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Field investigation of *Trypanosoma evansi* and comparative analysis of diagnostic tests in horses from Bahawalpur, Pakistan

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Abstract: In order to assess the prevalence of *Trypanosoma evansi*, a parasitological, molecular, and serological-based investigation was carried out in randomly sampled horses (n = 375) belonging to different age groups, sexes, and localities from the district of Bahawalpur, Pakistan. The diagnostic performance of applied tests was also compared. The prevalence was recorded as 0.5% with Woo's test, 1.3% with both ITS CF/BR PCR and RoTat 1.2 PCR, and 14.4% with CATT/*T.evansi*. Based on CATT/*T.evansi*, significant differences ($P \leq 0.05$) were observed for prevalence estimates according to different localities, sexes, body condition scores, and origins. A nonsignificant difference ($P \geq 0.05$) was observed among different age groups and variable parity numbers. Our study declares district Bahawalpur to be a high risk area for surra and proposes the potential use of CATT/*T.evansi* as a field test of choice for surveys in horses. However, the status of seropositive animals should be confirmed using a more sensitive molecular tool such as the satellite DNA target. A widespread status of anti-*Trypanosoma* antibodies calls for control measures and further investigation of various species (camels, cattle, sheep, goats, buffaloes) inhabiting the same area to identify reservoir status.

Key words: Horses, *Trypanosoma evansi*, Bahawalpur

1. Introduction

In Pakistan, horses are bred for different purposes, including agriculture activities, transportation, companionship, racing, display, and breeding. Some major reported ailments are caused by parasites, bacterial infections, lameness, gastrointestinal disorders, allergic dermatitis, bronchitis, hematuria, eye problems, and quidding (1).

Trypanosomosis (surra) is a serious disease of horses and camels in Africa and Asia and often leads to reduced productivity, mortality, and economic losses. It is caused by *Trypanosoma evansi*, a parasite of both intra- and extravascular fluids of multiple hosts, and is mechanically transmitted by hematophagous flies. Its geographical distribution is continuous from the northern part of Africa through the Middle East to Southeast Asia. In horses, *T. evansi* induces both acute and chronic forms of the disease, which is most often fatal and can last up to 3 months to 3 years in latter case. However, in some cases, horses may carry *T. evansi* without showing any clinical signs (2).

The outer coat of *T. evansi* is covered with variable surface glycoproteins (VSGs) that help it to evade the host's immune system by mean of antigenic variation. Owing to regular changes of its VSGs, it produces frequent relapses of parasitaemia and remittent clinical signs. The VSG RoTat 1.2 is expressed in the majority of *T. evansi* type A strains (3). Apart from clinical signs and symptoms, diagnosis in surra-infected animals should be carried out in the lab for confirmation. Serological and molecular tests based on RoTat 1.2 VSG are used as specific diagnostic tools for surra and have been employed in various studies in different countries and hosts, with variable performances (4,5). These include serological tests like direct agglutination test (CATT/*T. evansi*), indirect agglutination test (LATEX/*T. evansi*), immune trypanolysis, ELISA/*T. evansi*, and molecular tests like RoTat 1.2 PCR. CATT/*T. evansi* is considered to be a well-validated field test (6,7).

Since a recent outbreak of trypanosomosis was reported in different animals in the same vicinity, the objective of this study was to investigate the prevalence of *T. evansi* in horses of Bahawalpur, employing parasitological, serological, and molecular analysis including RoTat 1.2-based tests. The diagnostic performance of applied tests was also compared.

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2. Materials and methods

2.1. Study area and design

The district Bahawalpur lies in the south of Punjab at 29°24′0″N, 71°41′0″E with an area of about 24,830 km^2. It has a total horse population of 2657 (8). A total of 375 randomly selected horses were clinically examined at different markets from both rural and urban areas of the district throughout 2014. Every fifth animal was sampled to ensure representativeness and randomness. All horses used in the studies were managed under a traditional system of free grazing. Important study variables like sex, age (as recorded from the owners), body condition (9), history of previous abortions, castrated versus noncastrated, and parity number were also recorded. Animals showing clinical signs were treated with isometamidium chloride (Trypamidium, Mérial, France) via intramuscular injection of 0.5–1 mg/kg.

