

## Molecular detection of *Trypanosoma evansi* based on ITS1 rDNA gene in *Camelus dromedarius* in Sistan Region, Iran

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**Abstract.** Trypanosomiasis is a disease caused by a flagellate protozoon called *Trypanosoma* and can be mechanically transmitted by vectors to humans and animals. Various species of *Trypanosoma* are found in livestock and poultry, which include *Trypanosoma evansi*, *T. brucei*, *T. vivax* and *T. congolense*. The camel is the most sensitive livestock for *T. evansi*, so the exact identification of infection is very important for epidemiological studies and the design of control programs. The present study was conducted with the aim of molecular detection of camel trypanosomiasis in the Sistan region in 2015. Previous studies have shown that internal transcribed spacer one (ITS1) of the ribosomal DNA is a reliable genetic marker for carrying out systematic molecular studies of trypanosomes. In order to investigate infections of camels with *T. evansi*, a total of 113 blood samples were collected randomly and the presence of parasites in each sample was evaluated using the microscopic method and polymerase chain reaction (PCR) test. Genomic DNA was extracted and the ITS-1 was amplified by PCR. In comparison to the nucleotide sequence obtained with the sequences recorded in GenBank, it was determined that there is a 99% homology with the recorded sequence of *T. evansi*. The obtained sequence was registered in Gen Bank with kx900449 code. The *T. evansi* sequences from different countries such as India, Taiwan, Thailand, the Philippines, China and Argentina and etc., were extracted from the Gene bank and aligned using the ClustalW2 sequence alignment tool and MEGA software. In this study the prevalence of *T. evansi* infection using the molecular method was 6.19% and no positive samples were found by microscopic observation.

### INTRODUCTION

*T. evansi* is an important haemoparasitic protozoa that infects most mammals but causes more severe disease in camels and horses (Viseshakul *et al.*, 1990). The disease caused by *T. evansi* is known as Surra, which is particularly important in South America, Asia and Africa (Davison *et al.*, 1999; Omanwar *et al.*, 1999). Surra is one of the most important diseases in camels and causes economic damage in different countries. The chronic form of the disease is usually seen and exhibits clinical signs as anaemia, loss of weight and abdominal oedema. The acute form is characterized by fever and the presence of trypanosomes in

the blood vessels of the peripheral blood vessels and deep vessels. The animal loses weight and the humps break down quickly. Oedema sometimes forms on the abdominal area and the sexual organ pods. Abortion, reduced fertility and sometimes death in absence of treatment are observed as well in infected animals (Viseshakul *et al.*, 1990). Sistan and Baloochestan province is considered as one of the suitable habitats of this livestock in the country due to climatic conditions and camel breeding is important, as is the cultivation of other livestock. Nevertheless, there are few studies on trypanosomiasis in this region. Typically, microscopic, serologically and molecular techniques are used to identify pathogenic

protozoa. But despite the existence of high diagnostic tests, there are still problems with the detection methods for trypanosomiasis. For this reason, the polymerase chain reaction (PCR) is commonly used which is reliable and high-sensitivity diagnostic test. Previous studies have shown that the sequence of ITS-1 ribosomal DNA (rDNA) is a reliable genetic index for molecular studies of trypanosomes. According to the mentioned materials, since the molecular study of *T. evansi* in the camels of Sistan has not been carried out, this study was conducted to investigate the presence of infected camels with *T. evansi* in the region and estimate the prevalence of the infection. The findings of this study are the basis for more molecular studies on trypanosomiasis in the province, and further research is needed to understand enough about the distribution and frequency of Surra in this region.

## MATERIALS AND METHODS

To investigate the infection of camels with *T. evansi*, during the spring and summer of 2016, a total of 113 blood samples were collected from suspicious animals with clinical signs as anaemia and loss of weight, in different areas of Sistan, and from each camel a thin layer blood smear was prepared. Blood samples were collected in sterile numbered tubes containing EDTA solution, and then the presence of parasites in each sample was determined with PCR and microscopic examination. In the laboratory, blood samples were fixed with pure methanol and stained with Giemsa for 20 minutes. After staining, the blood smears were microscopically examined (100x) for the presence of parasites.

