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Research Article

Prevalence, relative risk factors and hemato-biochemical changes associated with equine trypanosomosis in eastern plane zone of Uttar Pradesh

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Abstract

Equine trypanosomosis has negative impacts on health and working efficiency of equines in different areas of the world including India. A cross sectional survey was conducted in thirteen districts of eastern plane zone of Uttar Pradesh, India during August to November, 2020 to evaluate the molecular and serological prevalence, relative risk factors and hemato-biochemical alterations of equine trypanosomosis. A total of 285 equine blood samples of both sex and different age groups were collected for the study. The overall prevalence of *T. evansi* infection in equines was 15.08 percent by Invariable surface glycoprotein (ISG) PCR and 19.29 percent by indirect-ELISA. The relative risk factors among different animals associated with PCR and ELISA techniques indicated that donkeys/mules were 27.08 and 34.65 times more prone to infection than horses. The young animals as well as parasite infected animals were also more susceptible to this infection than adult and parasite naive animals. Significant alterations in hemato-biochemical parameters (*viz.* Hb, PCV, TEC, MCV, MCHC, absolute lymphocyte count, TSP, albumin, globulin, serum glucose, serum enzymes, blood urea, creatinine and uric acid) were observed in infected animals as compared to infection free control.

Key words: Eastern plane zone, Equine trypanosomosis, Indirect-ELISA, Relative risk factors, *T. evansi*

INTRODUCTION

The causative agent of surra, *Trypanosoma evansi*, is transmitted mechanically by hematophagus flies such as *Tabanus* (horse flies) and *Stomoxys* (stable flies). In India, it is found as an endemic, mostly in north and north-western region (Yadav *et al.*, 2012). The overall serological prevalence of animal trypanosomosis is 11.36% in North and North-western states of India; in which maximum was recorded (19.69%) in Uttar Pradesh state (Kumar *et al.*, 2013). Animal trypanosomosis caused several sporadic outbreaks of equine in different parts of India (Yadav *et al.*, 2012). The clinical signs of equine trypanosomosis are intermittent fever, severe weight loss, progressive weakness, anaemia, hemoglobinuria, petechial hemorrage of mucous membranes, ventral and genital oedema, urticarial plaques, conjunctivitis and keratitis and sever neurological signs. The typical neurological signs of infection with *T. evansi* are ataxia and paralysis of the hind quarter and lips usually precede that results death of animal (Aregawi *et al.*, 2019). The severity of clinical signs of the disease depends upon the parasite strain, concurrent infection, stress, environmental factors and the immunological status of the host. Chronic form is most common in equine that persists for several months up to 2 years and is likely to present in association with secondary infection due to immune-suppression caused by *T. evansi* infection (Ahmed, 2008). Thus, surra has significant socio-economic importance and causes severe economic losses to the poor farmers in terms of poor production, sometimes abortion and death without treatment. The total annual loss caused by surra has been estimated to be INR 44740 million in India (Kumar *et al.*, 2017).

As per 20th livestock census, the equine population was found to be highest in Uttar Pradesh among the equine rearing states in

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India (Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, GoI). Generally, marginal people of this state rear equines for earning their livelihoods and use them in transport of goods, construction work, brick kilns, tourist and pilgrim places and marriage-ceremony etc. without following a definite system of management and husbandry practices. In Eastern Uttar Pradesh, detailed and systematic studies have not been conducted on equine trypanosomosis. Hence, the present study was carried out to know the prevalence of *T. evansi* and relative risk factors along with estimation of hemato-biochemical alteration associated with equine trypanosomosis in eastern plane zone of Uttar Pradesh.

MATERIALS AND METHODS

Ethical statement

An Institutional Animal Ethics Committee (IAEC) of Acharya Narendra Dev University of Agriculture and Technology, Kumarganj, Ayodhya, Uttar Pradesh granted an approval (IAEC/CVSc/2/P-32/2020/26) for conducting the present study.

