J. Parasitol., 104(5), 2018, pp. 539–543

PARASITOLOGICAL AND MOLECULAR DETECTION OF CANINE TRYPANOSOMIASIS FROM RIYADH PROVINCE, SAUDI ARABIA

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ABSTRACT: *Trypanosoma evansi* is the most widespread of the pathogenic salivarian trypanosomes; it causes a serious disease called surra that affects domestic animals such as camels, horses, and dogs, and often leads to reduced productivity and economic losses. Therefore, the objectives of the present study were to determine the prevalence rates of trypanosomiasis using 3 parasitological tests (wet blood film, Giemsa staining, and microhematocrit centrifugation technique) and polymerase chain reaction (PCR) among stray dogs from Riyadh Province, Saudi Arabia. In the current study, 117 dog blood samples collected from certain districts of Riyadh Province showed that 5 of 117 dogs (4.3%) were positive for the genus *Trypanosoma*. In addition, the findings indicated no effect of dog gender or age on parasite infection. For a more specific diagnosis, PCR amplification of the *RoTat 1.2 VSG* gene in 5 internal transcribed spacer1-positive samples diagnosed with *Trypanosoma* indicated that 2 were positive for *RoTat 1.2 T. evansi*. The absence of the *RoTat 1.2 VSG* gene in 3 of the 5 *T. evansi*-positive samples could be explained by the circulation of *T. evansi* type B in dogs from Saudi Arabia. Thus, this is the first study demonstrating *T. evansi* type B outside of Africa.

Trypanosoma evansi is the most widely distributed pathogenic species, affecting domestic and wild animals in tropical and subtropical countries, and it causes a significant disease called surra (OIE, 2010; Desquesnes et al., 2013). These parasites can be transmitted mechanically by several genera of hematophagous flies such as *Tabanus* and *Stomoxys* species (Luckins and Dwinger, 2004; Desquesnes et al., 2009). Canine trypanosomiasis is characterized by progressive anemia, anorexia, cachexia, fever, generalized weakness, edema of the head and throat, and finally, staggering gait (Chaudhuri et al., 2009; Eloy and Lucheis, 2009).

The diagnosis of *T. evansi* infection is still difficult because the clinical signs are varied and nonspecific (Reid, 2002; Herrera et al., 2004). Typically, *T. evansi* diagnosis is mainly based on finding the parasites in smears, wet blood films (WBFs), or by the microhematocrit centrifugation technique (MHCT) (Chowdhury et al., 2005; Gunaseelan et al., 2009; Prasad et al., 2015); however, these techniques are specific but less sensitive, particularly in detecting parasites during low levels of parasitemia. Serological methods have also been used for screening of *T. evansi* infection; nevertheless, antibodies may be missing because of serological latency (Reid, 2002; Birhanu et al., 2015).

Recently, DNA-based technologies including polymerase chain reaction (PCR) have been shown to be very specific and sensitive and have been widely used for the diagnosis of trypanosomiasis infection in camels, horses, cattle, and companion animals (Njiru et al., 2005; Ravindran et al., 2008; Salim et al., 2011; Rjeibi et al., 2015; Hussain et al., 2016). The rRNA internal transcribed spacer 1 (*ITS1*) and *ITS2* have been found to be reliable for phylogenetic separation of closely related species including piroplasms and their subspecies and trypanosomes (Fazaeli et al., 2000; Cox et al., 2005; Njiru et al., 2005). In addition, a variable surface glycoprotein gene of trypanosomes, *RoTat 1.2 VSG*, has been used as a confirmation test for *T. evansi* infection (Claes et al., 2004; Ngaira et al., 2004; Salim et al., 2011; Barghash et al., 2014; Tehseen et al., 2015).

Received 20 December 2017; revised 23 May 2018; accepted 9 July 2018.
DOI: 10.1645/18-16

In Saudi Arabia, *T. evansi* is enzootic in camels, with a prevalence up to 40% (Omer et al., 1998; Al-Khalifa et al., 2009; El Wathig and Faye, 2013; El Wathig et al., 2016) and has a prevalence of 3.0% in equines (D. A. Alanazi et al., unpubl. data). Since prevalence studies of canine trypanosomiasis are lacking in Saudi Arabia, the current study was designed to document the prevalence of trypanosomiasis in stray dogs from Riyadh Province using parasitological and molecular methods.

