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Clinico-hematological and Biochemical Studies on Naturally Infected Buffaloes with Trypanosomiasis in Deepor Beel Area of Assam

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ABSTRACT

Background: Trypanosomiasis, an arthropod-borne blood protozoan disease commonly known as Surra, has an adverse influence on the health and working capability of infected animals. Monitoring and identification of blood-borne parasitic infections in dairy animals are of vital importance to get the optimum production. There was a lake of the scientific report of incidence of trypanosomiasis in buffalo in the northeast region.

Methods: In the present study, a total of 100 buffalo was screened in the Deepor Beel area of Assam and 25 animals were suspected of trypanosomiasis based on clinical signs. The diagnosis was confirmed by microscopic examination and formol gel slide test which was further confirmed by PCR. The blood from twenty-one buffalo tested positive for the presence of *Trypanosoma evansi* was evaluated for clinico-haemato-biochemical alterations.

Result: The outbreak in the affected animals exhibiting high fever (105±1.0°F), hyperemic mucosa of eyes, corneal opacity, incoordination of the hindquarter, wasting condition of body and death, was confirmed based on examination of Wright-Giemsa stained blood smears, formol gel slide test and by polymerase chain reaction by using *T. evansi* specific primers yielding species-specific 227 bp PCR product. The infected bovines showed marked anaemic tendency as revealed by a significant decrease in Hb, PCV, RBC, Lymphocytopenia and leukocytosis along with marked thrombocytopenia. There is a marked increase in BUN, creatinine and total bilirubin level while random blood sugar shows a decreased level.

Key words: Assam, Buffalo, Clinical pathology, FGST, Trypanosomiasis.

INTRODUCTION

The parasitic diseases act as a major hindrance in the production of the livestock sector which also includes buffalo production and management. Due to various environmental changes as well as trans-border migration of livestock, some parasitic diseases which were absent in past, are gradually starting to emerge in this region. There is essentially no local knowledge on the prevalence of *Trypanosoma evansi* among any domestic animal of Assam. But at present, veterinarians are frequently encountering the menace of trypanosomiasis in cattle which leads to tremendous economic loss to the farmers. The disease trypanosomiasis has a significant impact on the economics of dairy farmers. Trypanosomiasis causes marked depression and suppression of the immune system resulting in increased susceptibility to different opportunistic infections in cattle and buffaloes (Claes *et al.,* 2004). Trypanosomiasis directly constrains the productivity of Buffalo by reducing birth rates, increasing abortion rates and increasing mortality rates. Scanty information is available about the prevalence of trypanosomiasis infection in buffalo. Trypanosomiasis in buffaloes was first documented by Lingard (1897) in India. But to date, there is no published report available on *T. evansi* from the buffalo population of NER India. Assam, situated in the North East region of India is known to have a substantial number of Buffalo, which is 421720 as per the 20th Livestock census, next to cattle in its utility in this region. Buffaloes contribute to a greater extent to the rural economy of Assam in terms of milk, meat, draught, hide and manure. Therefore, the

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present study was conducted to determine the presence of trypanosomiasis, clinico-hemato-biochemical changes in buffaloes kept under unorganized conditions in and around Guwahati, Assam. As per the author's knowledge, this report can be regarded as the first report of trypanosomiasis in buffaloes from Assam, NER India.

MATERIALS AND METHODS

Study area

The study was conducted in a private unorganized buffalo farm (known as khuti in local language), in the Deepor Beel area which is between the latitudes 26°05′ and 26°10′North and longitudes 91°42′East covering an area of 8.375 sq km, in the outskirt of Guwahati, the capital city of Assam (Fig 1).

All the swamp type buffalo were maintained in the semi-intensive system in the Deepor Beel area (Fig 2). Out of 100 buffalo (different age groups), twenty-five animals were having a complaint of lethargy, anorexia, emaciation, drops in milk yield and gradual loss of body conditions.

Sample collection

Blood samples from all the suspected as well as from six healthy buffaloes were collected by jugular vein puncture in properly labelled EDTA vials, appropriately packed in ice. 2 ml of the collected blood was kept in a vacutainer containing EDTA for parasitological as well as routine haematological analysis and another 2 ml in serum separator tube with clot activator for biochemical studies and FGST. Clinicopathological studies were done on the same day of blood collection and kept at 20°C until further use. Healthy buffaloes were included for comparison.

Microscopic examination of blood

The thin Giemsa stained blood smears were examined under oil immersion (100X) for detection of *T. evansi.* For thin blood smear, clean grease-free slides were taken and one drop of blood was put on the slide. The methanol fixed smears were then stained with Giemsa stain following standard protocol and observed under oil immersion lens (×100 magnification) for the presence of haemoprotozoa (Soulsby 1982). The parasites were identified based on characteristics morphology (Taylor *et al.,* 2016).