Study area

The eastern Uttar Pradesh covers an area of 85,804 square kilometer and situated between 23°50' N to 28°25' N and 81°10' E to 84°40' E. The present study was conducted in thirteen districts of eastern plain zone of Uttar Pradesh (UP), India. The climate is defined as moist sub humid, temperature ranges from 5.7°C to 41.4°C and average rainfall is 803 mm (https://farmech.dac.gov.in/FarmerGuide/UP/index1.html). In the study area, equines comprise of indigenous breed of horses, donkeys and mules of all ages and sex category. Animals are maintained under traditional extensive system that does not protect them from extreme weather conditions. The available feed resources constituted natural pasture, concentrates and crop residues. They are mainly used for transport, draught and pack work type.

Study design

A cross-sectional study was conducted between August to November, 2020, for the prevalence of trypanosome infection in equines in the study area. The study animals were selected by using simple random sampling method in which expected prevalence was taken as 50% with confidence interval (CI) of 95% (Thrusfield, 2005). A total of 285 animals comprising 220 horses and 65 donkeys/mules selected from the study area. Sampling method was based on a predesigned epidemiological questionnaire addressing the animal details like sex, age, type of work and managemental practices like helminthes infestation (Direct faecal examination method using standard procedure) to calculate the epidemiological risk factors associated with trypanosomes infection in equines in the study area. Conventional age categories were made as young (<2 years) and adult (>2 years).

Blood sample collection

About 5 mL of blood from jugular vein was collected aseptically from each animal in two collecting tube. One sample was collected with EDTA for extraction of DNA for PCR amplification and hematological studies while second was collected in clot activator vials for serum collection and stored at -20°C for serological examination and biochemical studies. Blood samples from animals were collected with utmost care with the prior consent of the owners.

Enzyme linked immuno sorbent assay (ELISA)

Blood samples of clot activator vials were centrifuged to generate serum and stored at -20°C. As a means to detect the antibodies against *T. evansi*, whole cell lysate (WCL) antigen specific indirect-ELISA was executed at National Research Center on Equines (NRCE), Hisar, Haryana, India as per the protocol standardized by Kumar *et al*. (2013). ELISA plates (Thermo Fisher Scientific) were coated with 50 µL diluted in 0.1M carbonate/bicarbonate buffer (pH 9.6) containing 500 ng WCL antigen (Kumar *et al*., 2013) per well. Plates were incubated overnight at 4°C then washed six times with phosphate buffer saline containing 0.05% Tween-20 (PBS-T). ELISA

plates were blocked with 100 µL blocking buffer (5% skimmed milk in PBS-T) then incubated at 37°C for 1 hour. After washing, 50 µL of 1:100 diluted field sera sample in 5% skimmed milk in PBS-T were loaded in duplicate. The plates were incubated at 37°C for 1 hour and washed with PBST followed by 50 µL of 1:15,000 dilution of anti-Horse IgG (Sigma- Aldrich) were added to the wells and again incubated for 1 hour at 37°C. Finally, 50 µL of 1:20 dilution tetra methyl benzidene substrate ((TMB, GeNei™)) was added per well. The reaction was stopped by adding 50 µL of 1M sulphuric acid. The absorbance was read at 450 nm on ELISA reader (Thermo Fisher Scientific, Finland) and results were expressed as mean OD 450 of duplicate samples.

