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Published in:
Journal of Veterinary Diagnostic Investigation

DOI:
10.1177/1040638718798688

Publication date:
2018

Brief Communication

Journal of Veterinary Diagnostic Investigation
1–4

DOI: 10.1177/1040638718798688
jvdi.sagepub.com

Molecular detection of equine trypanosomiasis in the Riyadh Province of Saudi Arabia

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Abstract. We conducted a cross-sectional study to detect trypanosome infections of horses and donkeys in the Riyadh Province of Saudi Arabia. DNA was extracted from blood samples collected from 368 horses and 142 donkeys, and subjected to universal first ribosomal internal transcribed spacer region (ITS1)-PCR followed by *Trypanosoma evansi* species–specific RoTat1.2-PCR. The universal ITS1-PCR revealed *T. evansi* infection in horses ($n = 12$; 3.3%) and donkeys ($n = 4$; 2.8%). There was no significant effect of sex or age on the prevalence of trypanosomiasis in horses or donkeys. Application of the RoTat1.2-PCR revealed that the RoTat1.2 *VSG* gene was absent from the positive ITS1-PCR samples of 3 horses and 1 donkey. This discrepancy could be explained by the circulation of *T. evansi* type B in Saudi Arabia; however, this suspicion requires confirmation.

Key words: Equine; ITS1; PCR; RoTat1.2; Saudi Arabia; trypanosomiasis.

Trypanosomiasis, or surra, caused by *Trypanosoma evansi*, is a serious disease that affects camels and horses in tropical and subtropical countries and often leads to reduced productivity and economic losses.[6] The parasite is found in both intra- and extravascular fluids of multiple hosts and is transmitted mechanically by several genera of hematophagous flies such as *Tabanus, Chrysops, Atylotus*, *Haematopota*, and *Stomoxys*.[11] In camels and horses, trypanosomiasis is found in acute and chronic forms, and clinical signs include intermittent fever, lacrimation, conjunctival petechiae, anemia, edema, enlarged lymph nodes, abortion, decreased fertility, and loss of body weight, which can result in death.[11]

In Saudi Arabia, the horse population is estimated to be >33,000 (http://www.fao.org/faostat/en/#data/QA, using filters: Saudi Arabia, Stocks, Horses, 2016), and more than 500 horses are imported annually from different countries such as the United Arab Emirates, the United States, and Europe. Horses are used for various purposes, including husbandry activities, transportation, racing, showing, and breeding. Diagnosis of equine trypanosomiasis relies on conventional parasitologic examinations and serologic assays.[2,3] DNA-based technologies including PCR have been widely used in the diagnosis of trypanosomiasis infection in camels, horses, cattle, and pets, given its sensitivity and specificity in detecting all stages of parasitic infection.[4,12,14,15,17] There is little information available on the prevalence or epidemiology of equine trypanosomiasis in Saudi Arabia. Thus, we aimed to estimate the prevalence of *Trypanosoma* spp. in horses and donkeys in the Riyadh Province of Saudi Arabia.

Our study was reviewed and approved by the Ethics Committee of the Department of Biological Science, Shaqra University (Riyadh, Saudi Arabia). This cross-sectional investigation was conducted from May 2015 to August 2017 in Riyadh Province, which is in the central part of Saudi Arabia between 24º38ºN and 46º43º E (Fig. 1). Riyadh Province has very hot summers, when the temperature approaches 50ºC. The average high temperature in July is 45ºC. Winters are cold but seldom dropping below 0ºC, with windy nights. The overall climate is arid, receiving very little annual rainfall (21.4 mm), with a relative humidity of 10–47% throughout the year. Riyadh Province is also known to have many dust storms (http://sdwebx.worldbank.org/climateportal/index.cfm?page=country_historical_climate&ThisCCode=SAU#).

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Figure 1. Map of Riyadh Province, Saudi Arabia.

Blood samples ($n = 510$) were collected from 368 horses and 142 donkeys. All of the animals were apparently healthy at the time of blood collection. Blood samples were collected from each animal (5–10 mL) from the jugular vein into blood collection tubes (BD EDTA Vacutainer tube, Gribbles Pathology, VIC, Australia) and transported to the parasitology laboratory at the Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, for DNA extraction.

Total genomic DNA was isolated (DNeasy blood and tissue kit, Qiagen, Hilden, Germany), eluted in 100 μL of elution buffer according to the manufacturer's instructions, and stored at −80°C prior to being sent to the molecular laboratory at the School of Biological and Marine Sciences, University of Plymouth (Plymouth, UK) for PCR analysis.

