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Equine trypanosomosis in central and western Punjab: Prevalence, haemato-biochemical response and associated risk factors

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ABSTRACT

The detection of *Trypanosoma evansi* in blood is intricate, primarily in chronic stage of infection, as the parasitaemia is often low and fluctuating. The climatic conditions of the target area of Punjab (a province of India with a total of 34,000 horses and ponies used for sports and transport) are conducive for the parasite propagation. The objective of present investigation was to assess the prevalence of *T. evansi* in central and western Punjab by PCR and card agglutination test (CATT/*T. evansi*) in relation to clinico-haematobiochemical alterations and risk factors associated with latent trypanosomosis. A total of 169 equine blood and serum samples tested by CATT/*T. evansi* revealed 16 cases positive, with 6.8% from central plain and 13.63% from western zone. To assess the specificity of serological test, PCR_1 was performed using established primer pair TR3 5′-GCG CGG ATT CTT TGC AGA CGA-3′ and TR4 5′-TGC AGA CAC TGG AAT GTT ACT-3′ for *T. evansi*. PCR_2 applied with primer pair *RoTat1.2F*: 5′-ATG TCA ACG ATG CCT GTT ACA TTA CGC AC-3′ and *RoTat1.2R*: 5′-TAA ATA TCA CTG TCA AGA CCT GCT GCG G-3′ to rule out the consensus between the finding of the two PCR assays and agglutination test for *T. evansi*, which displayed results in concordance with PCR_1. PCR assays showed 1.92 and 1.51% positive samples from central plain and western zone, respectively. With respect to PCR assay, CATT/*T. evansi* showed 100% sensitivity and 92.1% specificity. Microscopy showed a very low prevalence rate of 0.59% with only one sample positive with teaming parasitaemia. Comparison between sexes revealed higher positivity in mares by the three tests (BSE: 0.95%, PCR: 2.88%, CATT/*T. evansi:* 14.42%). The haemato-biochemical factors were found to be altered in PCR positive cases, while the mean value of vital parameters lied in normal range in seropositive cases. The female horse (RR = 0.0937, 95% CI = 1.388–190.223%) population was found to be at the highest risk of seropositivity for *T. evansi*, particularly in the unorganized farms (RR = 19.726, 95% CI = 2.918–400.221%).

1. Introduction

Trypanosoma evansi elicited trypanosomosis ('Surra') is a widely distributed disease in Asia, Africa and South America (Luckins, 1988), mainly affecting horses, camels, cattle, buffaloes and wild ruminants. Few reports of incidences of *T. evansi* in humans illustrate its significant zoonotic potential (Joshi et al., 2005). Camels and horses are comparatively more susceptible to the infection with high fatality (Claes et al., 2004). The disease renders negative effects on health, decreasing the productivity of livestock.

Generally, the disease is diagnosed on the basis of clinical evidences augmented with some parasitological or serological tests. However, the clinical signs like emaciation, fever, anaemia, lacrimation, corneal opacity and diarrhoea (Chaudhary and Iqbal, 2000) are not sufficient for diagnosis especially during the chronic stage of infection. At the same time, low and fluctuating parasitaemia renders the detection of haemoparasite difficult (Mahmound and Gray, 1980; Nantulya, 1990). Various antibody detection tests such as CATT/*T. evansi*, ELISA and LATEX/*T. evansi* make use of native variable surface glycoprotein (VSG) from the predominant *T. evansi* variable antigen type *RoTat* 1.2 (Verloo et al., 2001). Serological diagnosis based on antibody detection is unable to differentiate an on-going acute infection from chronic one (Luckins, 1977; Wastling and Welburn, 2011). As far as *T. evansi* infection is concerned, CATT/*T. evansi* is highly specific in detecting circulating antibodies with least false positive results (Bajyana and Hamers, 1988). On the other hand, polymerase chain reaction (PCR) assay, appear to be a promising molecular diagnostic technique for the efficient diagnosis of trypanosomal DNA in the blood samples (Gibson, 2009). PCR has been reported to be more sensitive than conventional parasitological techniques (Wuyts et al., 1995; De Almeida et al., 1997; Desquesnes, 1997; Masake et al., 1997; Bashir et al., 2014).

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Table 1
Prevalence of *T. evansi* in equines of central plain and western zone of Punjab.

Zones	District	Total		BSE (%)		CATT/*T. evansi* +++/++ (%)		PCR_1/PCR_2 (%)	
Central plain zone (103)	Amritsar	9	103	0	1 (0.97)	0	7 (6.8)	0	2 (1.92)
	Tarn-taran	24		0		2 (8.33)		0	
	Jalandhar	13		0		1 (7.6)		0	
	Ludhiana	57		1 (1.75)		4 (7.02)		2 (3.51)	
Western zone (66)	Sangrur	10	66	0	0	3 (30)	9 (13.63)	0	1 (1.51)
	Moga	10		0		0		0	
	Bathinda	13		0		1 (7.69)		0	
	Muktsar	33		0		5 (15.15)		1 (3.03)	
Total		169		1 (0.59)		16 (9.47)		3 (1.78)	
χ^2				1.977	0.645	8.680	2.196	2.708	0.042

However, most of PCR assays have not been validated yet under field conditions for the diagnosis of natural infection in equines (Wuyts et al., 1995; Clausen et al., 1998; Donelson et al., 1998) and may give false-negative results when the parasitaemia is very low and in these cases suspicion of potential carriers can be confirmed by serological examination (OIE, 2012).