2.2. Blood sampling

Blood (6 mL) was collected from the jugular vein and 3 mL of blood was placed into a tube containing EDTA for DNA extraction and stored in a cool box until transported to the laboratory. These samples were later aliquoted into microtubes and preserved at –80 °C until further analysis. For serological tests, 3 mL of blood was placed in a serum separator tube with clot activators. After centrifugation for 15 min at 2000 rpm, serum was aliquoted and stored at –80 °C.

2.3. Parasitological examination

2.3.1. Woo's test

Approximately 70 µL of blood was put into a heparinized capillary tube. The dry end was closed with plasticine and it was centrifuged at 14,000 rpm for 5 min. The capillary tube was then examined for parasites under a microscope at a magnification of 400× (10).

2.3.2. Giemsa staining

For the morphological examination of the possible agents in the samples, around 10 µL of samples positive by Woo's test was placed and spread on the center of a clean microscope slide. The smear was air-dried for 1 h followed by staining with Giemsa stain (pH 7.2) for 25 min. After washing with water, the stained smear was air-dried and observed under a microscope at a magnification of 500× with oil immersion (10).

2.4. Serological analysis

Sera were tested for the presence of anti-*T. evansi* antibodies using the card agglutination test for *T. evansi* (CATT/*T.evansi*) (Institute of Tropical Medicine, Antwerp, Belgium). Approximately 45 µL of the antigen was transferred onto the test card and mixed with 25 µL of the test sera diluted at 1/4 with PBS (pH 7.2). The card was rocked for 5 min at 70 rpm, and the reaction was checked in clear light. A reaction was scored as positive if blue agglutinates were visible by naked eye, as recommended by the manufacturer (11).

2.5. Molecular analysis

Genomic DNA was extracted from 250 μL of whole horse blood using a commercially available kit (PureLink Genomic DNA Mini Kit, Invitrogen) and was stored at –80 °C until further use. Two pairs of primers were employed for analysis. Details on the primers used in the molecular tests are shown in Table 1. Both ITS1 CF/BR PCR (12) and RoTat 1.2 PCR (7) were carried out in 25 µL of reaction mixture, which contained 1X Hot Star Taq Master Mix (Qiagen), 0.8 µM of forward and reverse primers, and 2.5 µL of template DNA. PCR conditions for ITS1 CF/BR PCR included activation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 90 s, with final extension at 72 °C for 5 min. For RoTat 1.2 PCR, the cycles included initial activation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. Final elongation was continued at 72 °C for 5 min. A positive and a negative control were included for each PCR reaction. Amplified products were analyzed by electrophoresis in 1.5% agarose gel and UV illumination after ethidium bromide staining of the DNA.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 15. The level of agreement between diagnostic tests was determined using kappa statistics (k) and was interpreted according to Landis and Koch (13). The influence on prevalence of various factors, such as sex, age, body condition, or parity number, was determined using the chi-square test along with odds ratios (ORs) and their 95% confidence intervals (CIs).

Table 1. Details on the primers used in the molecular tests.

Name	Specificity	Primer sequence	Amplicon size	Reference
ITS1CF/BR PCR	*Trypanozoon*	F:5'-CCGGAAGTTCACCGATATTG-3' R:5'-TGCTGCGTTCTTCAACGAA-3'	480 bp	Njiru et al. (12)
RoTat 1.2 PCR	*T. evansi*	F:5'-GCGGGGTGTTTAAAGCAATA-3' R:5'-ATTAGTGCTGCGTGTGTTCG-3'	205 bp	Claes et al. (7)

3. Results

A total of 375 horses were screened for *T. evansi* infection. The overall prevalence was recorded as 0.5% (95% CI: 0.01–2.01) by Woo's test and Giemsa thin smears (Figure 1), 14.4% (95% CI: 10.85–17.95) by CATT/*T.evansi*, and 1.3% (95% CI: 0.15–2.45) by ITS1 CF/BR PCR and RoTat 1.2 PCR (Figures 2 and 3). The animals identified as positive by Woo's test and PCRs showed clinical signs and were also positive by CATT/*T. evansi* test.