DNA Extraction and Polymerase chain reaction (PCR) assay

Genomic DNA was extracted from whole blood samples by Phenol chloroform isoamyl alcohol (PCI) method of DNA extraction with little modification of the method used by Sambrook and Russel (2001). DNA pellet, extracted by this method, was dissolved in 30-50 µL of nuclease free water (NFW) and stored at -40°C for further use. Extracted DNA was checked by agarose (0.5%) gel electrophoresis. PCR was performed using ISG-F/R (Invariable surface glycoprotein-forward/reversed) primers (ISG-F 5¹ AAAGCCACCGAAGATGCAGA 3¹ and ISG-R 5¹ TTGTCCCAATCCAGCCACTC 3¹) for amplification of 196 bp of repetitive sequence of mini-chromosome satellite (Masiga *et al.*, 1992; Kumar *et al.*, 2016). The DNA template was amplified in 25 µL of PCR reaction mixture using Top Taq master mix 2x of Qiagen (5 unit/µL Taq DNA polymerase, 3 mM $MgCl_2$ and 400 µM each dNTP), 0.4 µM of each primer and 2.0 µM template DNA (>40 ng/µM) were mixed and NFW was added to make a final volume of 25 µL PCR condition was optimized with ISG primers as follows: initial denaturation for 5 minutes at 95°C followed by 35 cycles at 94°C for 30 seconds (denaturation), annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds. The amplified PCR products were resolved by electrophoresis on 1.5% agarose gel and visualized under UV transilluminator mega Lum G gel imaging system (Aplegen Inc., USA) for detection of 196bp product size.

Interaction with hemato-biochemical parameters

PCR positive blood samples (might or might not be positive by indirect ELISA) were further analyzed for hemato-biochemical estimation with an equivalent non-infected control blood samples for comparative study. Total erythrocytes count (TEC), total leukocytes count (TLC), hemoglobin (Hb), packed cell volume (PCV), differential leucocytes count (DLC), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were estimated as described by Ghaffar *et al.* (2014). Different serum biochemical parameters such as total serum protein (TSP), serum albumin, serum glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), creatinine, bilirubin total (BIT), bilirubin indirect (BID), serum cholesterol, serum urea nitrogen, uric acid (UA) were estimated using Clinical Chemistry Analyzer (ERBAEM200).

Statistical analysis

The range of molecular and serological prevalence was determined at 95% confidence interval calculated using online calculator (http://www.mccallum-layton.co.uk/stats/ Confidence Interval CalcProportions.aspx), determination of epidemiological risk factors were statistically analyzed by chi-square test and data regarding hemato-biochemical parameters were done by Student's t-test, using Graphpad prism software (version 8.0.2) and the value of $p<0.05$ was considered as statistically significant.

