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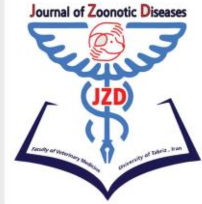

Molecular insights into *Trypanosoma evansi* prevalence in Dromedary camels of Northern Iran

Mohammad Fatemi[1], Gholamreza Mohammadi[1*], Mehran Ghaemi [2], Mohammad Azizzadeh[1], Mohammad Sadegh Golvajouei[3]

[1]Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[2]Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[3]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Iran

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Abstract
Camel trypanosomiasis, caused by various haemoprotozoan parasites, is a devastating disease with severe health impacts. *Trypanosoma evansi*, the most prevalent parasite in camels, causes surra disease, transmitted mechanically by biting flies without an intermediate host. Clinical manifestations include intermittent fever, anemia, loss of body condition, edema, and abortion in infected animals. This study utilized real-time PCR to detect *T. evansi* in dromedary camels in Golestan province, northern Iran. Using random cluster sampling, 48 blood samples were collected from camels in four counties: Gonbad-e-Kavoos, Kalaleh, Agh-ghala, and Gomishan. Real-time PCR detected *T. evansi* in 6 samples (12.5%; 95% CI: 3.2-21.8). In Gonbad-e-Kavoos, Agh-ghala, and Kalaleh, 2 out of 12 samples (16.6%) tested positive in each county, while no positive samples were found in Gomishan. High sensitivity and specificity diagnostic techniques are crucial for detecting and controlling the disease. This study confirms the prevalence of *T. evansi* in Golestan province and demonstrates the utility of real-time PCR for its detection and control.

Introduction

Blood parasites pose significant challenges to the camel farming industry, leading to decreased milk production, weight loss, treatment costs, abortion, reduced fertility, and death of infected camels, which can cause substantial economic losses for breeders (1-3). *Trypanosoma evansi* (*T. evansi*), the most important and prevalent protozoan disease in camels, causes surra. This widespread parasite can

***Corresponding author:** gmohamad@um.ac.ir
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infect various livestock, most frequently *Equidae*, *Camelidae*, and buffaloes (4).

Cattle, indigenous buffaloes, and numerous wild animals can serve as reservoirs for this parasite in *Camelidae* and *Equidae*. *T. evansi* is mechanically transmitted, most frequently by *Tabanus* and *Stomoxys* flies. It could be present in the blood and lymphatic system and infiltrate the central nervous system and joints (5, 6). *Camelidae* can develop acute, subacute, and chronic forms of the disease. The primary symptoms of the acute form are fever and severe anemia, with the parasite present in the blood, potentially causing rapid death. The subacute form is characterized by fever and edema (in the muzzle, chest, scrotum, and limbs) and can be fatal.

T. evansi causes chronic disease in *Camelidae*, characterized by weight loss, reduced hump size, intermittent fever, general muscle weakness (especially in the posterior limbs), decreased thirst tolerance, pale mucosal membranes, anemia, edema (particularly abdominal edema), and occasionally diarrhea. Conventional parasitological diagnostic techniques have low sensitivity and are only helpful in the acute form of the disease when parasitemia levels are high. Serologic tests are applicable in areas where other *Trypanosoma* species are absent due to their high sensitivity but low specificity (3, 7-9).

Polymerase chain reaction (PCR) has higher sensitivity and specificity for *T. evansi* diagnosis than other diagnostic methods (10) and is recommended for precise diagnosis in *T. evansi* survey and control programs (11). Real-time PCR has been commonly used to identify blood parasite infections in malaria, babesiosis, and theileriosis, proving an effective and appropriate technique (12).

In the northern province of Golestan, where the climate is semi-arid, pastures are poor, and the economic situation is precarious, there is an increasing trend toward camel husbandry. This primary farming choice within Golestan is a traditional practice for superior milk and meat production, bringing more attention to camel diseases. Due to the paucity of studies on the prevalence of *T. evansi* in Iranian camels using real-time PCR (13), this study aims to determine the prevalence of *T. evansi* in camels of Golestan province and highlight the importance of early diagnosis in reducing losses caused by this protozoan.

Materials and methods

Study area

Golestan province, located in northern Iran, spans 54° to 56° east longitude and 36.30° to 38.15° north latitude. It is bordered by Mazandaran, Semnan, and North Khorasan provinces, Turkmenistan, and the Caspian Sea to the west, south, east, north, and northwest (Fig. 1). Golestan features three distinct climates: mountain, temperate, and semi-arid. This research was conducted in the counties of Gonbad-e-Kavoos, Kalaleh, Gomishan, and Agh-ghala. The average annual precipitation in these counties is 425.4 mm. Golestan ranks third in camel husbandry in Iran, with a population exceeding 8,000 camels.