The scarcity of enough information in the available literature concerning latent equine trypanosomosis in Punjab (India) has prompted authors to evaluate the status of equine trypanosomosis in central plain and western zone of Punjab by polymerase chain reaction (PCR) and card agglutination test (CATT/*T. evansi*) in relation to clinico-haematobiochemical response and risk factors involved.

2. Materials and methods

2.1. Study areas

The province of Punjab covers a total area of 50,362 km^2 between 29′′30′ N to 32′′32′ N latitude and 73′′55′ E to 76′′50′ E longitudes. There are about 34,000 horses and ponies at the risk of infection due to *T. evansi* in Punjab (Fazili and Kirmani, 2011). The study was conducted in the two major agro-climatic zones of Punjab adjoining the Sutlej Basin (Table 1) as one of the previous study conducted in our laboratory revealed the higher risk of trypanosomosis in these areas (Singla et al., 2013).

2.2. Sampling frame

To study the status of molecular and serological prevalence of the disease, the expected prevalence to be 50% with confidence limits of 95% and a desired absolute precision of 5% to collect maximum number of samples was considered. The number of samples thus calculated was adjusted for finite population (Thrusfield, 2005) and correlated with 169 samples (65 horses and 104 mares) from of central plain zone and western zone of Punjab. About 5 ml blood was collected aseptically from the jugular vein of each animal in EDTA coated vials and glass vials for DNA isolation and serum separation, respectively. The extracted DNA and collected sera were stored at −20 °C for further analysis by CATT/*T. evansi* and PCR.

2.3. Blood film

Two thin blood films of each blood sample were prepared, dried and then fixed in absolute methyl alcohol for 1–2 min. The smear was immersed into diluted Giemsa stain for 30–45 min, and then washed with distilled water to remove excess of stain. After that the slides were left to dry and then examined under oil immersion lens (Coles, 1986). Micrometric measurements of parasites were carried out on microscope fitted with micrometry unit by using Software DPZ-BSW (OLYMPUS).

2.4. Serological test – CATT/T. evansi

CATT/*T. evansi* for antibody detection was originally described and converted into a test kit by the Institute of Tropical Medicine, Belgium (Bajyana and Hamers, 1988). Briefly, 25 μl of diluted sera was thoroughly mixed with about 45 μl of well-homogenized CATT antigen. The card was agitated in a circular motion using electric rotator at 60–70 rpm at room temperature for 5 min. Samples having blue granular agglutination were considered positive. Samples were read in comparison with the control wells according to the supplied instructions. Agglutination patterns were scored as − (negative), ± or + (suspected), and ++ or +++ (positive).

2.5. DNA extraction and PCR assay

Genomic DNA was extracted from the blood samples by using the protocol of HiPura™ Blood Genomic DNA Miniprep Purification Spin Kit. The PCR_1 reactions were performed by using forward primer TR3 5′-GCG CGG ATT CTT TGC AGA CGA-3′ and reverse primer TR4 5′-TGC AGA CAC TGG AAT GTT ACT-3′ specific for *T. evansi* (Wuyts et al., 1995), and amplify 257-bp fragment from the genomic DNA. PCR reaction mixture (25 μl) was constituted by 12.5 μl of KAPA 2G Fast HotStart Ready Mix (2× containing KAPA2G fast hotstart DNA polymerase, KAPA 2G fast hot start PCR buffer, 0.2 mM dNTP each, 1.5 mM $MgCl_2$), 1.5 μl of 10 pmol TR3/TR4 primers, 5 μl of template DNA and added 4.5 μl of nuclease-free water to complete the volume. The reaction was set in automated thermocycler with the following programme: initial denaturation at 95 °C (5 min), 30 cycles of denaturation at 94 °C (1 min), annealing at 57 °C (1 min), and extension at 72° C (1.5 min) and final extension at 72 °C (10 min). The amplified PCR products were separated by electrophoresis on 1% agarose gel and visualized under UV transilluminator for detection of 257 bp product size. For PCR_2, a pair of primers *RoTat 1.2 F*: 5′-ATG TCA ACG ATG CCT GTT ACA TTA CGC AC-3′ and *RoTat 1.2 R*: 5′-TAA ATA TCA CTG TCA AGA CCT GCT GCG G-3′ was designed in our laboratory for trypanozoan (JN797772, DQ200235, DQ200250, L07866, DQ200175, DQ200255, DQ200215, DQ200180) (Fig. 1a and b) that amplified 1571 bp fragment from the genomic DNA to rule out the consensus between the finding of the two PCR assays and agglutination test for *T. evansi*. The reaction mixture composition was the same as PCR_1 and the cycling conditions were: initial denaturation at 95 °C (10 min), 30 cycles of denaturation at 94 °C (30 s), annealing at 57 °C (45 s), and extension at 72 °C (75 s) and final extension at 72 °C (10 min).

2.6. Nucleotide sequence analysis

The 257 bp product obtained by the primers specific for *T. evansi* in PCR_1 were custom sequenced from Xcelris Genomics, Ahmedabad, India. The nucleotide sequences were subjected to BLASTn analysis (Altschul et al., 1990) for determining the similarity with