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Prevalence of *Trypanosoma evansi* in Maghrabi Camels (*Camelus dromedarius*) in Northern-West Coast, Egypt using Molecular and Parasitological Methods

[1]Safaa M. Barghash, [2]Tarek R. Abou El-Naga,
[3]Esraa A. El-Sherbeny and [4]Ahmed M. Darwish

[1]Parasitology Unit, Animal Health Department, Animal Production and Poultry Division, Desert Research Center, Cairo, Egypt
[2]Infectious Diseases Unit, Animal Health Department, Animal Production and Poultry Division, Desert Research Center, Cairo, Egypt
[3]Cyto-Genetics Unit, Plant Genetic Resources Department, Ecology and Dry Lands Agriculture Division, Desert Research Center, Cairo, Egypt
[4]Cell Biology Department, Genetic Engineering and Biotechnology Division, National Research Center, Giza, Egypt

Abstract: In Egypt, two local strains of dromedaries' camels (Sudanese and Maghrabi) are different from each other in some morphological, physiological properties and their origin. *Trypanosoma evansi* was well studied in imported and local Sudanese camels. No published studies exist on trypanosomosis in Maghrabi camels originating from North Africa, despite serious epidemics which have occurred in recent years. For the first time, an epidemiological survey was carried out from May 2012 to July 2013 in Northern-West Coast, Egypt to assess prevalence of *T. evansi* infection in Maghrabi dromedary camels. Some mainly parasite-related epidemiological factors that may play a role in the distribution and impact of trypanosomiasis were determined with special attention to the specific epidemiological situation found in the study area. Blood film examination (B. film) and polymerase chain reaction (PCR) using two specific primers (RoTat 1.2 and TBR1.2) based assays optimized for the detection of *T. evansi* were evaluated. Out of 249 camels examined for trypanosomiasis: 52 (20.9%), 164 (65.9 %) and 186 (74.7%) were positive by B. film, PCR based RoTat 1.2 and PCR based TBR 1.2, respectively. PCR based TBR 1.2 showed the highest sensitivity values, whereas PCR based RoTat 1.2 was the most specific tool. Age and sex are likely to be risk factors for trypanosomiasis in camels. The majority of trypanosomiasis in Maghrabi camel is apparently caused by a single *T. evansi* infection in the study area. It is concluded that *T. evansi* was prevalent in all districts sampled at high levels regardless of the test used. Our findings coincide with our previous suggestion of the presence of *T. brucei and T. equiperdum* infections at the borders of Egypt. *T. evansi* reveals a big problem that needed to be re-evaluated.

Key words: *Trypanosoma evansi* · Prevalence · RoTat1.2 · TBR1.2 · Camels · Egypt

INTRODUCTION

Trypanosoma is a tissue-blood parasite known as a haemoflagellate. *Trypanosoma evansi* is the agent of "Surra" trypanosomiasis, constituting as one of the major veterinary problems worldwide [1]. It is the most widely distributed pathogenic animal trypanosome, affecting domesticated livestock and wild animals in Asia, Africa, Central and South America, Europe and recently a case of human infection has been reported in India making it a potential human pathogen [2]. Trypanosomiasis continues to be described, in most cases, as either chronic or acute and symptomatic or asymptomatic in both experimental and natural infections. It can quickly become fatal, but some animals can develop also a sub-clinical infection and thus become reservoirs [3].

Corresponding Author: Safaa M. Barghash, Parasitology Unit, Animal Health Department, Animal Production and Poultry Division, Desert Research Center, 1-Mathaf El-Mataria St. 11753, El-Naam, P.O. Box 11753, Cairo, Egypt. Fax: +202 26357858.

Two general problems in *T. evansi* exist. One is that the actual risk of infection, high plasticity and the anticipated impact of *T. evansi* vary across districts and overtime. Besides climatic variation, management factor and movement patterns of animals may lead to evolving sub-populations of *T. evansi*, even at the local scale [4]. The second is that no effective vaccine is available up until now; existing treatments are challenged with problems comprising of drug resistance, toxicity and expensive/limited drugs. Despite the large amount of epidemiological studies, many questions remain unanswered. Some of these important factors are related to the characteristics of the trypanosomes themselves. Of particular importance are those questions related to the factors determining the distribution of the disease [5].

