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First molecular identification of *Trypanosoma evansi* from cattle in Syria

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Abstract

***Trypanosoma evansi*, the "surra" disease-causing agent, is a blood protozoan parasite that infects a wide range of mammalian species within an unlimited geographical region. It causes anemia, weight loss, and even death of the infected livestock that heavily affect animal husbandry. However, the full epidemiological information of *T. evansi* is lacking, especially in developing countries, and the risk of the disease is largely underestimated. In this study, 207 samples of blood DNA collected from Holstein Friesian crossbred cattle in the central region of Syria in May 2010 were screened for *T. evansi*, aiming to determine the prevalence of the parasite. *T. evansi* was screened by PCR targeting the internal transcribed spacer (ITS) 1 region, and 27 samples were found positive out of 207 (13%), which is relatively high considering that no clinical symptoms were observed. The ITS1 amplicons were later subjected to *RoTat1.2*-PCR for detection of *T. evansi* type A. This is the first report of molecular detection of *T. evansi* in Syria. Our study suggests that advanced investigations in cattle and other domestic animals are necessary in Syria.**

Key Words: Epidemiology, ITS1-PCR, Syria, *Trypanosoma evansi*

Introduction

Trypanosoma evansi, the causative agent of "surra" disease classified in the subgenus *Trypanozoon*, is a flagellated protozoan parasite[14)]. It shares some characteristics with other *Trypanozoon* species, including *Trypanosoma brucei* and *Trypanosoma equiperdum*, in terms of morphology and genome sequences[8,30)]. However, while *T. brucei* undergoes a complex cycle of differentiation and multiplication in tsetse flies, *T. evansi* does not have a vector stage and is only transmitted mechanically. Unlike *T. brucei*, *T. evansi* has lost the maxicircles of kinetoplastid mitochondrial DNA, which are required to undergo the procyclic form in tsetse flies. This makes *T. evansi* unable to reproduce in tsetse flies[27)]. *T. evansi* is transmitted mechanically by

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a wide range of biting flies including tabanids and stomoxes, vampire bats, and ingestion of raw meat[12,13]. Since *T. evansi* is no longer restricted to tsetse flies, it has the largest geographical distribution and host range among salivarian trypanosomes[11,29]. *T. evansi* infects a broad range of domestic animals and wildlife, which are more pathogenic to camelids and equids[14]. It has been described that the susceptibility of *T. evansi* is highly variable depending on the host species and presumably according to the region[12].

To differentiate *Trypanozoon* from other trypanosomes, polymerase chain reaction (PCR) targeting the internal transcribed spacer (ITS) 1 region of ribosomal RNA (rRNA) has been commonly used[16,33,41]. Identification of each species in *Trypanozoon*, including *T. evansi* and *T. brucei*, is complicated because of their twinning characteristics[9,30]. Although *T. evansi* has no maxicircles, they still possess short minicircle DNA that encodes guide RNAs for RNA editing[6,7]. Based on its minicircle restriction digestion profile, *T. evansi* is divided into type A and type B[6,34]. *T. evansi* type A is the most common species found in Africa, South America, and Asia[4]. In contrast, *T. evansi* type B is found in Eastern Africa. To date, the prevalence of *T. evansi* type B remains largely unknown, especially in Asia[3,6,18,32,41]. Further, it has not been studied whether the presence of the *Rode Trypanozoon antigen* type 1.2 (*RoTat1.2*) gene of *T. evansi* is related to pathogenesis or other biological phenotypes.

The variant surface glycoprotein (*VSG*) of *RoTat1.2* is specifically present in *T. evansi*, particularly in type A, but not in *T. brucei* strains, and has been utilized to differentiate *T. evansi* from other *Trypanozoon* members[9,49,50]. Because some of the diagnostic strategies for *T. evansi* rely on the presence of the *RoTat1.2*, such as *RoTat1.2* loop-mediated isothermal amplification (LAMP) and the serological card agglutination test for *T. evansi* (CATT)/RoTat1.2, only *T. evansi* type A, not type B, can be detected using these methods[2,15,422].