Notsignificant differences (P > 0.05) were observed between different age groups and variable parity numbers by different tests. Using CATT/*T.evansi*, prevalence estimates were significantly higher in females than males (χ^2 = 4.96, P = 0.030). A significant difference (P < 0.05) was also observed for horses in urban and rural areas using both PCRs (χ^2 = 11.04, P = 0.001) and CATT/*T.evansi* (χ^2 = 30.57, P = 0.000). Emaciated animals had significantly (P < 0.05) higher infection rates than those in good body condition by Woo's test (χ^2 = 17.8, P = 0.000), PCRs (χ^2 = 44.94, P = 0.000), and CATT/*T.evansi* (χ^2 = 81.55, P = 0.000). Emaciated animals were almost 18 times more likely to be suffering from surra than the ones in good health (OR = 17.5, 95% CI: 8.218–37.448). Significant differences (P < 0.05) were observed between animals bought outside and from a farm by Woo's test (χ^2 = 4.37, P = 0.04) and PCR (χ^2 = 11.04, P = 0.001) (Table 2).

The test agreement of Woo's test was moderate with both molecular tests (k = 0.57, 95% CI: 0.125–1.000), and it was slight with CATT/*T.evansi* (k = 0.053, 95% CI: –0.058 to 0.124). The agreement between both the molecular tests was perfect (k = 1), while they showed slight agreement with CATT/*T.evansi* (k = 0.13, 95% CI: 0.025–0.234).

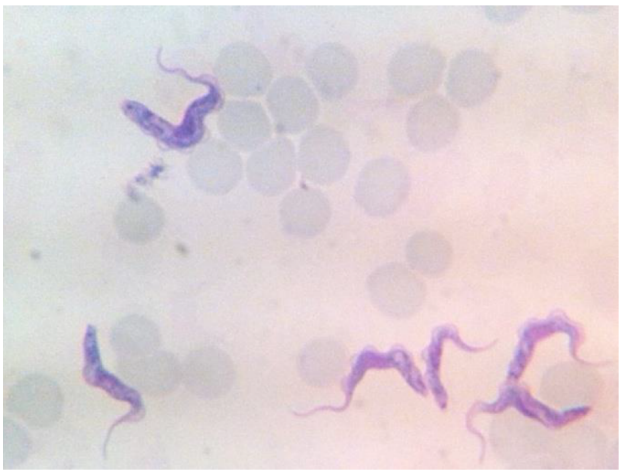

Figure 1. Giemsa-stained *Trypanosoma evansi* in horse blood (1000×).

4. Discussion

Various studies have reported surra in horses in Punjab, Pakistan (14,15). This is the first report of *T. evansi* using different techniques including those based on RoTat 1.2 VSG in this district. Woo's test is considered more sensitive than microscopic examination of stained thin smears. The low percent positivity of *T. evansi* by Woo's test in our studies can be attributed to the test's limited sensitivity and ability to detect animals with early and acute infections only. The lowest detection limit of Woo's test was estimated at 30 trypanosomes per milliliter of blood (16). Verloo et al. (6) reported that water buffaloes positive for *T. evansi* by the mouse inoculation test were negative by Woo's test.

PCR-based techniques have been claimed to be more sensitive and specific for detection than conventional microscopy and analytical sensitivities of 1–20 parasites/mL have been reported with highly repetitive satellite DNA targets (17,18). However, as in our case, the number of ongoing infections is underestimated by PCR and may generate false negatives owing to fluctuating levels of parasitaemia, inherent detection limits of the PCR, treatment with trypanocidal drugs, and quality and proportion of the DNA from the infectious agent relative

