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Comparative seroprevalence and risk factor analysis of *Trypanosoma evansi* infection in equines from different agro-climatic zones of Punjab (India)

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D. Sumbria [(1)], L.D. Singla [(1)*], R. Kumar [(2)], M.S. Bal [(1)] & P. Kaur [(1)]

(1) Department of Veterinary Parasitology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India

(2) National Research Centre on Equines, Haryana-125001, India

*Corresponding author: ldsingla@gmail.com

Summary

As parasitaemia is low and fluctuating during the chronic stage of infection, meticulous detection of *Trypanosoma evansi* in blood is difficult. The primary aims of this investigation were to assess for the first time the seroprevalence of *T. evansi* in all agro-climatic zones of Punjab, by indirect enzyme-linked immunosorbent assay (I-ELISA) and card agglutination test (CATT/*T. evansi*), and to evaluate the risk factors associated with latent trypanosomosis. A total of 319 equine serum samples collected from 12 districts of Punjab (India) belonging to different agro-climatic zones revealed 39 (12.23%) and 9 (2.82%) samples to be positive by CATT/*T. evansi* and I-ELISA, respectively. The highest prevalence was recorded from the Ludhiana district (42.86% and 7.14% by CATT/*T. evansi* and I-ELISA, respectively) in the central plain zone (for which the overall prevalence was 15% and 4.17%, respectively). There was fair agreement between the tests for the detection of *T. evansi* (kappa = 0.345). Species was the most influential risk factor for infection, with odds ratios (ORs) of 2.81 and 5.63 for donkeys/mules,

in comparison with horses, by CATT/*T. evansi* and I-ELISA, respectively. The female equine population (odds ratio [OR] = 3.13, 95% confidence interval [CI] = 1.32–7.67 [CATT]) was found to be at a higher risk of seropositivity for *T. evansi*, particularly on 'unorganised' (inappropriately managed) farms (OR = 3.18, 95% CI = 1.53–6.65 [CATT]) and among animals used for commercial purposes (OR = 2.51, 95% CI = 1.20–5.21 [CATT]). In conclusion, to declare disease-free status, use of the I-ELISA followed by retesting of suspect samples by CATT/*T. evansi* is suggested.

Keywords

Card agglutination test – Equine – Indirect enzyme-linked immunosorbent assay – Risk factor – Seroprevalence – *Trypanosoma evansi*.

Introduction

Trypanosoma evansi, the most prevalent pathogenic kinetoplastid haemoprotozoan, causes a devastating immunosuppressive disease called 'surra' (from the Hindi word mean 'rotten') in domestic, wild and laboratory animals throughout the tropical and subtropical areas of the world (1). It is endemic in most parts of the Indian sub-continent and is transmitted mechanically from infected carrier animals by haematophagous Dipteran insects belonging to genera *Tabanus*, *Stomoxys*, *Haematopota*, *Lyperosia* and *Hippobosca* (2). Although economic losses resulting from surra in India are believed to be large, particularly during epidemic outbreaks of the disease, the economic impact is difficult to assess because of incomplete epidemiological information and inaccurate data (3). On cattle ranches in the Brazilian Pantanal region, the estimated total losses due to *T. evansi* are about US $2.4 million/year (4). Although the parasite infects a wide range of domestic and wild animals, and even humans (5), the effects of the infection in different geographical locations vary according to the strain of the parasite, and the species and genetic makeup of the host(s) infected (5, 6).

The control of the disease is mainly based on recognition of infected animals by livestock keepers, who observe clinical signs and treat them on a herd basis or individually. This is an inaccurate and inefficient approach because many infected animals may remain undiagnosed and act as reservoirs of the parasite, given the periodically cryptic nature of the organism. Specific diagnosis of trypanosomosis is made on the basis of clinical evidence augmented by classical parasitological (blood smear examination), molecular (polymerase chain reaction [PCR] assays) or serological (card agglutination test [CATT/*T. evansi*], enzyme-linked immunosorbent assay [ELISA]) tests. Low and fluctuating parasitaemia renders the detection of the haemoparasite difficult on blood smear examination (7). In the diagnosis of natural infection in equines under field conditions, molecular testing may give false-negative results, especially when the level of parasitaemia is very low (8). This may be followed by flare-up of the infection under conditions of stress (9). These suspected potential carriers can be confirmed by serological examination (10). Among the different serodiagnostic tests, the ELISA (immunoglobulin [Ig]G based) is more likely to categorise truly uninfected animals correctly, whereas the CATT/*T. evansi* is more likely to classify correctly the truly infected animals (11). Camelids and equines are more susceptible to the infection than other species (cattle, buffalo, sheep and goats), and show high mortality (11, 12). An assessment of the risk factors may enhance the control of *T. evansi* infection because it can determine the factors associated with disease either at management or at host level.