MATERIALS AND METHODS

Study areas

The investigation was conducted from January 2016 to December 2017 in the Riyadh Province of Saudi Arabia. This province has an area of 404,240 km^2 and is located in the central part of Saudi Arabia (24–38°N, 46–43°E) (Fig. 1). Dogs from the stray populations in this province were trapped by live bait traps and randomly selected for inclusion in the study. A field examination station was set up at each location so the dogs could be examined for trypanosomiasis. Additionally, dogs visiting veterinary clinics were included in this study.

Sampling and blood collection

A total of 117 dogs (61 males and 56 females) varying in ages from 0 to $\leq$ 2 yr to >8 yr old was examined for trypanosomiasis. All the dogs were apparently healthy at the time of blood collection. Blood samples were collected from each dog (2–5 ml) from the cephalic vein into ethylenediamine tetra-acetic acid (EDTA) vacutainer tubes (BD Vacutainer® Tube, Gribbles Pathology, Victoria, Australia) and transported to the parasitological laboratory, Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, for DNA extraction.

Parasitological examinations

All blood samples were subjected to parasitological examinations by WBF, blood smear techniques using Giemsa staining as described by Jain (1986), and the MHCT as described by Schalm et al. (1975).

FIGURE 1. Map showing the location of the study area in Saudi Arabia. Color version available online.

DNA extraction

Total genomic DNA (gDNA) was isolated from the blood samples using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) and eluted in 50 or 100 µl of elution buffer as per the manufacturer's instruction. An aliquot between 50 and 100 µl of gDNA from each of the samples was stored at –80 C before being sent to the Molecular Laboratory, School of Biological and Marine Sciences, Plymouth University for PCR analysis. At the Molecular Laboratory, gDNA was stored at –20 C for up to 1 mo before molecular diagnostics.

Molecular analysis

Two-step PCR protocols were used to analyze the *Trypanosoma* spp. in DNA samples. First, the DNA samples were used to amplify a 250–700-base pair (bp) region of the *ITS* gene using the forward primer ITS1 CF (5′-CCGGAAGTTCACCGATATTG-3′) and reverse primer ITS1 BR (5′-TGCTGCGTTCTTCAAC GAA-3′); these primers are specific for parasites belonging to the genus *Trypanosoma* (Njiru et al., 2005). Then, all species-positive *ITS1 T. evansi* isolates (480 bp) were further subjected to a PCR test specific for *T. evansi*, in which a primer set that amplifies 151 bp of the *T. evansi RoTat 1.2 VSG* gene fragment was used (Konnai et al., 2009): TeRoTat920F (5′-CTGAAGAGGTTG GAAATGGAGAAG-3′) and TeRoTat1070R (5′-GTTTCG GTGGTTCTGTTGTTGTTA-3′). All PCRs were performed in a final volume of 50 µl containing 25 µl of Dream Taq DNA polymerase master mix 2× (Thermo Scientific™, Nalgene, U.K.), 0.4 µM (1 µl) of each primer, and 2 µl of DNA template. The reaction was brought to 50 µL total volume with PCR-grade water (Invitrogen, Paisley, U.K.). Positive and negative controls were included in all assays. Thermal cycling conditions for *Trypanosoma* species consisted of an initial 2-min incubation at 95 C; followed by 40 cycles of denaturation at 95 C for 30 sec, primer

TABLE I. Numbers and prevalence of trypanosomiasis infection in dogs using different methods. PCR, polymerase chain reaction; WBF, wet blood film; MHCT, microhematocrit centrifugation technique; ITS1, internal transcribed spacer 1.

		Parasitological examinations						PCR methods			
Gender	No. examine	Smear	%	WBF	%	MHCT	%	ITS1	%	Rotat VSG	%
Male	61	2	3.3	3	5	3	5	3	5	1	1.6
Female	56	1	1.8	1	1.8	1	1.8	2	3.6	1	1.8
Total	117	3	5.6	4	3.4	4	3.4	5	4.3	2	1.7

TABLE II. Age-based prevalence of trypanosomiasis infection in dogs using different methods. PCR, polymerase chain reaction; WBF, wet blood film; MHCT, microhematocrit centrifugation technique; ITS1, internal transcribed spacer 1.