A 2-step PCR protocol was used to detect *Trypanosoma* spp. in DNA samples. In the first step, first ribosomal internal transcribed spacer (ITS1)-PCR was performed to amplify a 250-700-bp fragment from the ITSI region using the ITS1 CF forward primer (5'-CCGGAAGTTCACCGATATTG-3') and the ITS1 BR reverse primer (5'-TGCTGCGTTCTTCAACGAA-3'), which differentiates trypanosome species by their respective band size, with 250, 400, 480, and 700 bp indicating *T. vivax, T. simiae, T. brucei* subspecies, and *T. congolense* savannah, respectively.[12] Then, all positive 480-bp ITS1-PCR samples were further subjected to *T. evansi* species–specific RoTat1.2-PCR using a primer set that amplifies 151 bp of the *T. evansi* RoTat1.2 *VSG* gene (TeRoTat920F: 5'-CTGAAGAGGTTGGAAATGGAGAAG-3'; TeRoTat1070R: 5'-GTTTCGGTGGTTCTGTTGTTGTTA-3').[10] The 2 PCRs were performed in a final reaction volume of 50 μL, containing 25 μL of 2× DNA polymerase master mix (Dream Taq, Thermo Scientific, Nalgene, UK), 0.4 μM (1 μL) of each primer, and 4 μL of DNA template; the reaction was brought to the final volume of 50 μL with 19 μL of PCR-grade water (Invitrogen, Paisley, UK). Positive and negative controls were included in each reaction. The thermocycling conditions consisted of an initial 2-min incubation at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 58°C for 30 s and extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min, after which the samples were held at 4°C. The PCR product was electrophoresed on 1.5% agarose gel containing 10 μL/mL SYBER Safe (Thermo Scientific) in Tris acetate–EDTA buffer at 100 V for 45 min and photographed under ultraviolet transilluminators (ImageQuant Laz4000, GE Healthcare Life Science, Hammersmith, UK). The size of each product was estimated by comparison with a GeneRuler 100-bp DNA ladder marker (Thermo Scientific).

To determine the trypanosome species, positive samples of *T. evansi* were sent to Macrogen Europe (The Netherlands) for sequencing of the ITS1 region, and the results were compared with sequences available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/). Phylogenetic analyses were constructed based on comparing the identified sequence in our study with the related sequences from GenBank by the neighbor-joining method with the distance algorithms available in MEGA v.7 (https://www.megasoftware.net/). Bootstrap values were determined with 1,000 replicates of the datasets. The sequence was deposited in GenBank under accession MH087231 (see Supplementary Figure 1).

Statistical analyses were performed (SPSS v.17.0, IBM, New York, NY). Animals were divided into 2 age groups: animals ⩽5-y-old (young) and animals >5-y-old (mature). The associations between the prevalence of *Trypanosoma* and risk factors such as sex and age were determined using the chi-square test; $p \leqslant 0.05$ was considered significant.

ITS1-PCR revealed that the prevalence of *T. evansi* infection was 3.3% in horses and 2.8% in donkeys (Table 1; Fig. 2). However, RoTat1.2-specific PCR revealed that 4 samples that tested positive by ITS1-PCR were negative for the *RoTat1.2* gene (Fig. 3). Phylogenetic data and BLAST results of ITS1 showed that *T. evansi* is closely related to *T. evansi* isolate STIB 780, accession KU552341, from Kenyan camels (Fig. 4). Both horses and donkeys in Riyadh Province were infected with a single trypanosome species (*T. evansi*), and no mixed-species infection was found. There was no significant effect of sex or age on the prevalence of *T. evansi* in horses or donkeys (Table 1).

We detected only *T. evansi* and not *T. vivax* using PCR with the generic primers ITS1 CF and ITS1 BR, which allow for the detection and differentiation of all pathogenic trypanosomes, as has been shown in previous studies.[12,15,16] In contrast, the *T. evansi* type A species–specific RoTat1.2-PCR confirmed the presence of parasite DNA in 12 ITS1-PCR–positive samples and its absence in 4 ITS1-PCR–positive samples. The latter finding could be attributed to the presence

Table 1. Chi-square analysis of the association of sex and age risk factors with *Trypanosoma* spp. infection in horses and donkeys in the Riyadh Region of Saudi Arabia.

	Horses				Donkeys			
Risk factor	*n*	Positive	χ^2	*p* value	*n*	Positive	χ^2	*p* value
Sex								
Male	127	3 (2.4)	0.481	0.496	55	1 (1.8)	0.567	0.327
Female	241	9 (3.7)			87	3 (3.4)		
Total	368	12 (3.3)			142	4 (2.8)		
Age								
⩽5 y	217	4 (1.8)	3.368	0.066	49	0 (0.0)	2.168	0.141
>5 y	151	8 (5.3)			93	4 (4.3)		
Total	368	12 (3.3)			142	4 (2.8)		

$p > 0.05$ = nonsignificant. Numbers in parentheses are percentages.