To date, no comprehensive assessment of the disease seroprevalence and risk factors has been conducted for *T. evansi* infection in equines in all agro-climatic zones of Punjab. Therefore, this study was designed to investigate the comparative seroprevalence of *T. evansi* using the indirect [I]-ELISA and CATT/*T. evansi* and to determine potential risk factors associated with the prevalence of the disease among equines in Punjab.

Materials and methods

Ethical aspects (consent statements)

The ethics committee for animal experiments from the Guru Angad Dev Veterinary and Animal Sciences University granted approval (IAEC/2014/46-73) for this work to be conducted. Prior consent was obtained from the owners of the equines. Measures were taken to avoid any accidental injury to each animal while collecting the blood samples.

Study areas and sampling frame

The province of Punjab covers a total area of 50,362 square kilometres between latitudes 29°30'N to 32°32'N and longitudes 73°55'E to 76°50'E. There are about 34,000 horses and ponies at risk of infection with *T. evansi* in Punjab (13).The study was conducted in all the agro-climatic zones of Punjab (Table I) because one of the previous studies conducted in the authors' laboratory had revealed a high prevalence of trypanosomosis in bovines in the province (14). Blood samples were collected from representative equines in 12 districts of the five major agro-climatic zones of Punjab. A total of 319 samples (133 male and 186 female) were randomly collected to screen for *T. evansi* infection. Blood (~3 ml) was collected from the jugular vein of each animal into clot-activator vacutainers for serum extraction. To study the serological prevalence of the infection, an expected prevalence of 50% with confidence limits (CI) of 95% and a desired absolute precision of 5% were used when deciding on the required number of samples (15). The number of samples thus calculated was adjusted for a finite population and 319 samples were collected. A predesigned epidemiological questionnaire, addressing the age (young: <2 years; adult: >2 years), gender, management and use of each equine, was used to analyse the risks associated with *T. evansi* transmission and was completed by the owner of each animal. The equine keepers following inappropriate management practices, such as rearing their stock in stables with kacha flooring, poor sanitation and unbalanced feeding programmes, were classified as 'unorganised', while those pursuing appropriate scientific management schedules were considered 'organised'.

Insert Table I

Blood films

Two thin blood films were prepared from each blood sample, dried on the spot, and then fixed in absolute methyl alcohol for 1–2 min in the laboratory. The smear was immersed in diluted Giemsa stain for 30–45 min, and then washed with distilled water to remove excess stain. The slides were left to dry and examined under an oil immersion lens (100× magnification) (16).

Serological tests

Card agglutination test/*T. evansi*

The CATT/*T. evansi* for antibody detection was originally described and converted into a test kit by the Institute of Tropical Medicine, Belgium (17). Briefly, 25 µl of diluted serum was thoroughly mixed with about 45 µl of well-homogenised CATT antigen. The card was agitated in a circular motion using an electric rotator at 60–70 rpm and room temperature for 5 min. Samples showing blue granular agglutination were considered positive. The samples were read in comparison with the control wells according to the instructions supplied. Agglutination patterns were scored as − (negative), ± or + (suspected), and ++ or +++ (positive).