In Egypt, *T. evansi* was well studied in imported and local Sudanese camels' origin from abattoirs [6-8] and under climatic conditions in deserts and various agro-ecological zones [9]. No published studies exist on trypanosomosis in Maghrabi camels originating from North Africa, despite serious epidemics which have occurred in recent years 2002, 2005, 2010, 2012 and 2013 as a result of continuous movement within this area. Furthermore, historical data have shown that trypanosomiasis in a particular area is constant and not subject to change over time in Egypt [9].

To our knowledge, by the end of the present study, the authors have completed a comprehensive survey of the extent of *T. evansi* damage in the deserts of Egypt, which was initiated in 2002 using different parasitological and molecular diagnostic tools. The application of these tools to the characterization of the etiological agents of trypanosomiasis has revealed a series of largely host-adapted species and genotypes that are maintained in distinct cycles of transmission. Moreover, it has led to better appreciation of health importance of *T. evansi* species/genotypes in various domestic animals and improved understanding of infection sources. Geographic, seasonal and socioeconomic differences in the distribution of *T. evansi* in domestic animals have been identified and have been attributed to differences in infection sources and mechanical transmission routes [9]. In addition, small ruminants acquired natural infections, suffer economic loss and appeared to constitute an enduring reservoir of *T. evansi* that infect domesticated livestock in Egypt. It probably plays a role in transmission of *T. evansi* to camels in Egypt [10].

Given the economic impact of the disease within the study area, the objectives of the present study aimed to determine the prevalence and distribution of *T. evansi* infection in Maghrabi camels, to compare between the tools used for the diagnosis of *T. evansi* infection and to improve the epidemiological factors determining the dynamics of the disease

MATERIALS AND METHODS

Study Area: Northern-West Coast, Egypt is considered as the major Egyptian Western entrance from Libya where camels are imported and exported legally. It is 240km west of Alexandria and 30km from Salloum, on the main highway from the Nile Delta to the Libyanborder. It has a hot desert climate, according to Köppen climate classification, but blowing winds from the Mediterranean Seagreatly moderate the temperatures, typical to Egypt's North Coast, making its summers moderately hot and humid, while its winters are mild and moderatelywet, when sleet and hail are also common. Summer is sunny and dry; while in the colder months in winter, there is a little rain and cloud. Three areas were selected (Namely: Mersa-Matrouh, Sidi-Berrani and El-Negeila).

Study Design and Sample Collection: Due to migratory communities which usually move from one place to another, it was difficult to adhere to a strict sampling frame. Thus, a spot survey in camels was conducted for *T. evansi* during the period from May 2012 to July 2013 in Northern-West Coast, Egypt. A total of 249 randomly selected camels of different ages and both sexes were obtained from selected regions and kept under nomadic conditions. The majority of animals were apparently healthy at the time of collection.

Blood Sample Collection: Blood samples were collected from the jugular vein of each camel using clean sterile Vacutainer tubes containing ethylene di-amine tetra acetic acid (EDTA). The whole blood samples were used for parasitological examination and for extraction of DNA as a target for PCR amplification. As recommended by QIAGEN, 100 µl of whole blood was mixed with an equal volume of Phosphate buffered saline (PBS) before processing of DNA, according to the protocol supplemented with the extraction kit. Samples were stored at-20°C until DNA extraction was performed.

Parasitological Examination: Two blood smears from each camel were stained with diluted Giemsa stain and examined with light microscopy (x40 and oil immersion objectives) according to Hoare [11].

Extraction of DNA from Blood Samples: Extraction of total genomic DNA was done using commercially available DNeasy Blood & Tissue Kit (QIAGEN Inc. Germany) according to the manufacturer's instructions. Briefly, 20 µl of proteinase K stock solution, 200 µl of whole blood with PBS and 200 µl of AL lysing buffer were pipetted into 1.5 ml Eppendorf tubes and the mixture was mixed, vortexed and incubated at 56°C for 10 minutes. 200 µl of absolute ethanol were added to the sample and the mixture was mixed by vortexing and spinning. The mixture was transferred to the QIAspin column and placed in a clean 2 ml collection tube, then centrifuged at 6000 xg for 1 minute. The QIAspin column was washed twice using 500 µl of washing buffers AW1 and AW2, respectively. The QIAamp spin column was then placed in a clean 1.5 ml Eppendorf tubes and the DNA was eluted with 200 µl of AE buffer and incubated for 1 minute at room temperature. Maximum DNA yield was obtained by spinning at 14,000 xg for 1 min. The DNA concentration was determined by spectrophotometer at wave length of 260 nm.