For specific diagnosis of *T. evansi* type B, several tests have been developed, including EVAB-PCR, targeting a type B-specific minicircle DNA sequence; a PCR; and LAMP targeting type B-specific *VSG JN 2118Hu*[32,35,36]. Once the presence of *T. evansi* type B infection is confirmed, CATT or *RoTat1.2*-PCR test should be avoided as the current CATT/*RoTat1.2*-PCR tests cannot detect *Rotat1.2*-negative *T. evansi* type B infection. In such cases, nucleic acid amplification methods targeting other genes, such as ITS1-PCR used in this study, are recommended to detect all *T. evansi* infections. *T. brucei* and *T. evansi* can switch their diverse transferrin receptors, and the two genes encoding the transferrin receptors, expression-site-associated gene (*ESAG*) *6* and *ESAG7*, display high genetic diversity[20-22,31,44,52]. This diversity has been speculated to contribute to sufficient iron uptake from different mammalian species and escape from anti-transferrin receptor antibodies[5,17,46]. However, differences in transferrin receptors of animals are not always responsible for differences in trypanosome growth in the sera[43]. In addition, the antibody against the transferrin receptor failed to prevent iron uptake of trypanosome[45]. Hence, it is not clear whether the genetic diversity of *ESAG6/7* within *T. evansi* is related to the biology or infectivity of the trypanosome in mammalian host and whether it is in any way related to countries/regions or host species[44,52].

It is widely believed that *Trypanozoon*, including *T. evansi*, originated in Africa and spread across the world[12]. It has been suggested that *T. evansi* evolved from a *T. brucei* infection in camels that had temporarily entered the sub-Saharan tsetse belt and adapted to mechanical transmission by biting flies[19]. However, a recent study explained the independent origins of *T. evansi* from *T. brucei* strains, where the ability of *T. evansi* to be transmitted mechanically occurred repeatedly[23]. The parasite spread from North Africa toward the Middle East, Turkey, India, up to Russia, across all of Southeast Asia, down to Indonesia and the Philippines, and was also introduced by the conquistadores into Latin

America[19,28,40]. However, there is no molecular study of *T. evansi* in Syria1). Syria, as one of the Middle Eastern countries situated between Africa and Asia and Europe might be a gate to learn about *T. evansi* evolution, where the parasite moved out of its place of origin and started to spread worldwide. Collectively, the aim of this study was to determine the prevalence and characterize the genetic diversity of *T. evansi* in Syria. This epidemiology study and genetic diversity information of the parasites will help in improving the control measures in this region and understanding host tropism and adaptation of *T. evansi* in different animals and regions worldwide.

Materials and Methods

DNA Samples: A total of 207 blood DNA samples were used, which were collected from clinically healthy Holstein Friesian crossbred cattle in the central region of Syria in May 2010. The sampling sites were Huleh (n = 51), Hama (n = 73), Qyser (n = 28), Ghab (n = 32), and Salmia (n = 23). The collection of field samples was approved by the Syrian government through the Ministry of Agriculture and supported by veterinarians and staff working at the Society for Protection of Animals Abroad in Syria. Sample collection methods were followed as described previously[47]. Readily prepared DNA samples were previously provided and used by Terkawi et al. (2012)[47]. In this study, microscopic parasite examination and serological tests for *T. evansi* diagnosis were not conducted.

PCR: PCR amplification of the ITS1 region of the rRNA was conducted to screen trypanosomes, including *Trypanozoon*. For amplification of the ITS1 region, the primer set CF 5'-CCGGAAGTTCACCGATATTG-3' and BR 5'-TGCTGCGTTCTTCAACGAA-3' was used[33]. Each reaction included 5 µl Ampdirect plus (Shimadzu, Japan), 0.05 µl BIOTAQ HS DNA Polymerase (5 U/µl) (Bioline, UK), 0.5 µl of each 10 mM primer, 2.95 µl RNase-free water, and 1 µl extracted DNA. The thermocycling profile started with an initial hold for 10 min at 95 °C, followed by 40 cycles at 94 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products were electrophoresed on 1.2% agarose S (Nippongene, Japan) in Tris-acetate EDTA buffer and stained using GelRed (Biotium, USA) dye before being visualized under UV light.