Indirect enzyme linked immunosorbent assay (I-ELISA)

The I-ELISA was conducted at the National Research Centre on Equines (NRCE), India. Briefly, the optimum dilutions of whole cell lysate antigen, conjugate (rabbit anti-horse IgG, whole molecule, horse radish peroxidase [Sigma Aldrich Co., USA]) and known positive reference serum were determined. Each serum sample was tested in duplicate. The ELISA plates were coated with 50 µl aliquots containing 500 ng protein antigen in 0.1 M carbonate/bicarbonate buffer (pH 9.6) per well. After overnight incubation at 4°C, the plates were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBST). The wells of the ELISA plate were blocked with 100 µl 5% skimmed milk in PBST

for 1 h at 37°C. After three further washes in PBST, 50 µl of 1:100 diluted sera with 5% skimmed milk in PBST were added to each well. After an incubation phase of 1 h at 37°C, the plates were again washed three times and 50 µl of a 1:10,000 dilution of rabbit anti-horse IgG–peroxidase conjugate (Sigma) were added and the plates incubated for 1 h at 37°C. Lastly, after washing three more times, 50 µl per well of 1:20 dilution tetra-methylbenzidene substrate (TMB/hydrogen peroxide, 20× concentration) was added. The reaction was stopped by adding 50 µl of 1 M sulphuric acid to each well. The plates were read at 450 nm on an ELISA reader (Bio Tek USA) and the results were reported as the average optical density at 450 nm (OD_{450}) of duplicate samples (18).

Statistical analysis

The prevalence of *T. evansi* with respect to various physical and biological factors was statistically analysed, employing Pearson's chi-squared test at $p \leq 0.05$. Potential risk factors were analysed using WinEpiscope software v. 0.1 and online software (statpages.info/ctab2x2.html). For the I-ELISA, any sample showing an OD_{450} above the mean + 4 standard deviations (SD) of three negative wells was considered positive. The SD of the OD_{450} of three negative wells was also calculated. Cohen's kappa was calculated to assess agreement between the tests.

Results

Blood film examination

Out of 391 animals screened, *T. evansi* was found in only one animal by classical thin blood smear examination.

Serological tests

Card agglutination test/*T. evansi*

Using this serodiagnostic assay, 39 samples showing +++/++ titres on the CATT/*T. evansi* were considered positive, while 89 samples showing +/± titres were considered to be suspected cases (Table I). Among the various

districts under study, the highest prevalence of positive titres was reported from district Ludhiana (42.86%, 95% CI = 21.38–67.41%) in the central plain zone (overall prevalence: 15%, 95% CI = 11.46–26.18), about which the farmers were informed immediately (Table I, Figure 1). The prevalence, based on the presence of anti-trypanosome antibodies, was found to differ non-significantly among the various districts as well as zones under study.

Insert Figure 1

Indirect enzyme linked immunosorbent assay (I-ELISA)

Out of 319 sera examined, only 9 samples showed a positive titre on the I-ELISA (Table I). Among the various districts under study, the highest prevalence of positive titres was again reported from district Ludhiana (7.14%, 95% CI = 1.27–31.47%) in the central plain zone (overall prevalence: 4.17%) (Table I, Figure 1). The prevalence, based on the presence of anti-trypanosome antibodies, did not differ significantly among the various districts and zones under study.

Risk factor analysis

The assessment of the odds ratio (OR) revealed the prevalence of *T. evansi* to be uniformly distributed among the equine population with respect to various risk factors (Table II). However, the difference in prevalence was significant in terms of the management (OR = 3.18, 95% CI = 1.53–6.65; OR = 7.8, 95% CI = 1.45–55.43) and use of the animals (OR = 2.51, 95% CI = 1.20–5.21; OR = 4.89, 95% CI = 1.06–25.28) by CATT/*T. evansi* and I-ELISA, respectively. The highest prevalence values were found on 'unorganised' farms (21.36%, 7.29%), and in equines used for commercial purposes (19.79%, 6.67%). The species of the host animal was apparently the most influential risk factor for infection, with ORs of 2.81 (positive) and 5.63 (suspect) by CATT/*T. evansi* and a prevalence of 26.09% in horses and 65.22% in donkeys/mules. The prevalence differed significantly between male and female animals (OR = 3.13, 95% confidence interval [CI] = 1.32–7.67 [CATT/*T. evansi*] for females). With regard to age, adult equines had a

higher prevalence of *T. evansi* infection, and the difference was significant for the CATT/*T. evansi* (Table II). There was fair agreement between the CATT/*T. evansi* and the I-ELISA for the detection of *T. evansi* (kappa = 0.345).

Insert Table II

Discussion

Blood smear examination is the gold standard technique for detecting haemoprotozoan infection but has low sensitivity (19). In this study, only one blood sample tested positive, with very low parasitaemia, thus supporting the fact that microscopic detection is not feasible until 2.5×10^6 parasites per millilitre of blood are present (20). In low dose infection, the intermission phase may be long and, even when symptoms are present, trypanosomes may still not be detectable in blood. This delays treatment and thereby increases the rates of morbidity and mortality in the animal population (21).