Primer Selection: For the detection of *T. evansi*, two PCR based-assays were performed and evaluated. These primers had already individually shown their ability to amplify *T. evansi* DNA from purified DNA, as well as in various host species [10, 12-15]:

- RoTat 1.2 primers amplify 205 bp; based on the sequenced gene coding for the *T. evansi* RoTat1.2 Variable Surface Glycoprotein (VSG), targeting the DNA within the region (608-812 bp) and lacking homology with any other known VSG genes sequence [16]. Primer sequences were as follows: F5'-GCG GGG TGT TTA AAG CAA TA-3', R5'-ATT AGT GCT GCG TGT GTT CG-3'.
- TBR1.2 primers amplify 164 bp; based on the highly repeated sequence of mini-chromosome satellite DNA [12]. Primer sequences were as follows: F5'-GAA TAT TAA ACA ATG CGC AG, R R5□-CCA TTT ATT AGC TTT GTT GC-3'.

DNA Amplification and Detection: PCR amplification reaction was performed in a total reaction volume of 25 µl containing 50 ng of template DNA and 12.5µl of commercially available PCR master mix (Promega, UK). The *T. evansi* species-specific primers (RoTat 1.2) were used at a concentration of 10 pmol/µl. The PCR amplifications were carried out in a Biometra thermocycler. Cycling conditions were as follows: pre- denaturation for 3 min. at 94°C, followed by 40 Cycles of 94°C for 1 min. (denaturation), 57°C for 1 min. (annealing) and 72 °C for 1 min. (polymerization) with a final extension at 72°C for 5 min. After amplification, 10 µl of PCR product were loaded in a 2% agarose gel; electrophoresis was done at 120 V for 1 h, with 50 bp DNA ladder (LAROVA®, Germany) as a standard molecular weight marker. Gels were stained with ethidium bromide and photographed under UV-light for examination. After determining that the band size of the amplicon was comparable to *T. evansi* species, a confirmatory PCR assay was performed by using *Trypanozoon* DNA species-specific primers (TBR1.2) with the same conditions as mentioned above, but annealing at 52°C. Subsequently, negative (distilled water) and positive (RoTat 1.2 DNA strain donated from Institute of Tropical Medicine, Antwerp, Belgium), controls were included in the PCR run.

Statistical Analysis: The obtained results of the survey were coded, tabulated and introduced for analysis. Samples were categorized in three ways: (a) District = samples from the three selected locations (b) Age = animals grouped into age group; $X \leq 4$ years, $4 \leq X = 10$ years and X 10 years (c) Gender (Sex). Statistical analysis was performed using SAS Users Guide: Statistics Ver. 9.1. [17]. The differences in prevalence between different regions and the influence of various factors, such as gender, age and locality were computed and determined using the F test. Means were separated using the Duncan Multiple range test procedure of the same statistical package. All statistics were considered significant at $P \leq 0.05$.

RESULTS

Prevalence and Distribution of *T. evansi*: The percentage of *T. evansi* in Maghrabi camels in each of the three locations are shown in Table 1 and Figures 1, 2. A total of 249 camels were examined with different techniques of which, 52 (20.9%), 164 (65.9 %) and 186 (74.7%) were positive by B. film, PCR based RoTat 1.2 and PCR based TBR 1.2, respectively. The highest prevalence rate of infections with *T. evansi* using B. film was recorded in Sidi Berrani (22.9%), followed with slight differences by Mersa Matrouh (21.6%) and finally El-Negeila (17.6%). In contrast, molecular tools revealed higher infection rates using PCR based RoTat 1.2 (71.2%, 65.7% and 57.4%) and TBR 1.2 (78.4%, 74.3% and 69.1%), in Mersa Matrouh,

Table 1: Prevalence and distribution of *Trypanosoma evansi* infections in Maghrabi dromedary camels based on locations, sex and age

