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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 in 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.
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 \((5–10 \text{ mL})\) 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 \(\mu\text{L}\) of elution buffer according to the manufacturer’s instructions, and stored at \(-80^\circ\text{C}\) 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 \(Tryptosoma\) 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 ITS1 region using the ITS1 CF forward primer \((5’-\text{CCGGAAGTTCCAGGATTTG}-3’)\) and the ITS1 BR reverse primer \((5’-\text{TGCTGCTTCTCAACGAA}-3’)\), 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 \((\text{TeRoTat920F: 5’-CTGAAGAGGTGGAAATGGAGAAG-3’; TeRoTat1070R: 5’-GTTCTGTTGTCTGGTTGTGTAA-3’})^{10}\). The 2 PCRs were performed in a final reaction volume of 50 \(\mu\text{L}\), containing 25 \(\mu\text{L}\) of 2× DNA polymerase master mix (Dream Taq, Thermo Scientific, Nalgene, UK), 0.4 \(\mu\text{M}\) (1 \(\mu\text{L}\)) of each primer, and 4 \(\mu\text{L}\) of DNA template; the reaction was brought to the final volume of 50 \(\mu\text{L}\) with 19 \(\mu\text{L}\) 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 a 1.5% agarose gel containing 10 \(\mu\text{L/mL}\) 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 \(\leq 5\)-y-old (young) and animals \(>5\)-y-old (mature). The associations between the prevalence of \(Tryptosoma\) and risk factors such as sex and age were determined using the chi-square test; \(p \leq 0.05\) 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.\(^{12,15}\) 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...
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 was 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

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.

| Risk factor | Horses | | Donkeys | |
|-------------|--------|---------|---------|
|             | n | Positive | χ² | p value | n | Positive | χ² | 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.
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.

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. However, other studies indicated that male and adult donkeys were more often affected by trypanosomiasis than female and young donkeys.

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