Formol gel slide test (FGST)

This test was performed as per the method described by Sarmah *et al.* (2018) with slight modification. Briefly, 100 µl test serum was placed in a cavity micro slide and 10 µl of concentrated formalin (37% formaldehyde) was added and thereafter mixed by gentle tilting of the slide. The observation was made for 20 min to visualize jellification of the test serum and simultaneous development of opacity if any. A positive test was indicated by the formation of gel that adhered to the slide and the development of opacity akin to the white of a boiled egg. Formation of precipitation at the bottom of the slide with a clear surface fluid running off the slide, when tilted as seen in the test using bovine serum (negative control) was considered negative.

Genomic DNA extraction and amplification

DNA extraction

The DNA from each blood sample was extracted from 100 µL of the EDTA-buffered whole blood using the commercially available DNA extraction Kit DNeasy Blood and Tissue kits, QIAGEN, Germany), according to the manufacturer's instructions with minor modifications. The extracted DNA was eluted in 100 µL elution buffer and stored at -20°C until further analysis.

DNA amplification

The primers for PCR (Bangalore Genei, India) from a repetitive sequence probe pMuTec 6.248 (Wuyts *et al.*, 1994) having forward and reverse sequence of 5′-TGCAGAC GACCTGACGCTACT-3′ and 5′-CTCC TAGAAGCTTCGG TGTCCT-3′, respectively, were used for amplification of a 227 bp fragment from *T. evensi* genomic DNA. All PCRs were performed in a final volume of 50 µl containing 25 µl of Dream Taq DNA polymerase master mix 23 (Thermo Scientific, Nalgene, U.K.), 0.4 lM (1 µl) of each primer and 2 µl of DNA template. The reaction was brought to 50 µL total volume with PCR-grade water. Thermal cycling conditions for *Trypanosoma* species consisted of an initial 2-min incubation at 95°C; followed by 40 cycles of denaturation at 95°C for 30 sec, primer annealing (58°C for 30 sec) and extension at 72°C for 1 min; and a final extension step at 72°C for 5 min, after which the samples were held at 4°C. Aliquots of 10 µl of PCR product were electrophoresed on a 1.5% agarose gel containing 10 µl/ml SYBR Safe (Thermo Scientific) in Tris-acetate-EDTA buffer at 100 V for 45 min and photographed under a UV imaging system. The size of each product was assessed by comparison with a Gene Ruler 100-bp DNA ladder marker (Thermo Scientific).

Haematological analysis

Haematological parameters *viz*. Hb, TEC, TLC, DLC, PCV, Platelet count, MCV and MCH were analysed by an automated haematological cell counter (MS 4e, France).

Biochemical analysis

For biochemical studies, serum was separated from blood by centrifugation at 3000 rpm for 15 min and was used for estimation of total and direct bilirubin, blood urea nitrogen, creatinine, SGPT, SGOT and Random Blood Sugar, using commercially available kits of Merck Specialties Pvt. Ltd. India with the help of clinical chemistry analyser (Bene Sphera clinical chemistry analyzer, model C61).

Statistical analysis

The data obtained in this study were subjected to statistical analysis by using SAS/STAT® 9.1. The relationship of age and sex with the incidence of disease was also determined by Chi-square analysis. Significance level $p<0.05$ was considered.

RESULTS AND DISCUSSION

The overall 7% presence of Trypanosomiasis infection based on microscopic smear examination, 14% with Formol Gel Test and 21% with PCR were recorded in buffaloes of Deepor Beel area of Assam (Table 1). Results obtained by various methods indicated a significantly ($p<0.05$) higher parasitological detection rate by molecular technique (PCR) than Formol Gel Test and Giemsa smear examination (Table 1).

The Giemsa-stained thin blood smears revealed extracellular leaf-like trypomastigote form of the flagellate haemoparasites *T. evansi* (Fig 3A). In the Formol Gel Slide