		Parasitological examinations						PCR methods			
Age (yr)	No. examine	Smear	%	WBF	%	MHCT	%	ITS1	%	Rotat VSG	%
<2	43	1	2.3	2	4.6	1	2.3	1	2.3	0	0
2–4	28	1	3.6	1	3.6	2	7.1	1	3.6	1	3.6
4–6	21	1	4.8	0	0	1	4.8	2	9.5	1	4.8
6–8	25	0	0	1	8	0	0	1	8	0	0
Total	117	3	5.6	4	3.4	4	3.4	5	4.3	2	1.7

annealing (58 C for 30 sec) and extension at 72 C for 1 min; and a final extension step at 72 C for 5 min, after which the samples were held at 4 C. Aliquots of 10 µl of PCR product were electrophoresed on a 1.5% agarose gel containing 10 µl/ml SYBR Safe (Thermo Scientific™) in Tris-acetate–EDTA buffer at 100 V for 45 min and photographed under a UV imaging system (ImageQuant Laz4000, GE Healthcare Life Science, Hammersmith, U.K.) The size of each product was estimated by comparison with a Gene Ruler 100-bp DNA ladder marker (Thermo Scientific™).

Statistical analysis

Statistical analyses were performed with the statistics package SPSS (v.17.0; IBM, New York, New York). The relationships between infection rate and risk factors such as gender and age were analyzed by the chi-square test of significance, which provided a P value ($P < 0.05$).

RESULTS

A summary of trypanosomiasis infection in male and female dogs is shown in Table I. All dog ages and the prevalence of infection with *T. evansi* are provided (Table II). The findings indicated that dog gender and age did not affect trypanosomiasis infection. The results obtained from PCR using the ITS1 CF/ITS1 BR primer set showed that 5 of 117 dogs (4.27%) were positive for the genus *Trypanosoma* (Fig. 2). For more specific diagnosis, PCR amplification was performed targeting the *RoTat 1.2 VSG* gene on the 5 *ITS1*-positive samples diagnosed with *Trypanosoma*. Results indicated that two were positive for *RoTat 1.2* and thus for *T. evansi* (Fig. 3).

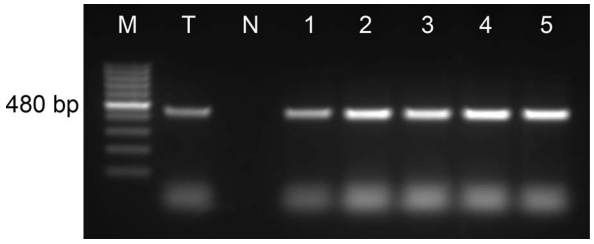

FIGURE 2. Agarose gel electrophoresis (1.5%) of amplified DNA from the collected samples using internal transcribed spacer (ITS1) CF/ITS1 BR. M, 100-base pair (bp) molecular size marker (GeneRuler); lane T, *Trypanosoma* spp. DNA positive control; N, negative polymerase chain reaction control (water); lanes 1–5, template DNA isolated from stray dog blood samples from Riyadh Province. Arrows indicate the 480-bp amplicons specifically generated using the ITS1 CF/ITS1 BR primer set.

DISCUSSION

This is the first parasitological and molecular diagnosis of trypanosomiasis in dogs in Middle Eastern countries. Nevertheless, *T. evansi* is a common protozoan infection in camels in this part of the world (Barghash et al., 2014). Previous studies from Saudi Arabia have estimated trypanosomiasis infection levels in camels ranging between 0 and 40% (Omer et al., 1998; Al-Khalifa et al., 2009; El Wathig and Faye, 2013; Al-Afaleq et al., 2015). Unfortunately, there were no available data to compare the results of the present study with local or regional studies on *T. evansi* infection in dogs because infection in dogs is not frequent; contamination could occur through the exposure of dogs to several hematophagous vector species or orally by the ingestion of contaminated meat (Raina et al., 1985).