### DNA extraction

DNA extraction was performed using a special kit (MSBT Iran DNA Extraction Kit). All DNA samples were stored at -20°C before they were used. The PCR reaction was then performed using the primers of the gene with this sequences TEF1: 5'CCGGAAGTT CACCGATATTG 3' and TER1: 5'TGCTGC

GTTCTTCAACGAA 3', the PCR product was 480 bp (Salim *et al.*, 2011). The PCR was performed on 50µl reaction volumes including, 4µl extracted DNA, 25µl Taq DNA polymerase 2× Master Mix (Pishgam Company, Iran), 50 pmol of each primer and sterile distilled water up to 50 ml in automated Thermocycler with the following program: 2 min incubation at 95°C to denature double strand DNA, 35 cycles of 30 s at 95°C, 30 s at 58°C (annealing step), 1 min at 72°C, and this was followed by final extension step at 72°C for 5 min. The PCR products were also electrophoresed through a 1.8% agarose gel to assess the presence of a special band of *T. evansi* (480 bp) (Fig. 1). Negative control (no template) was always run simultaneously with our PCR experiments.

Finally, Positive PCR product, with specific primers, was sent to Pishgaman Company for sequencing. 25µl of PCR product with 15µL of Forward and Reverse primers were also sent in separate tubes to Pishgam Company to determine the nucleotide sequence and sequencing was performed on both sides of the DNA strand. The nucleotide sequence was analysed by Chromas software.

ITS-1 sequences were then used to compare and calculate similarity scores. In this step, ITS-1 sequences of some Trypanosome isolates from other parts of Iran and other countries were also included. The ClustalW sequence alignment tool and Mega7 were used for all alignments and calculation of similarity scores. The phylogenetic trees were constructed based upon the ITS-1 sequences from Sistan.

## RESULTS

To investigate the infection of camels with *T. evansi*, a total of 113 blood samples were collected from animals **suspected to be infected** and a thin layer blood smear was prepared for each one of them. PCR analysis using TEF1 and TER1 primers showed that 7 out of 113 samples (6.19%) were infected with Trypanosome parasites; the 480 bp positive bands were clearly indicative of

Table 1. The names of the countries used for alignment with ClusterW technique

Parasite species	The code in the gene bank	Country name	Similarity Percentage
<i>Trypanosoma evansi</i>	KX900449	Present study	99
<i>Trypanosoma evansi</i>	KR858268	India _ camel	99
<i>Trypanosoma evansi</i>	AY912277	Thailand _cow	99
<i>Trypanosoma evansi</i>	AY912279	Thailand _deer	99
<i>Trypanosoma evansi</i>	FJ712712	China _ mule	99
<i>Trypanosoma evansi</i>	U75507	Thailand	99
<i>Trypanosoma evansi</i>	FJ712715	China Buffalo	99
<i>Trypanosoma evansi</i>	AB551920	Egypt _ camel	99
<i>Trypanosoma brucei</i>	D89527	Taiwan	99
<i>Trypanosoma evansi</i>	AY912270	Thailand _ Buffalo	99
<i>Trypanosoma evansi</i>	AY912278	Thailand _deer	99
<i>Trypanosoma evansi</i>	KR858270	India-cow	98
<i>Trypanosoma evansi</i>	FJ712716	China-camel	98
<i>Trypanosoma evansi</i>	KR858269	India-donkey	98
<i>Trypanosoma evansi</i>	KC988260	Argentina	99
<i>Trypanosoma evansi</i>	HQ593645	Philippines	98

the presence of parasites in camels' blood (Fig. 1), but nothing was found in the microscopic observation of 113 blood smears.

The sequences obtained in this study were compared using BLAST software with other GenBank sequences for *T. evansi*. It was found that there was a 99% consistency between the sequence obtained in this study and that of *T. evansi*. The sequence of the present study was registered in the Gen Bank with kx900449 code. Then the *T. evansi* sequences from different countries such as India, Taiwan, Thailand, the Philippines, China, Argentina and, etc., were extracted from the Gene Bank and aligned with ClustalW in the Mega7 software (Fig. 2).

The phylogeny tree was drawn using sequences derived from this research and sequences derived from the GenBank. The tree was drawn using the Mega 7 software (Fig. 4).

Species studied were 99% similar to *T. evansi* in China (FJ712712, FJ712715, FJ712716), Egypt (AB551920), Thailand (U75507, AY912270, AY912277, AY912278, AY912279), India (KR858268, KR858269, KR858270), Argentina (KC988260), Taiwan (D89527).