RESULTS

Molecular and serological prevalence

The samples were considered positive only

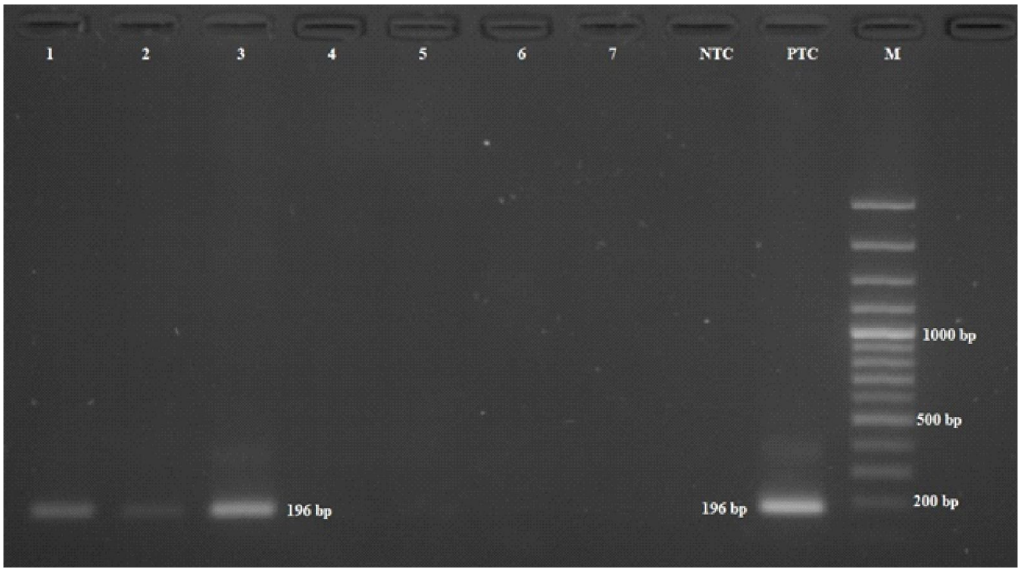

Fig. 1. Agarose gel (1.5%) electrophoresis of 196 bp of PCR product for *T. evansi*. Lane 1-7: Test samples, NTC- Negative template control, PTC- Positive template control, M- 100bp plus DNA ladder

showed single band of 196 bp size (Fig. 1) on agarose gel (1.5%) electrophoresis. The RPP (relative percent positivity) value of 15% was considered as cut-off threshold in indirect ELISA to estimate seropositivity against *T. evansi* infection (Kumar *et al*., 2013). Out of 285 samples examined, 43 (15.08% and 95% CL= 11.14–19.78) and 55 (19.29% and 95% CL=14.88–24.37) samples were found to be positive by ISG- PCR and indirect ELISA, respectively (Table 1). The seroprevalence of *T. evansi* ranged from 5.88% (Ambedkar Nagar district) to 51.06% recorded in Ghazipur district. The lowest or absent molecular prevalence was also observed in four districts (Sultanpur, Sant Ravidas Nagar, Pratapgarh and Ambedkar Nagar) of current study area (Table 1).

Evaluation of epidemiological risk factors

The prevalence of trypanosomes

Table 1. Prevalence of equine trypanosomosis in various districts of Eastern Plane zone, U. P.

District	Total samples	PCR positive samples (%)	95% CL	ELISA positive samples (%)	95% CL
Barabanki	17	2 (11.76)	1.46 - 36.44	3 (17.64)	3.80 - 43.43
Ayodhya	44	4 (9.09)	2.53 - 21.67	4 (9.09)	2.53 - 21.67
Sultanpur	17	0 (0.00)	0 - 19.51*	1 (5.88)	0.15 - 28.69
Jaunpur	19	2 (10.52)	1.30 - 33.14	2 (10.52)	1.30 - 33.14
Azamgarh	16	1 (6.25)	0.16 - 30.23	2 (12.50)	1.55 - 38.35
Mau	24	3 (12.50)	2.66 – 32.36	4 (16.66)	4.74 - 37.38
Ballia	21	5 (23.80)	8.22 - 47.17	6 (28.57)	11.28 - 52.17
Ghazipur	47	22 (46.80)	32.11 - 61.92	24 (51.06)	36.06 - 65.92
Varanasi	17	2 (11.76)	1.46 – 36.44	3 (17.64)	3.80 - 43.43
Santravidasnagar	9	0 (0.00)	0 - 33.63*	1 (11.11)	0.28 – 48.25
Pratapgarh	15	0 (0.00)	0 - 21.8*	1 (6.66)	0.17 - 31.95
Chandauli	22	2 (9.09)	1.12 - 29.16	3 (13.63)	2.91 - 34.91
Ambedkarnagar	17	0 (0.00)	0 - 19.51*	1 (5.88)	0.15 - 28.69
Total	285	43 (15.08)	11.14 – 19.78	55 (19.29)	14.88 –24.37

