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Hematological and molecular finding of *Trypanosoma evansi* in buffaloes of North Gujarat

J. G. Patel, B. I. Prajapati, R. S. Parmar, S. H. Raval, R. L. Patel, S. S. Patel, B. J. Patel, D. V. Joshi, K. M. Solanki, S. P. Modh, D. S. Patel

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Authors:
J. G. Patel ✉, R. S. Parmar, S. H. Raval, B. J. Patel, S. P. Modh, D. S. Patel
Department of Veterinary Pathology, College of Veterinary Science & A. H., Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, India

B. I. Prajapati, K. M. Solanki
Department of Veterinary Public Health, College of Veterinary Science & A. H., Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, India

R. L. Patel
Veterinary Officer, Dudhsagar dairy, Mehsana, Gujarat, India

S. S. Patel
Department of Veterinary Microbiology, College of veterinary Science & A.H., Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, India

D.V. Joshi
College of Veterinary Science & A. H., Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, India

✉ jasmi0102@gmail.com

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Abstract

Trypanosoma evansi is definitely one of the most common pathogenic trypanosomes in all over the world, obviously infecting domestic and wild animals. Before it contributes to even more economic losses, a fast and confirmatory early diagnosis is a must to curb the spread and development of the disease. The current study was aimed to identify *Trypanosoma spp*. in buffalo blood by blood smear analysis and polymerase chain reaction (PCR) as well as comparison of these two methods. This investigation was performed on 475 buffaloes with anaemia, recurrent fever, muscular weakness and loss of appetite. Out of 475 blood samples, 57 were found anaemic (Hb<8.5) through haematological examination. Wet, thin and thick blood smears as well as buffy coat smear examination and polymerase chain reaction (PCR) were performed on 57 blood samples to detect *trypanosoma*. Analysis of blood smears well as buffy coat smear(s) revealed that 5 (8.77 %) samples were found positive for *Trypanosoma*, while polymerase chain reaction analysis determined the infection in 20 (35.09 %) out of 57 animals.

Keywords buffalo, hematology, *Trypanosoma evansi*, PCR

Introduction

Variety of animals such as camels, buffaloes, horses, mules, cattle, goats, sheep and donkeys, are prone to *Trypanosoma evansi* (*T. evansi*) infection. As of late, *T. evansi* was additionally identified in influenced human in India, raising issues of the development of human infective strains in Asia [1]. According to economical point of view, Trypanosomiasis (Surra) is most important endemic disease throughout India caused by *Trypanosoma evansi*. It is transmitted mechanically by biting flies and has considerable medical and economic losses to the farmers in terms of abortion, infertility, loss of milk production, various neurological disorders, morbidity and mortality of the affected animals [2]. The disease is categorized by anemia, recurrent fever, oedema, muscular weakness, abortion and loss of appetite with 50-70% illness and death. Animals that show low unsteady parasite levels even after recovery/cure serve as vectors of the disease for years. Therefore, the detection of carriers is a key factor in disease control.

The diagnosis of *T. evansi* infection in ruminants, particularly in the field, is largely dependent on blood smear testing of affected animals. However, the level of parasitemia is often unsteady and low, particularly in the chronic stage such that the presence of trypanosome may go unnoticed [3]. In addition, typical clinical signs and pathognomic post-mortem lesions are lacking.

Therefore, more sensitive and specific technique is needed to detect *Trypanosoma* in animals. PCR is highly sensitive and specific method that could detect *T. evansi* infection earlier than blood smear examination and also help in the detection of carrier animals [4, 5]. Therefore, in this study, a successful PCR assay was used to identify the impact of Trypanosoma subclinical infection buffaloes.

Methodology

Collection of samples

475 blood samples from buffaloes with anaemia, recurrent fever, muscular weakness and loss of appetite were examined for this study. Blood samples received from the Teaching Veterinary Clinical Complex (TVCC) of Sardarkrushinagar Dantiwada Agricultural University (SDAU), Veterinary dispensaries, Dairy and field by the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, SDAU, Sardarkrushinagar were used for this study.

Hematology

Hematological parameters were performed with Auto Blood Analyzer. The following hematological parameters were studied (but not limited to): Hemoglobin estimation (Hb); Hematocrit (HCT); Differential Leucocyte Count (DLC); Total Leucocyte Count (TLC); Mean Corpuscular Volume (MCV); Mean Corpuscular Hemoglobin Concentration (MCHC); Total Erythrocyte Count (TEC); Mean Corpuscular Hemoglobin (MCH).

Direct microscopic examination

Wet blood films

Small drop of blood (2–3 µl) were placed on to a clean glass slide and cover-slip were placed on blood drop to spread the blood as a monolayer of cells. Smear was examined by light microscopy (200 X) to detect any motile trypanosomes.

Stained thick smears

A large drop of blood (10 µl) was kept in the center of a microscope slide and extended with the corner of another slide to an area of about 1.0–1.25 cm in diameter. Smear was air-dry for 1 hour or longer, fixed in methanol and was stained. Smear was examined by light microscopy at a magnification of 1000X with oil immersion.

Stained thin smears

Small drop of blood (3–5 µl) was placed on to a clean glass slide and thin blood smear was made in the usual way. Smear was briefly air dry and fix in methanol.

Haematocrit centrifugation technique (Woo's technique)

5 µl of blood was collected into three heparinised capillary tubes (75 × 1.5 mm). Wet end was closed with paraffin and was centrifuged at 12,000 rpm for 5 minutes. One capillary tube was broken at just above the junction between the buffy coat and the plasma and buffy coat was taken on glass slide and smear was prepared in the usual way. Two capillaries were broken at just above the junction between the buffy coat and the plasma and buffy coat was collected in a microtube and frozen and used for polymerase chain reaction (PCR).