## DISCUSSION

Sistan and Baluchestan Province is one of the suitable camel habitats in Iran. However, few studies have been conducted on trypanosomiasis in this region. Based on the problems encountered in the diagnosis of *T. evansi*, the early detection of infection not only plays an important role in epidemiology of disease and livestock health, but also it is economically important (Taylor *et al.*, 2008). In the present study, the camel infection with *T. evansi* was investigated in Zabol. Detection of infection usually occurs by finding the flagellate in the blood smear under the microscope. It is not always possible to find an organism even in symptomatic infections, which may be due to low levels of infection and even when the symptoms have increased, the trypanosomes may still not be detected in the blood. In the present study, no positive samples were found in microscopic observations. Miranzadeh examined thirty-seven slides of camel blood in Najaf Abad slaughterhouse to find *T. evansi*. Only two cases were observed positive and one case was infected with *Dipetalonema evansi* (Miranzadeh, 1994). In a survey conducted in 2007, of 100 blood

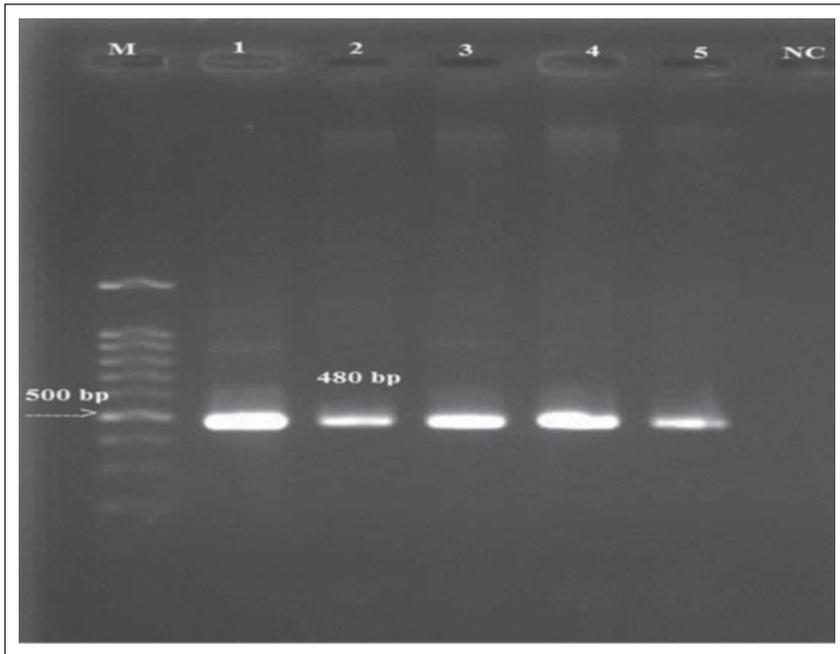


Figure 1. The band related to positive specimens of *T. evansi* (N represents negative control and M represents the marker, the weight of the bands is about 480bp).



Figure 2. Alignment with Version Mega 7 software, similar points marked with stars.