*One sided 97.5% confidence interval

infection was 9.09% and 11.82% in horses whereas in donkey/ mule, it was found to be 35.38% and 44.62% by PCR and indirect-ELISA test, respectively (Table 2). The variation in prevalence of infection among the equine species was statistically highly significant ($p<0.01$). Donkeys/mules population was at 27.08 and 34.65 times more prone to infection as compared to the horses. But no significant difference ($p<0.05$) was observed in respect of prevalence of trypanosomosis among sex of equines (Table 2). The young animals (<2years) showed significantly ($p<0.05$) higher incidence (27.9% and 32.55%) of *T. evansi* infection than adult animals (12.80% and 16.94%) by PCR and indirect ELISA test. They were at 2.17 and 1.10 times more risk of infection as compared to adult animals (Table 2). Helminths infested animals were observed significantly ($p<0.05$) more prone to *T. evansi* infection (21.18% and 25.42%) than the helminths free equines (10.77% and 14.97%) by PCR and indirect ELISA test shown in Table 2. The equines used for bricks kiln purpose had more prevalence (20.45% and 25.58%) of infection than those used for other transport (16.08% and 16.58%) work, but this variation was not statistically significant (Table 2).

Estimation of hemato-biochemical alterations

Assessments of different hematological parameters depicted that the mean value of Hb, PCV, TEC, MCV and MCHC were significantly ($p<0.01$) lower in PCR positive animals than control (Table 3). Absolute count of lymphocyte cells was decreased significantly in infected animals (Table 3). The value of TSP, albumin, globulin and serum glucose were recorded significantly ($p<0.01$) lower in infected equines than control (Table 3). A significantly ($p<0.01$) increased value of different serum biochemical parameters such as ALT, AST, GGT, blood urea, creatinine and uric acid was recorded in infected animals compared to infection free animals (Table 3).

Table 2. Equine trypanosomosis associated epidemiological risk factors diagnosed by PCR and indirect-ELISA in the study areas

Prevalence test	Variables	Category	Total animals	Infected animals (%)	$\chi 2$	RR	p-value
Diagnostic Polymerase chain reaction (PCR)	Animal	Horse	220	20 (9.09)	27.08		0.0001
		Donkey/ mules	65	23 (35.38)		3.89**	
	Sex	Male	111	16 (14.41)	0.06		0.79
		Female	174	27 (15.52)		1.07NS	
	Age	<2 years	43	12 (27.90)	6.49	2.17*	0.01
		>2 years	242	31 (12.80)			
	Helminthparasites	Present	118	25 (21.18)	5.846	1.96*	0.01
		Absent	167	18 (10.77)			
	Work	Brick kiln	86	18 (20.45)	3.28	1.66NS	0.07
		Transport	199	25 (16.08)			
Indirect-enzyme linked immuno sorbent assay (ELISA)	Animal	Horse	220	26 (11.82)	34.65		0.0001
		Donkey/ Mule	65	29 (44.62)		3.77**	
	Sex	Male	111	18 (16.22)	1.10		0.29
		Female	174	37 (21.26)		1.31NS	
	Age	<2 years	43	14 (32.55)	5.71	1.10*	0.016
		>2 years	242	41 (16.94)			
	Helminth parasites	Present	118	30 (25.42)	4.85	1.69*	0.02
		Absent	167	25 (14.97)			
	Work	Brick kiln	86	22 (25.58)	3.12		0.07
		Transport	199	33 (16.58)			

$\chi 2$= Chi square, RR= relative risk, NS-non significant (P>0.05), *significant (P<0.05), **highly significant (P<0.01)

Table 3. Hemato-biochemical parameters of *T. evansi* infected and non-infected equines