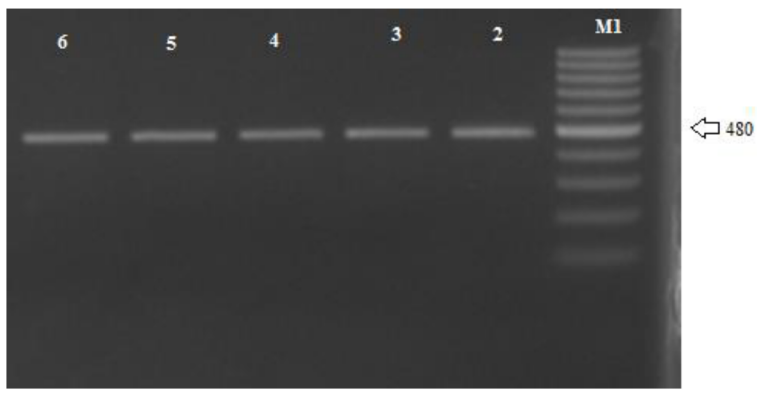

Figure 2. ITS1 CF/BR PCR products (lanes 2–6) and gene ruler (lane 1) on 1.5% agarose gel stained with ethidium bromide.

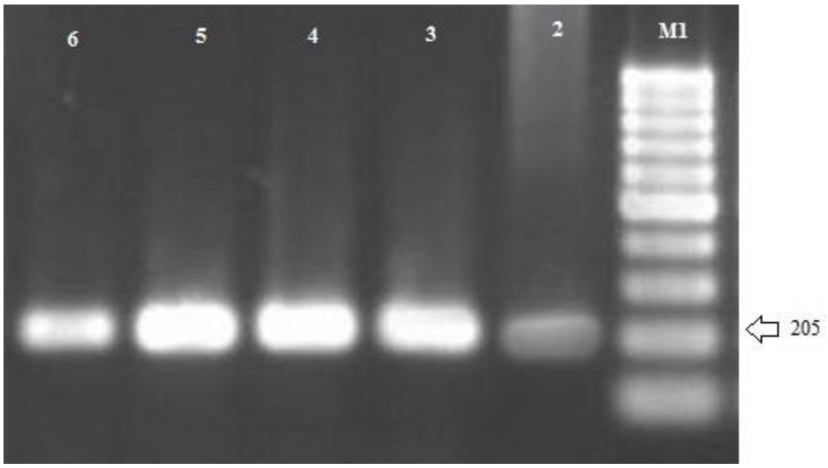

Figure 3. RoTat 1.2 PCR products (lanes 2–6) and gene ruler (lane 1) on 1.5% agarose gel stained with ethidium bromide.

Table 2. Number and percentage (%) of positive samples in the different diagnostic tests according to age, sex, area, body condition, origin, history of abortions, and parity number. Pos = positive, yrs = years, BC = body condition.

			Woo's test	ITS1 CF/BR PCR	RoTat 1.2 PCR	CATT
Variables		Total	Pos (%)	Pos (%)	Pos (%)	Pos (%)
Age	Adult (>6 yrs)	275	2 (0.7)	5 (1.8)	6 (1.8)	38 (13.8)
	Young (< 6yrs)	100	0 (0.0)	0 (0.0)	0 (0.0)	16 (16.0)
Sex	Male	156	0 (0.0)	0 (0.0)	0 (0.0)	15 (9.6)
	Female	219	2 (0.8)	5 (2.0)	5 (2.0)	39 (17.8)
Area	Urban	179	0 (0.0)	0 (0.0)	0 (0.0)	17 (9.5)
	Rural	196	2 (1.0)	5 (2.6)	5 (2.6)	37 (18.9)
BC	Good	337	0 (0.0)	0 (0.0)	0 (0.0)	30 (8.9)
	Emaciated	38	2 (5.3)	5 (13.2)	5 (13.2)	24 (63.2)
Origin	Born outside farm	118	2 (1.7)	5 (4.2)	5 (4.2)	22 (18.6)
	Born on farm	257	0 (0.0)	0 (0.0)	0 (0.0)	32 (12.5)
Parity number	>6	18	0 (0.0)	0 (0.0)	0 (0.0)	2 (11.11)
	<6	182	2 (1.1)	5 (2.7)	5 (2.7)	36 (19.8)

to the total DNA in the blood sample (19). The present study employed both RoTat 1.2 PCR and ITS CF/BR PCR to confirm the molecular prevalence. ITS1 CF and ITS1 BR have been evaluated for use in a universal diagnostic test for all pathogenic trypanosomes and cannot differentiate between different species, unlike RoTat 1.2 PCR, which is specific for *T. evansi*. The perfect agreement between different PCRs in the present study confirms the actual presence of parasite DNA in 5 samples and confirms the absence of strains that either lack RoTat 1.2 VSG genes or do not express it (20). Three PCR-positive animals were negative by Woo's test and this may be due to early infection or the chronic form of the disease with low parasitaemia. The gold standard (TBR1/2) PCR (21) targeting satellite DNA could be used in further studies to increase the sensitivity of DNA detection, and it could help to reach a conclusion on the status of samples positive by the CATT but negative by Woo's test and ITS1 or RoTat 1.2 PCR.