					Sex (Gender)						Age Groups								
		Total			Female			Male			X≤4			4X≥10			X10		
Factor																			
Location		*T1*	*T2*	*T3*	*T1*	*T2*	*T3*	*T1*	*T2*	*T3*	*T1*	*T2*	*T3*	*T1*	*T2*	*T3*	*T1*	*T2*	*T3*
Mersa Matrouh	*No.*	111			101			10			26			36			49		
	+Ve	24	79	87	23	74	82	1	5	5	8	11	11	8	27	30	8	41	46
	%	21.6	71.2	78.4	22.8	73.3	81.2	10	50	50	30.8	42.3	42.3	22.2	75	83.3	16.3	83.7	93.9
El-Negeila	*No.*	68			35			33			45			11			12		
	+Ve	12	39	47	9	26	27	3	13	20	4	18	26	2	9	9	6	12	12
	%	17.6	57.4	69.1	25.7	74.3	77.1	9.09	39.4	60.6	8.9	40	57.8	18.2	81.8	81.8	50	100	100
Sidi Berrani	*No.*	70			38			32			45			12			13		
	+Ve	16	46	52	9	24	26	7	22	26	6	26	30	6	9	9	4	11	13
	%	22.9	65.7	74.3	23.7	63.2	68.4	21.9	68.8	81.3	13.3	57.8	66.7	50	75	75	30.8	84.6	100
Total	*No.*	249			174			75			116			59			74		
	+Ve	52	164	186	41	124	135	11	40	51	18	55	67	16	45	48	18	64	71
	%	20.9	65.9	74.7	23.6	71.3	77.6	14.7	53.3	68	15.5	47.4	57.8	27.1	76.3	81.4	24.3	86.5	95.9

*No. = number of camels examined, the presence (+ve) and the percentage (+ve %) of *Trypanosoma evansi* positive animals in each of the three locations. Blood Film (T1) and two PCR assays [RoTat1.2 (T2), TBR1.2 (T3)] were used for this purpose.

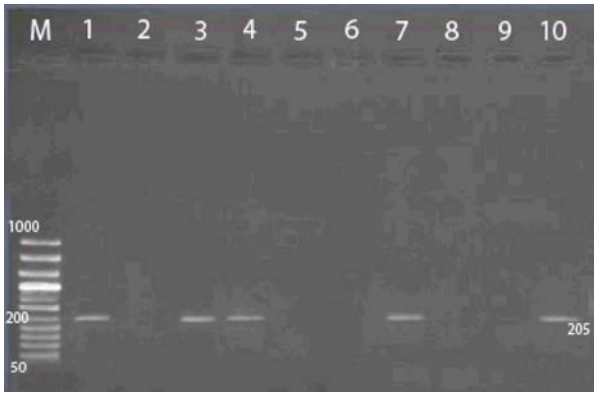

Fig. 1: Detection of *Trypanosoma evansi* PCR product using RoTat1.2 specific primers in naturally infected Maghrabi camels. It amplified *T. evansi* at 205 bp based on RoTat 1.2 VSG gene. Lane M: DNA molecular size marker (LAROVA®, Germany). Lanes (1, 3, 4 and 7) are RoTat 1.2 VSG gene positive. Lanes (2, 5, 6 and 8) are RoTat 1.2 VSG gene absent. Lanes 9 and 10 are negative and positive controls, respectively.

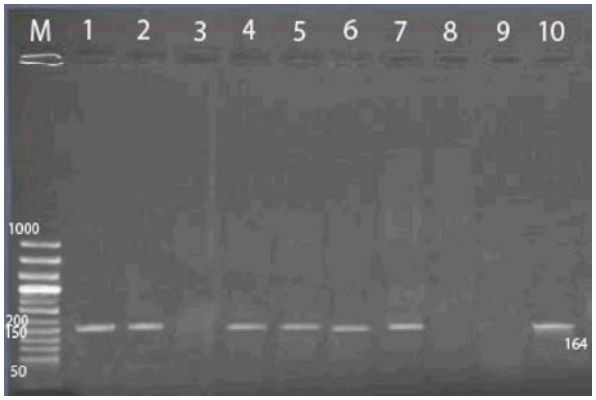

Fig. 2: The amplified PCR products were electrophoresed on (2%) agarose gels, stained with Ethidium bromide and visualized under UV light. PCR amplification of a 164 bp amplicon using *T. evansi* TBR1.2 primer on purified *T. evansi* DNA on tested samples (lane 1, 2, 4, 5, 6 and 7), Negative samples (lane 3 and 8), Negative control (lane 9) and positive control (lane 10).