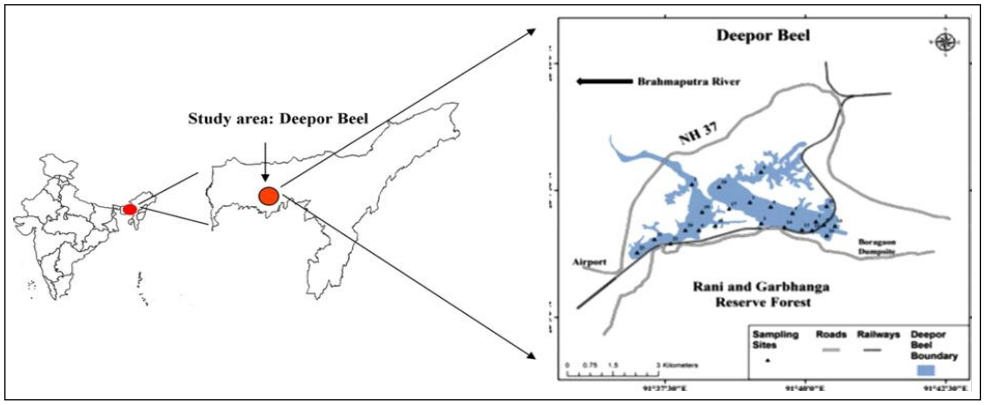
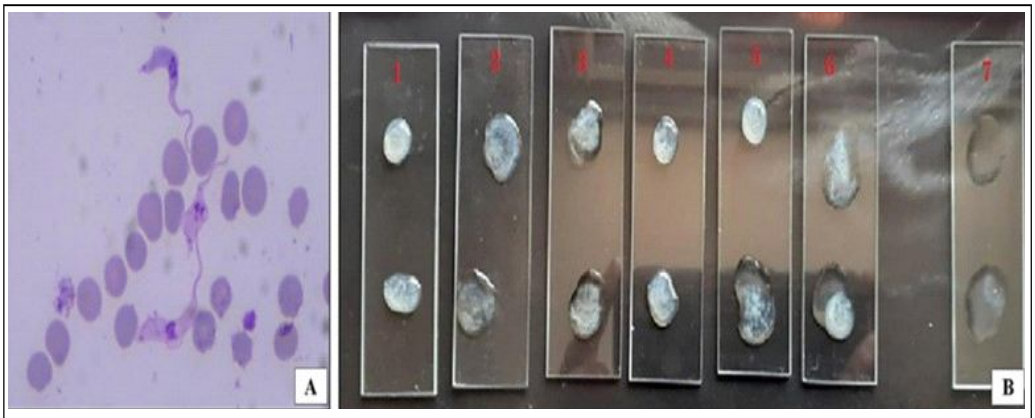


Fig 1: The red colour circle indicated the study area.



Fig 2: Semi-intensive rearing system of buffalo (Khuti) in Deepor Beel area, Guwahati, Assam.



A. Giemsa stained blood smear Showed the presence of *Trypanosoma evensi.*
B. Formol Gel Slide Test results: 1-6 Positive test serum showing gel formation and opacity like white of a boiled egg; 7- Negative test serum showing precipitation with a clear surface.

Fig 3: Diagnosis of trypanosomiasis in buffalo.

Table 1: Comparison of the result of the different tests for detection of Trypanosomiasis in buffalo.

Test	Total examined	Positive	Incidence (%)	χ 2	P-value
Giemsa smear examination	100	7	7.00	6.1636	0.045877
Formol Gel Test	100	14	14.00		
Polymerase chain reaction	100	21	21.00		