Sampling

The sample size was determined using the formula $n = Z^2 \times P_{exp} (1-P_{exp})/d^2$, where (n) is the sample size, P_{exp} denotes the expected prevalence, and (d) is the desired absolute precision (14, 15). Z is the normal deviation (1.96) at a 95% confidence level. Based on similar studies, the expected prevalence using the PCR method is 2.1% (16), with (d) set at 0.05, resulting in a sample size of 32.

This cross-sectional study was conducted from August to September 2019, using random cluster sampling to collect 48 camel blood samples from four counties in Golestan province (12 from each

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county) (14). The selected counties were Gonbad-e-Kavoos, Kalaleh, Gomishan, and Agh-ghala. All sampled camels were female and over two years old. Blood samples were collected aseptically from the jugular vein into tubes containing the anticoagulant EDTA, with the date, sample number, and sampling region recorded on the labels. The samples were transported in a cold state to a laboratory and stored at -70°C until DNA extraction and real-time PCR analysis.

Fig. 1. Golestan province in the north of Iran, where the study area

DNA Extraction

DNA from blood samples was extracted using a DNA extraction kit (Betagen, Iran) according to the manufacturer's instructions. The quality of the extracted nucleic acids was evaluated by electrophoresis on a 1.5% agarose gel (17). The quantity of extracted DNA was measured using a NanoDrop spectrophotometer (BioTek Instruments, USA).

Real-time PCR

A primer pair for the Rode Trypanozoon antigen type 1.2 Variable Surface Glycoprotein (*RoTatVSG*) gene of *T. evansi* was used in real-time. Primer pair sequences were TeRoTat920m 5′-CTGAAGAGGTTGGAAATGGAGAAG- 3′ and TeRoTat1070m 5′-GTTTCGGTGGTTCTGTTGTTGTTA -3′ (18). For each sample, a total volume of 20 µl was mixed, containing 10 µL of 2X SYBR Green master mix (Ampliqon, Denmark), 5.2 µL of DNase-free water, 4 µL of DNA template, and 0.4 µL of each primer (10 µM). For no-template control (NTC) and positive controls, DNase-free water and DNA of two microscopically confirmed positive samples (Fig. 2) were added to the microtubes containing the prepared master mix, respectively. Real-time PCR was performed using a LightCycler® 96 (Roche, Germany). The thermal amplification protocol was 10 min at 95°C, followed by 45 cycles of 95°C for 10 s, 62°C for 30 s, and 72°C for 20 s. Finally, a melting curve analysis was conducted to determine the specificity of the real-time PCR products.

Standard Curve Plotting

To determine the efficiency of the real-time PCR assay, a standard curve was plotted using the decimal serial dilution of extracted DNA from a

positive sample. The standard curve was generated by plotting the threshold cycle values (Ct value) against the log concentrations of copy numbers using LightCycler® 96 software.

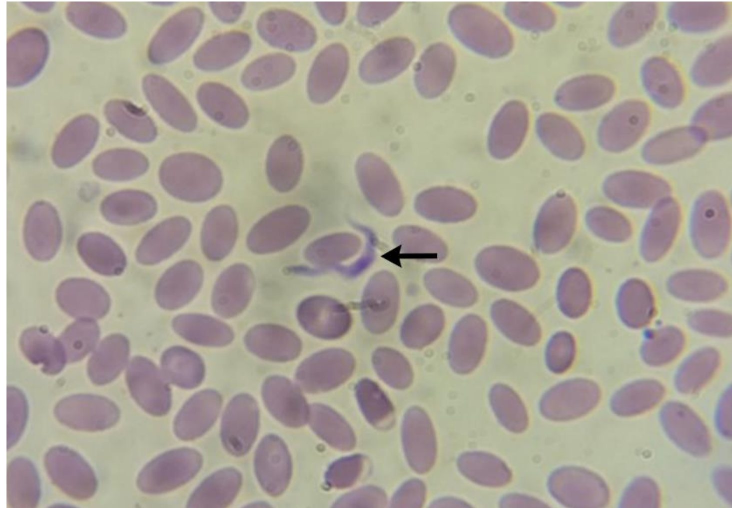

Fig. 2. Microscopic picture of Giemsa-stained blood extension showing a *T. evansi* indicated by a black arrow

Results

DNA samples were subjected to a SYBR Green real-time PCR test to detect *T. evansi*. The real-time PCR graphs (Supplementary Figure 1) show precise sigmoid curves for positive samples, while negative samples show no upward rise. Melting curve analysis confirmed that the positive results corresponded to a single desired PCR product. As shown in Supplementary Figure 2, melting peaks at 84.5°C indicate the accuracy and specificity of the test. Also, the efficiency of the real-time PCR test was determined to be 85% (Supplementary Figure 3).