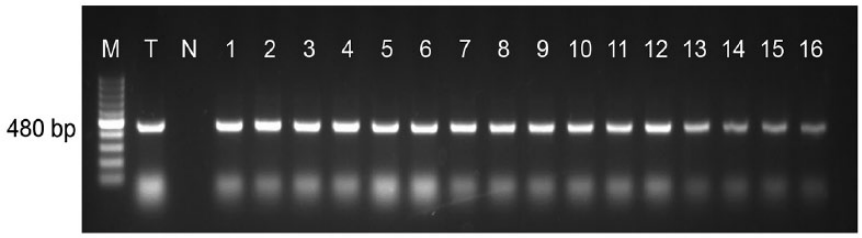

Figure 2. Results of the 1.8% agarose gel electrophoresis showing amplified DNA of the 480-bp band that is specific for *Trypanosoma brucei* subspecies (*T. evansi*) using ITS1-PCR. Lane M = 100-bp molecular size marker (GeneRuler); lane T = *Trypanosoma* spp. positive control DNA; lane N = negative PCR control (water); lanes 1–12 = template DNA isolated from horse blood samples; lanes 13–16 = template DNA isolated from donkey blood samples.

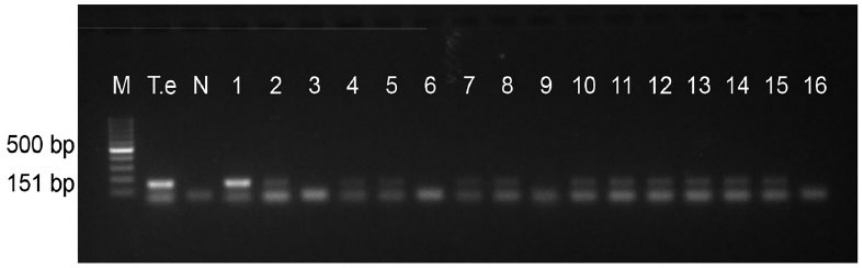

Figure 3. Results of 1.8% agarose gel electrophoresis separating the amplicons of the RoTat1.2 *VSG* gene of *Trypanosoma evansi*. Lane M = 100-bp molecular size marker (GeneRuler); lane T.e = *T. evansi* positive control DNA; lane N = negative PCR control (water); lanes 1–12 = template DNA isolated from horse blood samples; lanes 13–16 = template DNA isolated from donkey blood samples. All samples were weakly positive except samples 3, 6, 9, and 16, which were negative. The 151-bp band is specific for *T. evansi* using the TeRoTat920F/TeRoTat1070R primer set.

of non–RoTat1.2 *T. evansi* type B outside of Africa, although this should be confirmed by further investigation.[17]

Overall, *T. evansi* was more prevalent in horses ($n = 12$; 3.3%) than in donkeys ($n = 4$; 2.8%) in our study, but the prevalence in horses was lower than identified in other studies. In Jordan, the prevalence of *T. evansi w*as reported to be 9.6% ($n = 83$) in horses and 0.0% ($n = 40$) in donkeys.[1] In Sudan, trypanosomes were more prevalent in horses 12.7% ($n = 393$) than in donkeys (3.4%, $n = 116$).[15] However, in Gambia, the prevalence of trypanosome infection was 93.4% ($n = 183$) in horses and 82.7% ($n = 58$) in donkeys.[8] In Israel, the seroprevalence of *T. evansi* was 4.6% ($n = 614$) in horses.[5] In Pakistan, the prevalence of *T. evansi* was 0.5% ($n = 375$) in horses with the Woo test, 1.3% with both ITS1 CF/BR PCR and RoTat1.2 PCR, and 14.4% with a card agglutination test for trypanosomiasis (CATT)/*Trypanosoma evansi.*[17] However, in India, PCR testing indicated that 6.8% ($n = 44$) of donkeys were infected with *T. evansi.*[13]

Figure 4. Phylogenetic relationships based on the partial nucleotide sequences (4983-bp) of ITS1 ribosomal DNA of *Trypanosoma* (Riyadh equine) with other variants of *T. evansi* in GenBank. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model. Evolutionary analyses were conducted in MEGA v.7. Numbers at nodes are bootstrap values derived from 1,000 replicates.

Differences in results from previous studies may be attributed to management and environmental differences of the studies as well as sample sizes and methodology (i.e., microscopic, serologic, and PCR techniques). The lower prevalence of infection in donkeys than horses could be related to the feeding preference of vectors for horses over donkeys or may be related to the behavior of donkeys, which prevent flies from feeding by their head movements.[8]

The nonsignificant difference between the prevalences of infection according to sex or age group is in agreement with previous studies, which demonstrated that sex and age groups are equally exposed to and affected by trypanosomiasis.[7,17] However, other studies indicated that male and adult donkeys were more often affected by trypanosomiasis than female and young donkeys.[9]

Acknowledgments

We thank the staff members of the Biological Science Department, Faculty of Science and Humanities, Shaqra University, and the staff members of the molecular laboratory at the School of Biological Science, University of Plymouth, UK for their technical support.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This study was supported by the Deanship of Scientific Research at Shaqra University.

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