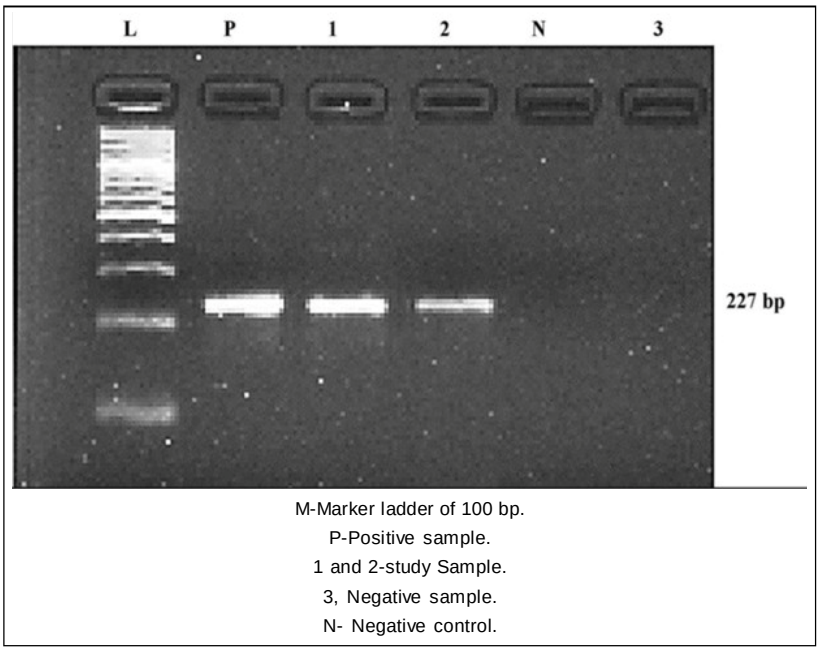


Fig 4: PCR amplified product 227 bp specific to *T. evansi.*

Test, out of 25 samples, 14 samples showed distinct gel formation which is characterized by white boiled egg, adhered to the micro slide, indicated positive for trypanosomiasis (Fig 3B). The species was further confirmed by PCR. Amplification of DNA from whole blood yielded a PCR product of 227 bp which confirmed the species of the parasite to be *T. evansi*. (Fig 4). Infection of *T. evansi* resulted in non-significant variation based on gender and age (Table 2). The study revealed that the incidence was non-significantly higher in the 3 to 5 years age group of female buffaloes.

Various clinical ailments in buffaloes such as high fever (105°F±1.0°F), hyperemic mucosa of the eyes, lacrimation, corneal opacity, pale mucus membranes and incoordination of the hindquarter. The animals were found to be cachectic with prominent bones and rough body coats and skin, dull and depressed (Fig 5).

The routine haematological investigation showed a significantly ($p<0.01$) decrease in the level of Hb, PCV, TEC and platelet count (Table 3). The leukocytopenia, lymphocytopenia and neutrophilia were the characteristic haematological findings observed in infected buffaloes as compared to healthy ones (Table 3).

The biochemical investigation of the blood reveals significantly ($p<0.01$) increased levels of blood urea nitrogen, creatinine and total bilirubin but blood sugar level was significantly ($p<0.01$) decreased as compared to healthy (Table 3).

Trypanosoma evansi is highly pathogenic to domestic animals. The various mechanism adopted by this parasite to evade the host's immune response results in a chronic infection leading to damage of vital organs due to the multiplication of the parasite. A positive result in the formol gel slide test was indicated by the formation of gel that adhered to the slide and the development of opacity akin to the white of a boiled egg. Sarmah *et al*. (2018) has recorded FGST to be a rapid and simple low-cost traditional tool that may still find a place in the tentative diagnosis of clinical trypanosomiasis. The positive result in FGST is indicative of a recent or previous infection of *T. evansi*. In the present study, results showed a lower prevalence of trypanosomes examined by Giemsa smear examination (7.00%) as compared to Formol Gel Test (14.00%) and molecular investigation (21.00%) suggesting high sensitivity and specificity of molecular techniques. However, these findings are higher than the prevalence reported. Laha *et al*. (1989), Das *et al.* (1998), Rao and Hafeez (2005) have recorded a lower prevalence of 2.69%, 2.71% (Guntur) and 7.28% (Godavari) respectively in buffaloes from various parts of India. The high prevalence of trypanosomiasis detected in the buffalo population of the region could be explained by favorable environmental conditions for the breeding of

Table 2: Relationship of age and sex-wise incidence of *Trypanosoma evansi* in buffaloes based on different tests.

Risk factors		Total examined	Positive	Incidence (%)	χ^2	P-value
Giemsa smear examination						
Age						
	< 1 year	13	1	7.69	1.6015	0.65905
	1-3 years	25	1	8.00		
	3-5 years	35	4	20.00		
	>5 years	27	1	11.11		
Sex						
	Male	25	2	20.00	0.0442	0.833427
	Female	75	5	10.66		
Overall		100	7	7.00		
Formol gel test						
Age						
	< 1 year	13	2	15.38	1.6096	0.657215
	1-3 years	25	3	12.00		
	3-5 years	35	7	28.57		
	>5 years	27	2	7.41		
Sex						
	Male	25	5	28.00	0.727	0.393844
	Female	75	9	13.33		
Overall		100	14	14.00		
Polymerase chain reaction						
Age						
	< 1 year	13	2	15.38	3.748	0.289988
	1-3 years	25	4	16.00		
	3-5 years	35	12	34.29		
	>5 years	27	3	11.11		
Sex						
	Male	25	7	28.00	0.6196	0.431201
	Female	75	14	18.67		
Overall		100	21	21.00		

vector flies in low lying areas due to the presence of water basins of Deepor Beel in the region.

The results of the present study revealed a non-significant difference in the presence of trypanosome infection in buffaloes based on age and gender. A similar finding was also observed by Mulugeta *et al*. (2013) who has found that the infection rate of trypanosomiasis in buffaloes has no bearing on any of the sex. The present study showed that the 3-5 years age group were mostly infected with trypanosomiasis which might be due to the predisposing stress factors *viz*. lactation, pregnancy, nutritional and climatic changes *etc*.