Sidi Berrani and El-Negeila, respectively. The results of the present study exhibited the applicability of PCR assays for epidemiological surveillance of trypanosomiasis in the study area. Furthermore, this study reports the first case of *T. evansi* infection in Maghrabi camels from Northern-West Costal belt area, Egypt.

Factors Affecting *T. evansi* Infection: Results of prevalence of *T. evansi* were found to be significantly different between both sexes in relation to locations and between tests used. There was considerable variation in values within age category reflected in a high significant variation between the older (X 10) and each of the younger groups (4 X≥10, X ≤ 4). Results of prevalence of *T. evansi* infection increased directly proportional and

progressively with age in all herds studied: X 10 years of age recorded the highest infection, especially in El-Negeila and Sidi Berrani, while a slightly lower prevalence of *T. evansi* infection was recorded in Mersa Matrouh (Table 1).

With respect to gender, significant differences have been found between male and female camels examined regardless of the test used (Table 1). A total of 41,124 and 135 out of 174 females were *T. evansi*-positive compared to (11, 40 and 51) out of 75 males using B.film, RoTat 1.2 and TBR 1.2, respectively. While the infection was higher in females in Mersa Matrouh (22.8%, 73.3% and 81.2%), males recorded the highest prevalence in Sidi Berrani (21.9%, 68.8 and 81.3%), according to B. film, RoTat 1.2 and TBR 1.2 positive results, respectively. Concerning locations, results showed that *T. evansi* was found to be prevalent in all districts sampled at high levels with no significant difference regardless of the test used.

Evaluation of Parasitological and Molecular Tests Used: Statistically, a good correlation between PCR based TBR1.2 and PCR based RoTat1.2 results reached to 80.8%, while the correlation between B. film and PCR based TBR1.2 results was 27.6% and between B. film and PCR based RoTat1.2 results was 32.8%. Molecular methods gave no significant difference in each property (nearly similar). TBR1.2-PCR primers proved to have the highest sensitivity values. Consequently, it was easy to compare with other diagnostic methods particularly PCR based RoTat1.2. Anyway, the present results showed that TBR1.2 and RoTat1.2-PCR based assays could be used to identify *T. evansi* with a clear amplification and good reproducibility. Wherever the RoTat1.2-PCR yielded only 205 bp fragment as a higher specific marker for *T. evansi*, TBR1.2-PCR yielded 164 bp as expected value for *Trypanozoon* that concluded *T. brucei*, *T. equiperdum* and *T. evansi* subspecies (Fig. 1, 2). Surprisingly, not all TBR1.2-PCR positive results were positive by RoTat1.2-PCR in this study whereas from 186 TBR1.2 positive results, only 164 were positive with RoTat1.2.

DISCUSSION

To face the worldwide threat of trypanosomiasis caused by *T. evansi*, international organizations have stressed the need to evaluate and standardize diagnostic tools. PCR detection of *T. evansi* has known a great expansion during the last 20 years, but primer sets are often insufficiently assessed and compared [18].

In the present study area, a great variation existed in the feeding and management among herds associated with season, age and sex which had an effect on the prevalence of new and recurrent infections. Possible causes of this variation could be attributed to the fact that Trypanosoma parasites circulate within a wide and diverse host community in this ecosystem. According to information acquired from animals' owners and veterinary personnel, the main reported problem affecting animals in this area is *T. evansi* infection (locally named as Guphar) and is commonly associated with bacterial and concurrent infections, which complicates clinical diagnosis. People could not specifically identify trypanosomiasis, but based their assessments on clinical signs, including anemia, emaciation, diarrhoea and sometimes abortion. They attributed the occurrence of high levels of newly and active infections to regular transportation of animals to neighboring governorates for grazing, wherever the close contact of camels with other animal species act as a serious source of infection and transmission as previously reported [19, 20]. In spite of this, the present study revealed camels are largely kept without close association with other carrier animals, such as sheep and goats. So, transmission could take place by biting flies especially Stomoxys species and hard ticks which are present in large numbers. This is explaining why transmission may occur at any time.

However, the present results of *T. evansi* infections using PCR based RoTat 1.2 showed 65.9%, compared to our previous studies near the borders of Egypt using the card agglutination test (CATT/ *T. evansi*) were recorded as: 73.5% in Halaib, Shalateen and Abu-Ramad in Southern East, 90% in Siwa Oasis in the West and 46.7% in Maryout in the North of Egypt, where no common use of a control program on a large scale is present. This variation between results may be attributed to several factors such as differences in techniques used, localities and consequently difference in climatic conditions which to a large extent affect the vector activity [6, 9].