Regarding an efficient pen-side test, the literature provides contradictory opinions about the use of the CATT/*T. evansi* targeting the RoTat 1.2 antigen (22). Prior to any international movement or during quarantine, the IgG-based I-ELISA would be appropriate for verifying that animals are free from infection (9), but in situations where there is overt disease, and to monitor treatment of animals with trypanocidal drugs, the CATT/*T. evansi* can be used. For declaring disease-free status, use of the I-ELISA followed by retesting of suspect samples by the CATT/*T. evansi* is recommended (9). In this study, 89 samples with agglutination scores of +/± were considered only suspicious for infection because slight reactions on the CATT/*T. evansi* can be observed in uninfected horses; therefore, the cut-off was set at a reaction score of ++ (23). Using both tests, the seroprevalence of *T. evansi* was found to be highest in the Ludhiana district of the central plain zone; the farms in this zone are in the vicinity of paddy fields which are conducive to the breeding of tabanid flies. This may have led to the high prevalence of *T. evansi* in this area, which is in agreement with other studies (24).

The assessment of various risk factors demonstrated that the prevalence of infection in female equines (OR = 3.13, 2.11 by CATT/*T. evansi* and I-ELISA, respectively) was greater than that in their male counterparts, probably due to their use as both draught and breeding animals (19, 25). In this study, a markedly lower prevalence was observed in equines less than 2 years of age, when compared with adults. Maternal antibodies provide passive immunity to the young until the age of 3–6 months (26); this immunity diminishes from 6 months to 2 years of age, and their chance of encountering the infection also increases (OR = 1.81, 2.40) when the animals are used for sports and/or work as draught animals. Similar findings have been reported in camels, showing a higher prevalence of surra in the age group above 4.5 years when compared with the age group of 1.5–4.5 years (27). As donkeys and mules are kept mainly outdoors under poor conditions during daily work, their chance of exposure to vectors increases, resulting in an increased risk of haemoparasitic infection in these species (in this study, OR = 2.81, 5.63) (25, 28). Owing to suboptimal management practices, animals on 'unorganised' farms had a higher risk of infection (OR = 3.18, 7.80) with *T. evansi* because the likelihood of direct contact with vectors is higher on these farms (19, 25). Advanced management and disease control programmes reduce the chance of infection in equines kept for recreational purposes, while open grazing practices in equines used for commercial purposes increase the risk of *T. evansi* infection (OR = 2.51, 4.89) (29).

Conclusion

This investigation indicated that, in the early stage of infection, both techniques (CATT/*T. evansi* and I-ELISA) may be used to determine the seroprevalence of *T. evansi* and to evaluate the effectiveness of drugs. In order to declare disease-free status, use of the I-ELISA followed by retesting of suspect samples by CATT/*T. evansi* is suggested.

Competing interests

The authors declare that they have no competing interests.

