not expressed in any of the merozoite developmental stages. There are differences between EMA-1 sequences (about 10%) and the antigenicity of *T. equi* strains isolated from various countries [11].

To our knowledge, this is the first molecular study of *T. equi* infection based on EMA-1 in horses breeding in Iran. PCR based on EMA-1 has been used to detect a considerable number of *T. equi* carriers among horses imported into Mexico [41]. EMA-1 PCR has been found to be able to detect the parasite in blood with a corresponding parasitemia of 0.000008% or 0.000006%, equal to six infected cells out of 108 erythrocytes [42,43]. Also, it has been suggested that the use of EMA-1, improves the sensitivity and specificity of PCR assays and may assist in the improved detection and diagnosis of equine piroplasmiasis [44].

The results presented in the current study supports that EMA-1 is a strong tool for molecular diagnosis of *T. equi*. Given the importance of horse breeding in various parts of Iran, especially in the study area, and the economic losses caused by *T. equi*, a more sensitive diagnostic tool is needed; in the present study a clear difference was revealed between positive and negative controls with the use of PCR assay based on *T. equi* EMA-1.

Our findings suggest further molecular and serological studies based on the surface proteins of *T. equi*, especially EMA-1, are needed to design a proper diagnostic method and a vaccine against this parasite.

### Acknowledgements

We wish to thank all the horse owners to donate their animals to the current study. Also, the authors gratefully appreciate Veterinary Unit of Piranshahr for the assistance. The present study was financially supported by Shahid Chamran University of Ahvaz.

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Received 07 July 2017

Accepted 10 November 2017