When infection was correlated with host age groups, it showed a progressive increase with age in adults [21-23]. However, owners prefer to graze their animals in open fields' where they become more exposed to vector bites especially in Sidi Berrani. Young animals become susceptible to infection after weaning, losing the maternal immunity transferred to them through colostrums. Moreover, in the study area animals of different age groups were reared together permitting transmission of infection from adults (carriers) to

susceptible young animals, as explained by Singh *et al.* [24]. The increase in acquiring *T. evansi* infections in females compared to males could be explained on bases that many of male samples were aged X≥4 and the majority of samples were from pregnant females, related to the stress of lactations and successive pregnancies.

As expected, B. film proved to be of limited value in diagnosis of subacute, chronic cases and ongoing infections as the level of parasitaemia is often low and fluctuating [25, 26]. In contrast, a high sensitivity with purified DNA was confirmed and was successful in applying the two primers on field samples from Maghrabi camels. Consequently, it was easy to compare with parasitologic methods [8, 14, 27].

In order to address the importance of the extraction technique in our observations where dimers and non-specific bands were absent, it is therefore likely to be due to the extraction process. However, the DNeasy kit (Qiagen) extraction and purification methodology maximized DNA yield from blood and provided a highly efficient method compared to other methods (Promega®, Gentra®) which were used in our previous studies [9, 10, 15]. To both evaluate and compare TBR1.2 and RoTat 1.2 PCR primer sets: TBR1.2 proved to have a higher sensitivity and superiority compared to RoTat1.2 a specific marker for different worldwide *T. evansi* strains. This might be due to TBR1.2 depending on identifying portions of DNA targeting sequences found in the nucleus and not in the kinetoplast, making results independent of the parasite kinetoplastic state and avoiding the problem of failure targeting kDNA as reported in South America [27, 28]. TBR1/2 should be used in any primer development and evaluation, as a gold standard for the detection of *Trypanozoon* DNA by PCR. It is essential to carry out proper performance evaluation during primer development and to use systematically TBR1/2 as a reference [18].

To determine the trypanosomes subspecies circulating in the study area, our results show clearly that we face a predominant *T. evansi* infection, but the question is whether it is alone or not. The present study revealed the absence of RoTat 1.2 VSG gene in 22 *T. evansi-positive* samples with TBR1.2. Three main hypotheses could explain this result [a] occurrence of mutants due to mixed genotyping occurred between some *T. evansi* isolates with other *Trypanozoon* [b] presence of *T. brucei* and/ or *T. equiperdum* infection in the Northern West border area of Egypt which coincides with our previous studies in Siwa oasis located to the South of Mersa Matrouh [c] the present of *T. evansi* lacking the RoTat 1.2 VSG gene in the study area as reported in Kenya [29]. It must be noted that, in our previous studies [9], RoTat 1.2 a specific marker for *T. evansi* strains, failed to differentiate some Egyptian strains that have been previously identified as *T. evansi*. Natural populations of *T. evansi* in Egypt were identified with degrees of heterogeneity, describing for the first time that natural populations of *T. evansi* in Egypt revealed the presence of higher levels of intra-specific genetic variability of *T. evansi.* Also, it was reported that mixed genotyping has occurred between some *T. evansi* isolates with *T. brucei* and *T. equiperdum* in the border areas of Egypt and evidence was produced that strain difference might occur between Egyptian sub-populations and reference strains of *T. evansi* donated from the Institute of Tropical Medicine in Antwerp, Belgium [15].

CONCLUSION

In conclusion, the absence of trypanosomiasis signs revealed sub-clinical and /or chronic infections with low parasitaemia. *T. evansi* infection (provoked with stress factors) leads to a degree of economic impact on the camel rearing industry in this area and revealed a big problem that needed to be re-evaluated; in particular it was present associated with bacterial and parasitological secondary infections [30-32]. Maghrabi camel trypanosomiasis in the study area is apparently caused by not only *T. evansi* but also other *Trypanozoon* sub species. Finally, *T. evansi* is highly prevalent in the study area, which strengthens the need to change control policies and institute measures that help prevent the spread of the parasite and its vectors.

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