The generalized and most prominent symptom observed was depression and emaciation of the animal, which may be because the parasites utilize glucose and oxygen which leads to degenerative and inflammatory changes in the host (Shivajothi *et al.*, 2014). Hypoglycaemia observed in the present investigation may be attributed to the consumption of a large amount of blood sugar by Trypanosomes. There may be depletion of body muscle which results in emaciation of the animal. Another significant symptom was corneal opacity which is also recorded by various workers (Reddy and Shivajothi, 2017).

Trypanosomes also invade the nervous system by penetrating the blood-brain barrier (Chandratre *et al.*, 2019), which may be a cause of the incoordination of the hindquarter along with other causes like hypoglycemia and other nutritional deficiency.

The most prominent haematological finding in this study is anaemia (normocytic normochromic type) which is following previous workers (Chandratre *et al.*, 2019). The anaemia may be caused due by inhibition of the haemopoietic system by the toxin liberated by the parasites failing in the production of the red blood cells. The lowered RBC counts can be attributed to the rapid multiplication of trypanosomes leading to the destruction of RBCs either mechanically or chemically due to the release of toxic metabolites by the parasites and inefficient erythropoietic mechanism. Anaemia may also be due to extravascular hemolysis leading to erythrophagocytosis. It may further attribute to the utilization of glucose of red blood cells by parasites for its survival. An increase in some biochemical parameters like SGOT and SGPT points out the proliferation of trypomastigotes stages of parasites in vital organs such as the liver, muscle including heart and nervous system. The same findings were also recorded by the previous



A. Cachectic with prominent bones; B. Rough body coat and skin, dull and depressed; C. Pale mucous membrane; D. Lacrimation with corneal opacity.

Fig 5: Various clinical ailments were observed in trypanosomiasis infected buffalo.

Table 3: Haemato-biochemical alteration of trypanosome infected buffalo in comparison to healthy buffalo.

Parameters	Infected (n=21)	Healthy (n=6)	T value
Haemoglobin (g/dl)	7.14±0.08	11.00±0.48	-6.947**
Packed cell volume (%)	22.9±0.23	35.2±0.89	-10.804**
Total erythrocyte count (Million/µl)	4.55±0.07	6.77±0.08	-20.567**
Total leukocyte count (Thousand/µl)	5.50±0.05	11.29±0.46	-11.443**
Mean corpuscular volume (MCV) (Femtolitre)	37.51±0.12	41.91±0.92	-3.944
Mean corpuscular hemoglobin (pg)	16.53±0.10	16.55±0.20	0.311
Mean corpuscular haematocrit (g/dl)	31.50±0.06	32.08±0.25	-2.403
Thrombocyte (Thousand/µl)	52.05±0.50	149.83±12.04	-7.551**
Lymphocyte (%)	16.69±0.08	66.45±1.09	-40.670**
Monocytes (%)	1.3±0.10	4.83±0.72	-4.824*
Neutrophil (%)	74.48±0.11	20.78±0.65	80.260**
Eosinophil (%)	6.64±0.11	7.06±0.35	-0.808
Basophil 9%)	0.89±0.03	0.87±0.05	46.115
BUN (mg/dl)	47.42±0.23	21.17±0.38	-11.306**
Random blood sugar (mg/dl)	30.36±0.31	51.87±1.79	0.870**
SGPT/ALT (IU/L)	45.53±0.66	44.35±1.27	-2.412
SGOT/AST(IU/L)	72.39±0.50	77.46±1.99	-0.460
Direct Bilirubin (mg/dl)	0.20±0.0.4	0.22±0.06	8.730
Total Bilirubin (mg/dl))	0.60±0.02	0.13±0.02	6.640**
Creatinine (mg/dl)	3.40±0.06	1.75±0.19	46.115**

*Significant at 0.05 level, **Significant at 0.01 level.

worker (Chandratre *et al.,* 2019). BUN and creatinine levels were significantly increased in infected buffaloes which were also observed by Jani and Jani (1993) and Singh *et al.* (2011). This change in BUN and creatinine level might be due to either altered nitrogen metabolism or reduction in renal circulation leading to kidney damage (Jani and Jani, 1993).

CONCLUSION

The present study gives the first information about the occurrence of *Trypanosoma evansi* in buffaloes from Assam, India. This study gives a detailed account of different clinical symptoms along with biochemical and haematological alterations. Further, epidemiological studies will also help to figure out the prevalence rate of *T. evansi* infection

in buffaloes and this may raise an alarm to the field veterinarians on the threat of dormant *T. evansi* infection in buffalo and its possible exacerbation under different stress-related conditions.

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No potential conflict of interest was reported by the authors.

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