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Table I

Seroprevalence of *T. evansi* in equines in five agro-climatic zones of Punjab

Zones **	Districts	Samples		CATT/*T. evansi*						I-ELISA		
				+++/++ (%)		95% CI	+/± (%)		95% CI	Positive (%)		95% CI
		(A)	(B)	(C)	(D)		(C)	(D)		(C)	(D)	
SMZ	Hoshiarpur	55	38	6 (10.91)	4 (10.53)	4.17–24.13	16 (29.09)	14 (36.84)	23.38–52.72	1 (1.82)	0 (0)	∞ (0.0–∞)
	Pathankot		17		2 (11.76)	3.29–34.34		2 (11.76)	3.29–34.34		1 (5.88)	1.05–26.98
UZ	SBS Nagar	53	22	7 (13.21)	4 (18.18)	7.31–38.52	11 (20.75)	6 (27.27)	13.15–48.15	2 (3.77)	1 (4.55)	0.80–21.80
	Mohali		31		3 (9.68)	3.35–24.90		5 (16.13)	7.09–32.63		1 (3.23)	0.50–16.19
CPZ	Amritsar	120	23	18 (15.00)	1 (4.35)	0.77–20.90	42 (35.00)	8 (34.78)	18.81–55.11	5 (4.17)	1 (4.45)	0.70–20.90
	Jalandhar		10		1 (10.00)	1.79–40.40		4 (40.00)	16.82–68.73		1 (10.00)	1.79–40.42
	Ludhiana		14		6 (42.86)	21.38–67.41		6 (42.86)	21.38–67.41		1 (7.14)	1.27–31.47
	Patiala		73		10 (13.70)	7.61–23.41		24 (32.88)	23.19–44.27		2 (2.74)	0.70–9.45
WZ	Moga	28	20	4 (14.29)	3 (15.00)	5.24–36.04	10 (35.71)	5 (25.00)	11.19–46.87	1 (3.57)	1 (5.00)	0.80–23.61
	Bathinda		8		1 (12.50)	2.24–47.09		5 (62.50)	30.57–86.32		0 (0)	∞ (0.0–∞)
WPZ	Ferozepur	63	37	4 (6.35)	2 (5.41)	1.50–17.70	10 (15.87)	6 (16.22)	7.65–31.11	0	0 (0)	∞ (0.0–∞)
	Fazilka		26		2 (7.69)	2.14–24.14		4 (15.38)	6.15–33.53		0 (0)	∞ (0.0–∞)
Total			319		39 (12.23)	9.07–16.28		89 (27.90)	23.26–33.06		9 (2.82)	1.49–5.27
Chi-squared				3.14	17.03		8.46	18.96		3.06	7.38*	
Kappa						0.345						

CATT: card agglutination test
CI: confidence interval
I-ELISA: indirect enzyme-linked immunosorbent assay
SBS: Shaheed Bhagat Singh
*$p \leq 0.05$
**SMZ: Sub-mountain undulating zone, UZ: Undulating zone, CPZ: Central plain zone, WZ: Western zone, WPZ: Western plain zone
(A): Zone-level samples, (B): District-level samples, (C): Zone-level prevalence, (D): District-level prevalence

Table II

Distribution of variables used to investigate the risk factors associated with CATT/*T. evansi* and I-ELISA seroprevalence in equines in Punjab, India

Factors	Variables	Samples	CATT +++/++ (%)	Odds ratio (95% CI)	CATT +/± (%)	Odds ratio (95% CI)	I-ELISA (%)	Odds ratio (95% CI)
Sex	Male	133	8 (6.02)		26 (19.55)		2 (1.523)	
	Female	186	31 (16.67)	3.13 (1.32–7.67)	63 (33.87)	2.11 (1.21–3.69)	7 (3.91)	2.56 (0.48–18.15)
	χ^2		8.20*		7.91*		1.44	
Age	More than 2 years	267	35 (13.11)		81 (30.34)		8 (3.09)	
	Less than 2 years	52	4 (7.69)	1.81 (0.58–6.31)	8 (15.38)	2.40 (1.03–5.79)	1 (1.96)	1.58 (0.19–34.31)
	χ^2		1.19*		4.84*		0.18	
Species	Horses	296	33 (11.15)		74 (25)		5 (1.72)	
	Donkeys/Mules	23	6 (26.09)	2.81 (0.92–8.30)	15 (65.22)	5.63 (2.13–15.18)	4 (21.05)	12.25 (2.50–58.94)
	χ^2		4.44		17.16*		0.05	
Management	‘Organised’	216	17 (7.87)		58 (26.85)		2 (0.93)	
	‘Unorganised’	103	22 (21.36)	3.18 (1.53–6.65)	31 (30.10)	1.17 (0.68–2.03)	7 (7.29)	7.80 (1.45–55.43)
	χ^2		11.83*		0.37		8.77*	
Use	Recreational	223	20 (8.97)		47 (21.08)		3 (1.36)	
	Commercial	96	19 (19.79)	2.51 (1.20–5.21)	42 (43.75)	2.91 (1.68–5.04)	6 (6.67)	4.89 (1.06–25.28)
	χ^2		7.33*		17.15*		5.89*	
	Total	319	39		89		9	

*$p \leq 0.05$

CATT: card agglutination test

CI: confidence interval

I-ELISA: indirect enzyme linked immunosorbent assay

Fig. 1

Relative prevalence of seropositive and suspected cases of *T. evansi* infection among equines in the districts of Punjab under study