The ITS1-positive samples were subjected to PCR specific for *T. evansi* (type A), which amplifies 151 bp of the *RoTat1.2 VSG* fragment by using the primer set TeRoTat920F 5'-CTGAAG AGGTTGGAAATGGAGAAG-3' and TeRoTat1070R 5'-GTTTCGGTGGTTCT GTTGTTGTTA-3'[25]. The *RoTat1.2* amplicons were electrophoresed on a 2.2% agarose gel. PCR amplification of the 740 bp fragment of *ESAG6* was performed using *ESAG7* F455 5'-CATTCCAGCAGGAGTTGGAGG-3' and *ESAG6* R1045 5'-TTGTTCACTCACTCTCTTTGACAG-3' primers as described by Isobe et al.[21]. *ESAG6* amplicons were electrophoresed on a 1.2% agarose gel. The reaction was performed for 35 cycles at 58 °C annealing temperature in *RoTat1.2*-PCR and 40 cycles at 60 °C in *ESAG6*-PCR; the other thermocycling conditions were as mentioned above. The master mix conditions were the same as those for ITS1-PCR. Increased sensitivity of *ESAG6* amplification was achieved using the newly designed nested PCR. The second round of PCR was performed using the inner primer set, forward 5'-GCAGGAGTTGGAGGAAATGA-3' and reverse 5'-TGAGCTCAGCCTCTTTCTGC-3. The reaction mixture was the same as that used for the initial *ESAG6*-PCR. The thermal cycling conditions used were the same as that for the initial PCR except for the modification to 35 cycles.

Sequence and phylogenetic analysis of ESAG6: The *ESAG6*-PCR products were purified by ExoSAP-IT (GE healthcare/USB, USA) following the manufacturer's instructions. Purified PCR products were sequenced using the Big-Dye Terminator v3.1 (Applied Biosystems, USA) on an automated capillary sequencer (Applied Biosystems 3130 Genetic Analyzer; Applied Biosystems Japan Ltd., Tokyo, Japan). DNA sequences were edited using ApE[10]. The DNA sequence data were aligned against 47 sequences of *ESAG6* deposited in GenBank using the ClustalW program in the MEGA7 software[26]. A phylogenetic tree was constructed using neighbor-joining (NJ) algorithms. For the trees provided by NJ methods, bootstrap branch supports were calculated from 1000 pseudo-replicates following the rule of branch consistency.

Statistical analysis: Chi-square test was used to evaluate significant differences ($P < 0.05$) in the infection rate in animals of different ages, locations, anemia, and co-infection with *Babesia* spp. Statistical analysis was conducted to compare the infection rate of *T. evansi* in animals and co-infection with *Babesia* spp. using the data from Terkawi et al. (2012)[47].

PCR sensitivity test: Sensitivity of the PCR systems for ITS1, *RoTat1.2*, and *ESAG6* was validated using primer sets of CF and BR, TeRoTat920F and TeRoTat1070R, and *ESAG7* F455 and *ESAG6* R1045, respectively. The detection limit was examined in four replicates using 10-fold serial dilutions of DNA extracted from *T. evansi* IL3354 isolate cultured *in vitro*.

Results

Molecular detection of T. evansi by PCR: DNA samples were subjected to PCR amplification of the ITS1 region and *RoTat1.2* for molecular identification and *ESAG6* for genetic diversity characterization of *T. evansi*. Sanger sequencing was carried out to validate the results of ITS1-PCR, and it was confirmed that all 27 ITS1-positive samples contained *T. evansi*. The present study showed a 13.0% prevalence of *T. evansi* infection in cattle by ITS1-PCR screening (Table 1). *RoTat1.2*-PCR amplified in 17 samples that were ITS1-positive, but it failed to amplify in the other 10 samples.