Parameters	Control	Infected animals	t- test	P value
Hemoglobin (g %)	10.53±0.28	4.76±0.17	17.36**	0.0001
Haematocrit (%)	34.16±0.61	20.00±0.62	16.14**	0.0001
TEC (x10³ cells/µL)	7.44±0.45	2.65±0.17	9.88**	0.0001
MCV (fl)	47.88±3.04	79.66±6.4	4.47**	0.0001
MCHC (g/dL)	30.87±0.85	24.03±1.04	5.08**	0.0001
TLC (x10³ cells/µL)	8.07±0.37	7.52±0.40	0.95NS	0.1739
Lymphocyte (x10³ cells/µL)	3.90±0.21	2.82±0.20	3.70**	0.0006
Neutrophil (x10³ cells/µL)	3.47±0.19	3.85±0.28	1.11NS	0.1380
Monocyte (x10³ cells/µL)	0.25±0.01	0.31±0.02	1.59NS	0.0628
Eosinophil (x10³ cells/µL)	0.32±0.02	0.39±0.04	1.31NS	0.1019
Basophil (x10³ cells/µL)	0.12±0.01	0.15±0.01	1.24N S	0.1130
TSP (g/dL)	5.83±0.12	2.08±0.41	8.71**	0.0001
Albumin (g/dL)	2.57±0.07	0.73±0.12	12.64**	0.0001
Globulin (g/dL)	3.26±0.15	1.35±0.30	5.62**	0.0001
Glusoce (mg/dL)	69.76±1.61	27.69±1.20	20.91**	0.0001
ALT (U/L)	34.21±1.12	69.88±3.46	9.79**	0.0001
AST (U/L)	167.69±5.30	210.00±12.49	3.11**	0.0025
GGT (U/L)	21.93±2.50	34.64±1.04	4.68**	0.0001
Blood urea (mg/dL)	19.50±1.48	38.55±6.11	3.03*	0.0031
Creatinine (mg/dL)	1.37±0.08	1.97±0.09	4.59**	0.0001
Uric (mg/dL)	0.33±0.02	0.69± 0.00	3.62**	0.0008
BID (mg/dL)	0.23±0.01	0.17±0.07	0.79NS	0.2166
BIT (mg/dL)	0.470±0.05	1.00±0.22	1.26NS	0.1104
Triglycerides (mg/dL)	17.00±2.21	19.33±3.09	0.61NS	0.2731
Cholesterol (mg/dL)	80.33±2.98	78.66±3.08	0.38NS	0.3507

NS- non significant (P>0.05), *significant (P<0.05), **highly significant (P<0.01)

DISCUSSION

The current study demonstrated the prevalence of *T. evansi* infection in equines diagnosed by ISG-PCR and indirect-ELISA method and also the epidemiological risk factors along with hemato-biochemical alteration associated with equine trypanosomosis in the study area. The overall prevalence of *T. evansi* infection in equines was observed 15.08% and 19.29% by molecular and serological methods, respectively. The present seroprevalence data corroborates well with the findings of Kumar *et al*. (2013) who reported 19.69% seroprevalence of *T. evansi* in equines of Uttar Pradesh state, India.

A strong correlation was also observed between equine trypanosomosis and environmental as well as epidemiological risk factors in exposed animals in the study area. Prevalence of trypanosomosis was increased significantly (p<0.01) in donkeys/mules population than the horses. Similar finding was also observed by Camoin *et al*. (2019) and Kouam *et al*. (2010). Comparatively higher risk of trypanosomosis in donkeys/ mules might be due to traditional extensive rearing system with poor management practices and used for haulage and transport for which they are more exposed to flies than horses. Occurrence of trypanosomosis showed no statistically

significant difference among sex of equines. This finding is in line with the earlier investigations (Bekele and Nasir, 2011; Tafese *et al*., 2012; Sumbria *et al*., 2014). This might be due to an equal chance of exposure to the infection and similar distribution of parasite between the sexes. The young animals showed significantly ($p<0.05$) higher prevalence of *T. evansi* infection means they had at 2.17 and 1.10 times more risk of infection as compared to adult animals. Similar findings have been reported by Abebayehu *et al*. (2011) in animal trypanosomosis. Sumbria *et al*. (2014) reported that young equines were supposed to be thrice at the chance of *T. evansi* infection as compared to the adults. This could be due to the fact that young animals do not have strong immune responses against trypanosomes infection and therefore they are not able to control the parasitemia during infection. Helminths infested animals had 1.96 and 1.69 times more risk of infection as compared to non-infested animals. This result is in line with the earlier work (Sumbria *et al*., 2014). This could be due to poor management and open housing system where wasted feed and their manure would not be cleaned regularly. Thus, the chance of infection was high due to more exposure to insect or flies as compared to helminths free equines. The equines used for bricks transport especially donkeys were seen at high risk of infection. This record is in agreement with previous workers (Burn *et al*., 2010). The current study suggested that the animals used in bricks transport were more likely to have skin injury, soil surface abnormalities and more risk of ectoparasite or fly infestation than those equines used for other transport purposes.