KR858268	A	A	G	T	C	G	T	A	A	C	A	A	G	G	T	A	G	C	T	G	T	A	G	G
AY912277	A	A	G	T	C	G	T	A	A	C	A	A	G	G	T	A	G	C	T	G	T	A	G	G
AY912279	A	A	G	T	C	G	T	A	A	C	A	A	G	G	T	A	G	C	T	G	T	A	G	G
FJ712712	A	A	G	T	C	G	T	A	A	C	A	A	G	G	T	A	G	C	T	G	T	A	G	G
U75507	A	A	G	T	C	G	T	A	A	C	A	A	G	G	T	A	G	C	T	G	T	A	G	G
FJ712715	A	A	G	T	C	G	T	A	A	C	A	A	G	G	T	A	G	C	T	G	T	A	G	G
AB551920	A	A	G	T	C	G	T	A	A	C	A	A	G	G	T	A	G	C	T	G	T	A	G	G
D89527	A	A	G	T	C	G	T	A	A	C	A	A	G	G	T	A	G	C	T	G	T	A	G	G
AY912270	A	A	G	T	C	G	T	A	A	C	A	A	G	G	T	A	G	C	T	G	T	■	G	G
This study	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
KR858268	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
AY912277	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
AY912279	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
FJ712712	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
U75507	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
FJ712715	G	A	T	G	A	G	G	G	A	T	G	■	T	A	■	A	■	A	T	A	G	T	T	C
AB551920	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
D89527	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
AY912270	G	A	T	G	A	G	G	G	A	T	G	■	T	A	■	A	■	A	T	A	G	T	T	C
AY912278	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
KR858270	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
FJ712716	G	A	T	G	A	G	G	G	A	T	G	■	T	■	T	A	■	A	T	A	G	T	T	C
KR858269	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
KC988260	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
HQ593645	G	A	T	G	A	G	G	G	A	T	G	■	T	A	T	A	■	A	T	A	G	T	T	C
This study	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
KR858268	T	G	T	T	A	T	T	T	T	C	T	A	■	T	A	T	G	T	A	T	G	T	G	T
AY912277	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
AY912279	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
FJ712712	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
U75507	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
FJ712715	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
AB551920	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
D89527	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
AY912270	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
AY912278	T	G	T	T	A	T	T	T	T	C	T	A	■	T	A	T	G	T	A	T	G	T	G	T
KR858270	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
FJ712716	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
KR858269	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
KC988260	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
HQ593645	T	G	T	T	A	T	T	T	T	C	T	A	T	■	A	T	G	T	A	T	G	T	G	T
AY912278	A	T	A	C	A	G	T	A	G	T	■	A	T	A	A	C	A	C	A	G	A	G	A	A
KR858270	A	T	A	C	A	G	T	A	G	T	A	A	T	A	A	C	A	C	A	G	A	G	A	A
FJ712716	A	T	A	C	A	G	T	A	G	T	A	A	T	A	A	C	A	C	A	G	A	G	A	A
KR858269	A	T	A	C	A	G	T	A	G	T	A	A	T	A	A	C	A	C	A	G	A	G	A	A
KC988260	A	T	A	C	A	G	T	A	G	T	A	A	T	A	A	C	A	C	A	G	A	G	A	A
HQ593645	A	T	A	C	A	G	T	A	G	T	A	A	T	A	A	C	A	C	A	G	A	G	A	A
This study	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
KR858268	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
AY912277	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
AY912279	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
FJ712712	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
U75507	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
FJ712715	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
AB551920	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
D89527	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
AY912270	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
AY912278	A	■	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
KR858270	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
FJ712716	A	■	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
KR858269	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
KC988260	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
HQ593645	A	T	A	T	A	T	A	T	T	T	A	T	G	T	A	T	A	T	A	T	G	C	T	A
KR858270	T	G	G	C	T	T	C	C	T	A	T	T	T	C	G	T	T	G						
FJ712716	T	G	G	C	T	T	C	C	■	A	T	T	T	C	G	T	T	G						
KR858269	T	G	G	C	T	T	C	C	T	A	T	T	T	C	G	T	T	G						
KC988260	T	G	G	C	T	T	C	C	T	A	T	T	T	C	G	T	T	G						
HQ593645	T	G	■	C	T	T	C	C	T	A	T	T	T	C	G	T	T	G						

Figure 3. Sampling alignment using the Mega7 software ClustalW method (colour points show the genetic difference).