Prevalence of T. evansi *according to location, packed cell volume (PCV) value, age, and co-infection with* Babesia *spp.*: The highest prevalence of *T. evansi* was observed in Ghab (34.4%, $n = 11$), followed by Qyser (25%, $n = 7$) and was significantly higher than that in the other regions ($P < 0.0001$, Table 2). In contrast, *T. evansi* infection in cattle had no correlation ($P > 0.05$) with anemia (Table 3), age (Table 4), and *Babesia* spp. co-infection (Table 5).

Sequencing and phylogenetic analysis based on ESAG6: Sixteen samples were positive in *ESAG6*-PCR, and these were successfully sequenced. Phylogenetic analysis based on the *ESAG6* revealed that Syrian sequences clustered together, with the majority in clade 10 (9 sequences) and clade 9 (4 sequences), while the remaining sequences were distributed in clade 1 (1 sequence) and clade 5 (2 sequences). This indicated that some of the major genotypes were shared within the three sampling locations in Syria, and genetic diversity was observed in our samples (Figure 1).

Analytical sensitivity test of ITS1, RoTat1.2, *and* ESAG6-*PCR:* The minimum amount of DNA detectable by PCR was 1×10^{-4} ng for ITS1-PCR (two positives out of four experiments), 1×10^{-2} ng for *RoTat1.2*-PCR (one positive out of four experiments), and 1×10^{-4} ng for *ESAG6* (one positive out of four experiments; Supp Fig 1 and Supp Table 1). ITS1-PCR and *ESAG6*-PCR showed higher sensitivity than *RoTat1.2*-PCR.

Discussion

In the present study, we described for the first time the prevalence of *T. evansi* infection among cattle in Syria. ITS1-PCR was used to determine