Out of 48 blood samples tested for *T. evansi* using the SYBR Green real-time PCR test, 6 samples were positive. Thus, the prevalence of Surra disease in camels in Golestan province was determined to be 12.5% (95% confidence interval: 3.2-21.8). The disease prevalence rates in Gonbad-e-Kavoos, Kalaleh, and Agh-ghala counties were 16.6%, while no positive samples were detected in Gomishan (Figure 3).

Discussion

Surra has a global distribution and is one of the primary reasons for the decline of camel products in Iran. The reported prevalence of *T. evansi* varies across different regions of Iran, possibly due to the diagnostic methods employed and/or the study locations (2). Sazmand and Joachim (2017) reported varying levels of *T. evansi* prevalence in various districts, with the lowest in Tehran and Najafabad (0.0%) and the highest in Zabol (19.47%) (19).

In limited studies, real-time PCR has been used to detect *T. evansi* infection in camels. Ghaemi et al. (2019) investigated the prevalence of *T. evansi* in the provinces of North Khorasan (10.25%), Razavi Khorasan (9.43%), and South Khorasan (1.6%) using a real-time PCR test. This study revealed that the average prevalence of surra disease in northeast Iran was 6.5%. They concluded that the varying prevalence of *T. evansi* in these provinces could be attributed to annual rainfall and vector fly activity (18). Golestan province, located west of North Khorasan, has a higher mean annual precipitation than Khorasan provinces. The present study reported a *T. evansi* prevalence of 12.5% in Golestan province, higher than the prevalence

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reported by Ghaemi et al. (2019) for North Khorasan province (The western neighbor of Golestan province), suggesting that rainfall may affect the prevalence of Surra. Bahari et al. (2021) reported an 8% prevalence of *T. evansi* in camels of Qom province, which receives less annual precipitation than Golestan (20). Khosravi et al. (2011) conducted a parasitological microscopic study in Rafsanjan County, Kerman Province, finding a 2.1% prevalence of *T. evansi* confirmed via PCR testing (16). Sazmand et al. (2011) reported a 15.5% prevalence of *T. evansi* in Yazd province using a parasitological microscopic technique (21). Ahmadi Hamedani et al. (2014) examined 21 camels in Semnan province, finding a single case (4.76%) of *T. evansi*. This study also revealed that affected camels' red blood cell parameters were significantly lower than those of healthy camels (22). The difference in reported prevalence between Ahmadi Hamedani et al.'s study and the present investigation may be attributable to different diagnostic methods and annual rainfall. Most research on the prevalence of *T. evansi* in Iran's camels has been conducted in the central and southern regions, where camel breeding is common (Table 1).

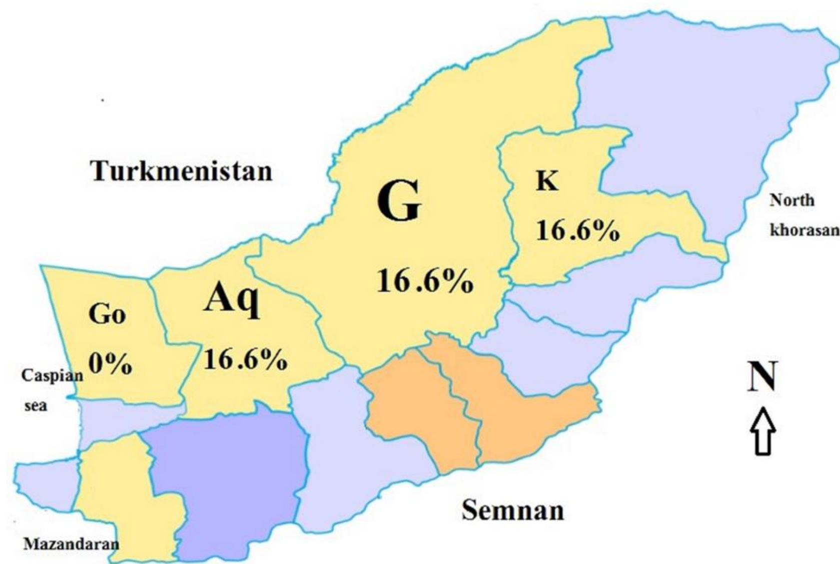

Fig. 3. Prevalence of *Trypanosoma evansi* investigated in Gomishan (Go), Agh-ghala (Aq), Gonbad-e-Kavoos (G), and Kalaleh (K) four counties of Golestan province in Iran.