For medium-sized routine field surveys on surra, CATT/*T.evansi* is a well-validated test; however, it can

overestimate the prevalence as antibodies may persist in the animals for up to several months and possibly due to nonspecific agglutinations owing to crude extracts of the parasite (22). A seroprevalence of 47.7% by CATT/*T. evansi* was recorded in camels in the same locality by us, using CATT/*T.evansi* for antibodies against surra (23). Since this is the first time that CATT/*T.evansi* has been used for analyzing the seroprevalence of surra, we cannot compare our results with any other study from Pakistan. Aslam et al. (24) and Nadeem et al. (25) reported 21.6% and 6% seroprevalence in equine samples from Lahore and Gujranwala using ELISA and immunofluorescence. ELISA is more suitable for large-scale surveys due to automation of the technique. As per OIE recommendations (10), an animal that tested positive for surra should also be tested with CFT-dourine. Moreover, Claes et al. (26) proposed that CATT/*T.evansi* can replace laboratory-based tests, such as the complement fixation test (CFT) and the horse CFT for equine trypanosomosis. Therefore, the presence of antibodies against *T. equiperdum* cannot be ruled out. A high percent positivity of antitrypanosomal antibodies is indicative of the fact that this is an endemic area and there is a high threat to equine populations.

Regarding the nonsignificant difference between age groups, our study is in concordance with the study of Eyob et al. (27), suggesting that all age groups are equally exposed and effected by surra. The higher infection rates in females in general and those having parity number ≤6 are in accordance with the studies of Sumbria et al. (28) and can be attributed to pregnancy and lactation, which may render females more susceptible to infection (29). The lower prevalence in urban areas can be attributed to proper management and availability of veterinary professionals. Horses with poor body scores may be more susceptible to the disease due to stress, poor nutrition, workload, other infections, and, hence, compromised immunity. Our results are in accordance with the results of Gari et al. (30), who reported a similar trend for dourine-suspected horses. Animals bought from outside a farm were more susceptible to the disease, probably owing to the fact that animals reared on a farm are better managed in terms of nutritional status and care by veterinary professionals.

The slight to moderate agreement between different tests can be attributed to the difference in sensitivity and specificity of the applied tests. The low agreement between parasitological tests and others is attributed to low sensitivity and alternating parasitemic waves of *T. evansi* in the host. Although both RoTat 1.2 PCR and CATT/*T. evansi* are based on RoTat 1.2 VSG, the low agreement can be attributed to the intermittent phase of parasitaemia and absence of antibodies in the early onset of the disease. Perfect agreement between the two PCRs confirms the presence of *T. evansi.*

In conclusion, Bahawalpur is at a high risk of surra, showing a high disease status in various groups. Since these are preliminary studies and no test is 100% sensitive or specific, more studies should be carried out to assess the current disease status and comparative diagnostics applying multiple tests. For routine field surveys in horses, the CATT/*T.evansi* test can be used to target animals for treatment, but the decision to treat animals not only depends on CATT positivity but also on the clinical aspect and history of trypanocidal treatment. For large-scale surveys ELISA should be preferred, owing to its subjectivity and reproducibility in borderline cases and ability to detect aparasitemic animals efficiently. Additionally, for possible nonspecific agglutinations in card agglutination tests, the status of the seropositive animal should be confirmed before concluding on the CATT/*T.evansi* performances. Alternatively, the more sensitive gold standard PCR test using TBR primers could be used to confirm the status of seropositive but parasitologically negative samples. Continuous surveillance measures by national veterinary services and control of biting insect populations should be carried out simultaneously to prevent disease dissemination. Further studies should be carried out in multiple species inhabiting the same area to monitor infection and the reservoir status.

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