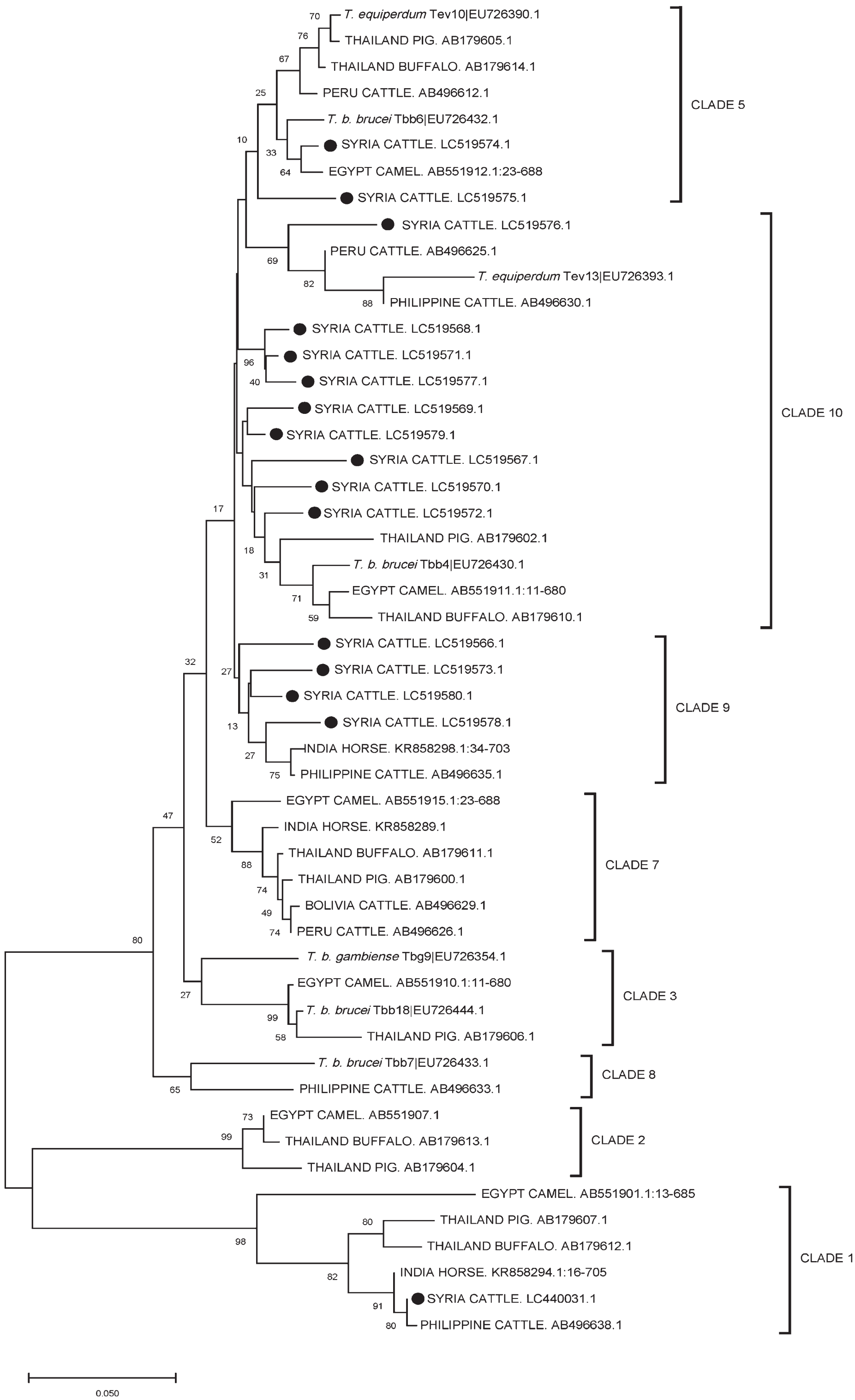

Figure 1

Phylogenetic tree of the *ESAG6* was constructed based on the nucleotide sequences of *Trypanosoma brucei* and *Trypanosoma evansi*. The entry data were represented according to their countries of origin—Peru, Philippine, Egypt, India, Thailand, Bolivia, and Syria; the hosts—cattle, camel, horse, donkey, buffalo, deer, and pig; and the GenBank accession numbers. The dotted entry names are the nucleotide sequences obtained from this study. The bootstrap confidence values at the nodes illustrate the percentage of times the group occurred out of 1000 trees, and the bar depicts the genetic distance.

Table 1. PCR screening result for *T. evansi*

ITS1	*RoTat1.2*	*ESAG6*	Number of samples	
+	+	+	9	10
		-	1	
	-	+	7	17
		-	10	
-	-	-	180	

(+) = PCR positive, (-) = PCR negative.

Table 2. Comparisons of the prevalence of *T. evansi* infections on the basis of sampling region.

City	Total number of samples	ITS1 positives with 480bp (putative *T. evansi*)	Positive rate
Huleh	51	1	2.0%
Hama	73	6	8.2%
Qyser	28	7	25.0%
Ghab	32	11	34.4%
Salmia	23	2	8.7%
Total	207	27	13.0%

Table 3. Comparisons of the prevalence of *T. evansi* infections on the basis of PCV value.

PCV	Status	Total number of samples	ITS1 positives with 480bp (putative *T. evansi*)	Positive rate
<24%	Anemic	9	2	22.2%
24-46%	Normal	198	25	12.6%
Total		207	27	13.0%

Table 4. Comparisons of the prevalence of *T. evansi* infections on the basis of age.

Age (years)	Category	Total number of samples	ITS1 positives with 480bp (putative *T. evansi*)	Positive rate
1-2	Young	55	6	10.9%
3-5	Old	99	14	14.1%
Above 5	Older	53	7	13.2%
Total		207	27	13.0%

Table 5. Comparisons of the prevalence of *T. evansi* infections on the basis of coinfection with *Babesia* spp.