In the present study, PCR positive samples were used for hemato-biological estimation against the non infected control because PCR is a very high sensitive and enough to detect the early stage of active *T. evansi* infection (Rudramurthy *et al*., 2013). Indirect ELISA is not sensitive to detect the early stage of infection. Moreover, in treated cases, also the anti-trypanosomal antibodies persisted over 6 months or even more (Monzon *et al*., 2003; Yadav *et al*., 2012). A significantly devaluation of blood cellular variable noticed in *T. evansi* infected animals suggested that lyses of erythrocytes due to RBC destruction by the flagellar movement of trypanosomes, indiscriminate phagocytosis by activated macrophage, hemolysis of RBCs by hemolysin released by parasites and non compensatory erythropoiesis (Bal *et al*., 2014). Previous workers were also noticed lymphopenia in animals infected with trypanosomosis (Abubakar *et al*., 2005). Lymphopenia could be a result of immuno-suppression due to shrunken of lymphoid organs (Abubakar *et al*., 2005). Abo-Aziza *et al*. (2017) and Hota *et al*. (2019) have also been reported lower level of TSP, albumin, globulin and serum glucose in the animal infected with surra. The hypoalbuminaemia might be occurred due to uptake of albumin bound fatty acid and lipoproteins, while hypoglobulinaemia due to increased hepatocellular damage take place during the infection of trypanosomosis (Mishra *et al*., 2017; Hota *et al*., 2019). Previous investigators have been also reported hypoglycemia in trypanosomosis (Habila *et al*., 2012) and it has been suggested that it might be due to rapid consumption of the blood glucose by trypanosomes for their metabolism during infection (Bal *et al*., 2014) and also the increased metabolic rate of host during fever as well as hepatocyte degeneration (Garba and Mayaki, 2018). Elevation of ALT and AST enzymes could be due to increased levels of systemic antigen–antibody immune complexes and their consequent accumulation in the vital organs. This might play a role of tissue breakdown and inflammation in the host body (Sivajothi *et al*., 2015). Centrilobular degeneration consequences hypoxia and severe oxidative stress induced by *T. evansi* infection (Abd El-Baky and Salem, 2011). The level of GGT recorded significantly ($p<0.05$) higher is suggestive of tissue breakdown and inflammation in the animal's body or might be due to lysed trypanosomes at different stages

of the infection (Parashar *et al*., 2018). The level of blood urea, creatinine and uric acid observed significantly ($p<0.05$) higher in PCR positive animals might be due to acidosis or primary renal insufficiency caused by decreased renal blood flow (Seleim, 2003). All other hemato-biochemical variables in infected animals were not statistically significant from non-infected equines.

The present study revealed that 15.08% of equines were positive for trypanosomosis by diagnostic PCR and around 19.29% by in direct-ELISA that indicates subclinical trypanosomosis which is constantly prevalent in equine population and affect small number of animals on post monsoon season in the study area. Evaluation of relative risk factors with respect to molecular and serological diagnostic methods suggested that donkeys/ mules were at more risk of trypanosomes infection than horses. The young animals and helminth-infested animals were also more susceptible to this infection. Finally, significant changes in hemato-biochemical parameters in subclinically infected animals could be helpful in the diagnosis of *T. evansi* in equines. Therefore, more surveys on equine trypanosomosis should be designed and implemented in different seasons and rest of the agro-climatic zones of eastern part of Uttar Pradesh in future.

Conflict of interest: Authors have no conflict of interest in this study.

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