In the current study, the results showed that diagnosis with parasitological examinations was insufficient to confirm trypanosomes to the species level, in contrast with PCR methods. The *ITS1* PCR test is increasingly used in surveys for *T. evansi* confirmation in dogs (Aref et al., 2013; Rjeibi et al., 2015; Jaimes-Dueñez et al., 2017). However, this method has drawbacks such as the low analytical sensitivity of the *ITS1* CF and BR primers, which reach approximately 10 pg (100 trypanosomes) for *Trypanozoon*, and their inability to differentiate between the different subgroups of *Trypanosoma* (Cox et al., 2005; Njiru et al., 2005). Therefore, the variable surface glycoprotein of trypanosomes, RoTat 1.2 VSG, was used as a target to distinguish *T. evansi* from other *Trypanosoma* species (Urakawa et al., 2001; Salim et al., 2011, 2014; Barghash et al., 2014; Birhanu et al., 2016; Tehseen et al., 2017). The low positive numbers of *T. evansi*

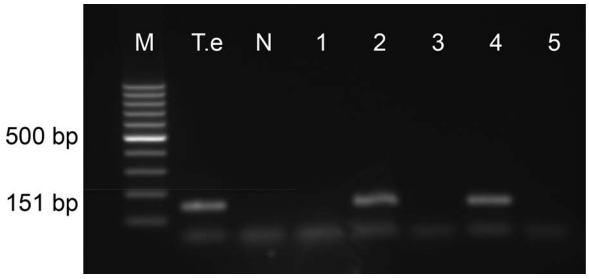

FIGURE 3. Agarose gel electrophoresis (1.5%) of amplified *RoTat 1.2 VSG* gene of *T. evansi* using the specific primers TeRoTat920F and TeRoTat1070R. M, 100-base pair (bp) molecular size marker (GeneRuler); lane T.e, *T. evansi*-positive control DNA; N, negative polymerase chain reaction control (water); samples 2 and 4 are weak positive; samples 1, 3, and 5 are negative. Arrows indicate the 151-bp amplicons specifically generated using the TeRoTat920F/TeRoTat1070R primer set.

infections detected in the current study based on the *RoTat 1.2 VSG* gene compared with the results of the *ITS1* PCR agreed with the results of previous studies (Njiru et al., 2005; Salim et al., 2011; Elhaig et al., 2013; Barghash et al., 2014; Tehseen et al., 2015). Nevertheless, mixed infection by *T. evansi* type A and type B is also possible, since these strains have been identified from Kenya (Ngaira et al., 2004; Njiru et al., 2006), Sudan (Salim et al., 2011), and Ethiopia (Birhanu et al., 2015, 2016). This study reported for the first time *T. evansi* type B outside of Africa; the reason could be related to the export of livestock from eastern Africa, particularly from Sudan, Djibouti, and Somalia, to Saudi Arabia.

In the present study, the results revealed that *T. evansi* infection was not affected by the sex of dogs; both male and female were equally susceptible. These results are in agreement with those of previous studies from other parts of the world (Chowdhury et al., 2005; Prasad et al., 2015). In addition, the results of the present study showed that all ages were similarly susceptible to *T. evansi* infection, in agreement with the findings of Rashid et al. (2008), Gunaseelan et al. (2009), Prasad et al. (2015, and Rjeibi et al. (2015).

CONCLUSION

In the present study, *T. evansi* infection in Saudi dogs was reported for the first time, and the detailed diagnostic PCR protocol was provided. Additionally, this study demonstrated the presence of *T. evansi* type B outside of Africa for the first time. Further studies are necessary to estimate the prevalence rates of *T. evansi* infection in Saudi dogs and to prevent parasite infection.

ACKNOWLEDGMENTS

This study was revised and approved by the Ethics Committee, Department of Biological Science, Shaqra University, according to the ethical principles of animal research.

This project was kindly supported by Shaqra University, Deanship of Scientific Research. The author thanks the staff members of the Biological Science Department, Faculty of Science and Humanities, Shaqra University, for technical support.

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