Category	Total number of samples	ITS1 positives with 480bp (putative *T. evansi*)	Positive rate
Absence of *Babesia* spp.	160	19	11.9%
Presence of *B. bovis*	16	1	6.2%
Presence of *B. bigemina*	31	7	22.6%
Total	207	27	13.0%

the presence of *T. evansi* in the studied samples because Syria is not a habitat of the tsetse fly, with the *T. brucei* vector found only in sub-Saharan Africa[51]. The positive rate determined by ITS1-PCR screening was 13.0% and relatively high, considering that no clinical symptoms were observed. In our analysis, 37% of the ITS1-PCR positive samples could not be detected by *RoTat1.2*-PCR possibly owing to low parasitemia, presence of *T. evansi* type B, or the performance gap between ITS1-PCR and *RoTat1.2*-PCR in our samples. To confirm the *RoTat1.2* negativity in ITS1-PCR positive samples, further analyses are required, including microscopic parasite examination, serology assay to detect RoTat1.2 antigen, and highly sensitive nucleic acid detection system.

Geographic factors played a role in the infection rate distribution in this study. Ghab, followed by Qyser, were the most prevalent areas of *T. evansi* infection in this study. Both these regions were green areas dedicated to the agriculture sector in the country, where the population of livestock was dense and animal movements were frequent. Poor farm hygiene and weather conditions in the areas provided the best environment for horseflies (tabanus flies) to reproduce extensively. Farmers in Ghab and Qyser practiced a free-range management system where animals were released during the day to graze freely and returned home at dusk to sleep, which increased the exposure to horseflies. These factors elevated the possibility of disease transmission in Ghab and Qyser. Given the significant differences in occurrence of *T. evansi* among the studied regions, it is necessary to investigate the geographic distribution of horseflies as parasite vectors in Syria. The higher altitude of Hama has possibly become a geographical barrier for wildlife trespasses, which might explain the difference in prevalence[47]. In addition, the indoor management farm in Salmia contributed to lesser animal contact with fly vectors, which was one of the transmission factors.

In Asia, Holstein Friesian cattle are susceptible to *T. evansi* infection, and infected cattle frequently exhibit a significant decrease in PCV profiles and body weight as well as a negative effect on milk yield and fertility, including abortion[24,37-39]. In this study, the cattle samples were a mixed breed of Syrian local and Holstein Friesian. Syrian local cattle are known to be resistant to trypanosome infection. This may explain the lack of correlation between infection in cattle and anemia. Considering the observed high prevalence and mild symptoms, these cattle might be one of the potential reservoirs of *T. evansi* in Syria.

We observed that all age groups of cattle were equally exposed and affected by surra. The studied regions were endemic to other blood parasites of *Babesia* spp.; therefore, we also analyzed the correlation between *Babesia* spp. and *T. evansi* using the molecular analysis data of Terkawi et al.[47] in the same samples collected. We did not find any significant correlation in co-infection of *Babesia* spp. and *T. evansi*. This is possibly because of limited interaction in terms of vector, lifecycle (one is an intracellular parasite and the other is extracellular), and immunogenicity (presumably acquired immunity does not cross-react with each other).

Sequence analysis of the *ESAG6* of *T. evansi* in Syria showed genetic diversity. The major genotypes were clades 10 and 5, and the genotypes were found in three sampling locations. An association of genotypes with countries, regions, or host species was not observed in our study. The diversity of transferrin receptors has been shown to possibly relate with the need for antigenic variation to escape from host immune responses[48]. This is the first report of molecular detection of *T. evansi* in Syria. Therefore, additional epidemiological study of the parasites is necessary. Further investigations in cattle and other livestock animals is also required to improve the control measures against *T. evansi* in Syria.

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Compliance with ethical standards

The authors declare that they have no conflicts of interest.

Availability of data and materials

The obtained sequences are available at GenBank under the following accession numbers: LC440031.1, LC519566.1, LC519567.1, LC519568.1, LC519569.1, LC519571.1, LC519572.1, LC519573.1, LC519574.1, LC519575.1, LC519576.1, LC519577.1, LC519578.1, LC519579.1, and LC519580.1.

Supplemental data

Supplemental data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.68.2.117

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