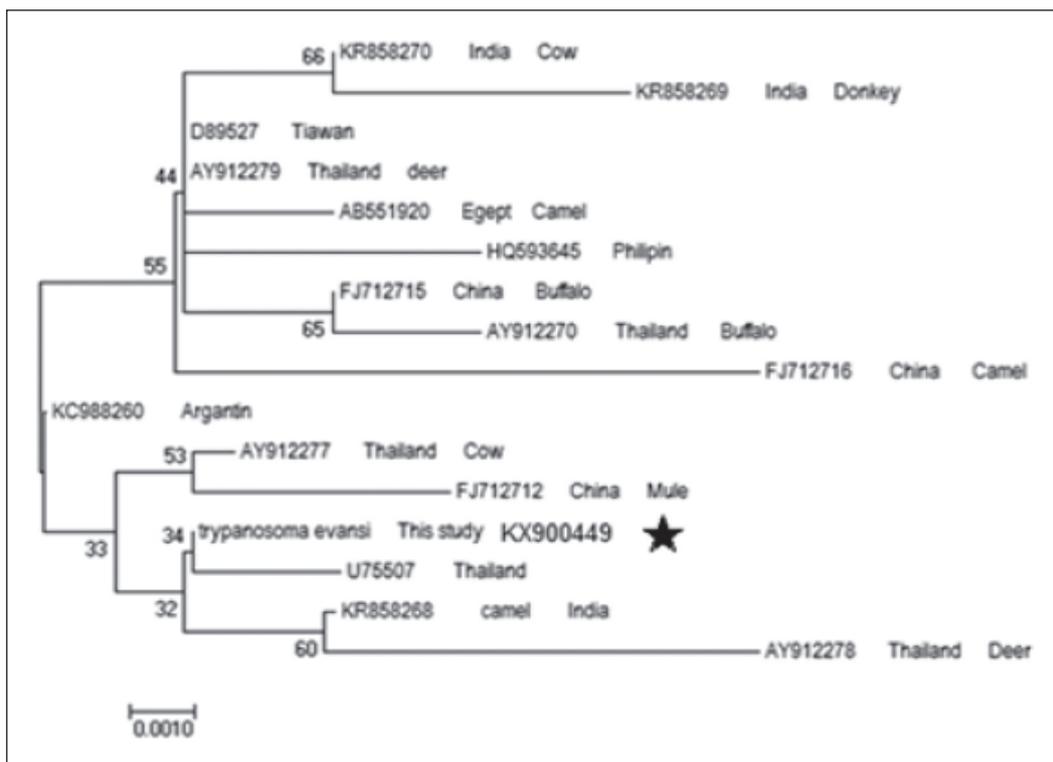


Figure 4. The phylogeny tree is drawn using the Neighbor-Joining method and the P-distance model. Relationship of species is seen in the percentage of trees repeating. In the Bootstrap test (500 repetitions), the cluster is shown next to the branches. The tree is drawn to scale.

samples from camels slaughtered in Mashhad, it was reported that 5% of cases were infected with *T. evansi* (Aghaei Qamasari, 2007). In the study of 117 blood samples from the seemingly healthy camels from different areas of Yazd, 4 positive samples were reported using Giemsa staining method (Ahmadi Hamedani, 2012). Although the microscopic observation of Giemsa stained blood smear is the most commonly used for the diagnosis of this protozoa, due to its low sensitivity, it is not commonly used in epidemiological studies. In fact, the lack of a reliable diagnostic method is a major problem in effective disease control. Although the microscopic examination is a specific method, it is not sensitive enough in the early stages of the disease, and the PCR method seems to be of sufficient sensitivity. Previous studies have shown that the sequence of ITS-1, ribosomal DNA (rDNA) is a reliable genetic marker for molecular studies on trypanosomes. The results of this study

showed that 7 out of 113 samples (6.19%) were infected with *Trypanosoma* as identified by the PCR method. AL-Tagi states that 115 camels in Kuwait tested positive for trypanosomes by the PCR method, and it was found that 2 of the camels (1.7%) were infected with *T. evansi* (Ahmadi Hamedani, 2012). Salim *et al.* (2011) examined the epidemiology of trypanosomiasis in camels by studying the ITS1 rDNA and RoTat 1.2 VSG genes in Sudan, and concluded that the trypanosomiasis in Sudan was caused only by *T. evansi* species, and they did not detect other species of *Trypanosoma* (Salim *et al.*, 2011).

Muieed *et al.* in a study in 2010, compared the sensitivity of PCR reaction and microscopic observation for the detection of *T. evansi* in horses. In this study, the number of positive samples in the PCR reaction was 16% and in microscopic studies was 5% (Muieed *et al.*, 2010). The findings show that the PCR method compared with

the microscopic method is an indispensable indicator for detecting *T. evansi* in horses in the early stages of infection. In India, Reghu *et al.* (2008) examined the prevalence of *T. evansi* infection in camels, donkeys and dogs by PCR and microscopic methods. Out of 131 blood samples (camel: 61 donkeys: 44 dogs: 26), 26 were positive by the PCR method (camel: 21 donkeys: 3 dogs: 2). By microscopy only 2 samples were positive (camel: 2 donkeys: 0 dogs: 0). In a comparative study that was conducted in Egypt in 2009, the percentage of PCR infection was 93.8% and wet smear was 13.8% (Reghu *et al.*, 2008). The results of this study and other researchers have shown that the Giemsa staining method has low sensitivity. The studies clearly show the higher sensitivity of PCR-based techniques to other techniques, such as serology and blood staining method. Accurate diagnosis, combined with extreme sensitivity in identifying carriers without specific disease symptoms, is definitely an important tool for epidemiological studies. The application of PCR and early detection of a disease can help to limit infectious reservoirs and the risk of keeping camel herds together, despite the presence of mechanical carriers around them. As there is a lack of studies on this parasite and its pathogenicity in this region, more recognition must be given to it to stop transmission of the disease among the camels.

#### CONCLUSION

In the present study, the prevalence of *T. evansi* was reported 6.19% by molecular method. Sistan and Baluchestan Province is one of the suitable camel habitats in the country, however, only a few studies have been conducted on trypanosomiasis in the region. Surra causes economic damage, therefore early diagnosis will prevent significant economic loss to the region. The findings of this research provides a basis for more molecular studies on trypanosomiasis in this province to have better diagnostic methods and control programmes.

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