Mirshekar et al. (2019) analyzed blood samples from 370 dromedaries in Sistan-va-Baluchestan province using the micro-hematocrit centrifugation technique (MHCT) and PCR. The prevalence of *T. evansi* through MHCT was 11.89%, while PCR revealed a prevalence of 31.35%, much higher than other studies. Most positive samples were identified in the province's northern region, which receives the least annual precipitation (39). This finding may be due to the illegal transportation of camels from Pakistan to Iran. Sazmand et al. (2016) found a 0.5% prevalence of *T. evansi* in camels of Sistan-va-Baluchestan and Kerman provinces using both microscopic and PCR methods. They concluded that differences in the study population, such as host age, seasonal migration length, and sampling season, could account for these varying results (36). Comparing the results of studies on the prevalence of *T. evansi*, it appears that the diagnostic method employed may influence the reported results. Fernández et al. (2009) compared parasitological and PCR tests for diagnosing *T. evansi* in

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experimentally infected mice, demonstrating that PCR could detect parasites in the blood before parasitological methods. This suggests that PCR is suitable for diagnosing infections in apparently healthy animals (40). Tehseen et al. (2015) showed that PCR has greater sensitivity and specificity than microscopic examination for trypanosomiasis detection and identification, making it more valid for prevalence studies (41).

Table 1. The prevalence of *Trypanosoma. evansi* in camels in different regions of Iran using different diagnostic methods (* LM refers to the light microscopy)

Province(s)	Camel number	Prevalence	Diagnostic tool(s)	Reference and published year
Tehran	127	9.5%	LM*	[23]
Tehran	196	7.7%	LM	[21]
Isfahan	37	5.4%	LM	[25]
Bushehr	333	9.5%	LM	[26]
Kerman	60	1.6%	LM	[27]
Fars	285	14%	LM	[28]
Fars	100	9%	PCR	[29]
Razavi Khorasan	262	0.58%	LM	[30]
Sistan-va-Balouchestan	113	19.5%	LM	[31]
Yazd	110	15.5%	LM	[21]
Yazd	117	3.4%	LM+PCR	[32]
Semnan	21	4.8%	LM	[22]
Isfahan	278	1.1%	PCR	[33]
Kerman	95	2.1%	LM+PCR	[16]
Isfahan & Yazd	227	3.96%	LM	[34]
Tehran	100	0.0%	LM	[35]
Kerman & Sistan-va-Balouchestan	200	0.5%	LM+PCR	[36]
Khuzestan	300	19%	LM+PCR	[37]
Sistan	113	6.2%	LM+PCR	[38]
Sistan-va-Balouchestan	370	11.89%	MHCT	[39]
		31.35%	PCR	
North Khorasan	39	10.25%	Real-Time PCR	[18]
Razavi Khorasan	53	9.43%		
South Khorasan	60	1.6%		
Qom	100	8%	PCR	[20]

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In the present study, the prevalence of *T. evansi* was estimated to be 12.5% in Golestan province through real-time PCR. *T. evansi* may result in economic losses for the camel industry, including costs associated with treating infected animals, weight loss, abortion, infertility, and mortality (1-3). Given the importance of camel husbandry to the rural economy in Golestan province, early diagnosis of surra disease using sensitive methods, such as real-time PCR, may help minimize economic losses. Additionally, real-time PCR may aid in evaluating disease transmission from animal reservoirs to Camelidae, Equidae, and potentially humans (mainly in rural areas) (42). Employing sensitive diagnostic methods for Surra also facilitates the evaluation of drug efficacy in treating animals. As horse breeding is highly prevalent in Golestan province, further research on other host species and carriers, as well as risk factors associated with *T. evansi*, is recommended to develop effective preventive policies.

Conclusions

This study highlights the significant prevalence of *Trypanosoma evansi* in dromedary camels in Golestan province, northern Iran, with a detection rate of 12.5% using real-time PCR. The findings underscore the importance of employing sensitive and specific diagnostic methods, such as real-time PCR, for early detection and control of surra disease. The economic impact of *T. evansi* on the camel farming industry, including treatment costs, weight loss, abortion, infertility, and mortality, necessitates prompt and accurate diagnosis to mitigate losses. The study also suggests that environmental factors, such as annual rainfall, may influence the prevalence of *T. evansi*, indicating the need for further research on the epidemiology of this parasite. Given the critical role of camel husbandry in the rural economy of Golestan province, implementing effective diagnostic and control measures is essential for sustaining camel health and productivity. Future research should focus on other potential host species, carriers, and risk factors associated with *T. evansi* to develop comprehensive preventive strategies.

Acknowledgments

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Ethical approval

The project was conducted according to the ethical principles and the national norms and standards for conducting Medical Research in Iran (IR.UM.REC.11398. 041).

Conflict of interest statement

We declare that we have no conflict of interest.

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