Staining of blood smear

Smear was stained with Eosine Methylene blue (Field) stain in the usual way.

Detection of Trypanosoma evansi by PCR

Buffy coat was used for molecular detection of *Trypanosoma evansi* by Polymerase Chain Reaction (PCR). DNA was isolated from Buffy coat using a DNA extraction kit (DNeasy blood and tissue kit, Qiagen) and all the steps were followed according to instructions provided in the kit.

The primer were got synthesized from BioInnovations, Mumbai. Specifications of primers are given in Table 1. Primer sequences (in 5'-3' direction) and annealing temperatures were as follows: RoTat 1.2 Forward GCG GGG TGT TTA AAG CAA TA, Tann. 59°C and RoTat 1.2 Reverse ATT AGT GCT GCG TGT GTT CG, Tann. 59°C. Amplification products were separated by agarose gel electrophoresis (2% agarose in 1× TAE) and DNA bands were visualized using UV trans-illuminator or Gel documentation System and photographed.

Table 1: Specifications of primers

Target	Primer ID	Sequences 5'–3'	Product size
T. evansi	RoTat 1.2 forward	GCGGGGTGTTTAAAGCAATA	205 bp
	RoTat 1.2 reverse	ATTAGTGCTGCGTGTGTTCG	

Comparative assessment of PCR and the blood smear examination for detection of Trypanosoma spp. in buffalo blood samples

There was comparison of the results of PCR and blood smear tests to detect Trypanosoma in buffalo blood samples. Both PCR and the blood smear examination tested a total of 57 blood samples from anaemic buffalo. As per method described by Martin (1977) [6], cross tabulation of PCR and the blood smear analysis considering PCR as reference test was reported for determining relative sensitivity and specificity of blood smear examination by following formula as given below.

$$\text{Sensitivity (\%)} = \frac{\text{(PCR and blood smear examination positives) X 100}}{\text{PCR positives}}$$

$$\text{Specificity (\%)} = \frac{\text{PCR and blood smear examination negatives X 100}}{\text{PCR positives}}$$

Results and Discussion

A total 475 blood samples were scanned for haematological abnormalities especially anaemia and blood protozoa based on haematology and blood smear examination. Out of 475 samples, 57 were found anaemic (Hb <8.5). Wet, Thin and thick blood smears as well as buffy coat smears were examined and 5 (8.77 %) were found positive for *Trypanosoma*. Microscopic examination of thin blood smears showed that *Trypanosoma* is an extra-cellular organism that resembled a leaf with a single flagellum submerged in oil (Figure 1). Under the high-power objective, approximately 3-20 organisms were discovered in each microscopic field. Wet blood smear revealed motile organism with rapid twisting motion.

The average value of Haemoglobin (Hb), Packed Cell Volume (PCV) and Total Erythrocyte Count (TEC) in anaemic animals are 5.78 ±1.579, 16.29 ± 4.887 and 3.75 ± 1.457 respectively (Table 2). The mean values of MCV, MCHC and MCH in anaemic animals are 45.42 ± 8.068, 35.78 ± 2.201 and 16.23 ± 2.741 respectively (Table 2). Details of mean values of Total Leucocyte Count (TLC), Lymptocyte, monocyte, Neutrophil, Eosinophil and Basophil in anaemic animals are 7.06 ± 4.522, 3.38 ± 2.309, 0.63 ± 0.429, 2.84 ± 2.929, 0.13± 0.232 and 0.00 ± 0.000 respectively (Table 2).

Bovine trypanosomiasis is a vector born hemoprotozoan disease that imposes heavy economic losses such as drop in meat production and milk yield, irregular estrous, stillbirth, abortion and low quality semen [7]. (It also causes haematological and biochemical changes in calves [8].

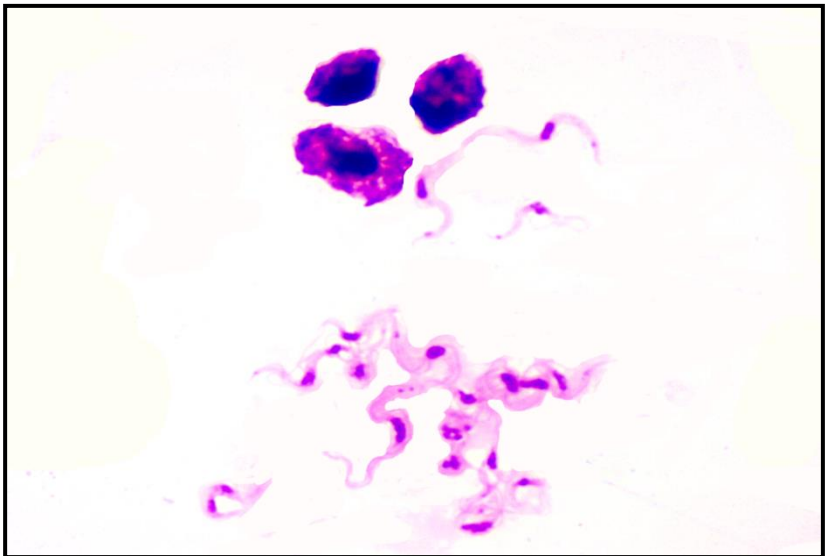

Figure 1. *Trypanosoma evansi* seen in buffalo blood smear stained with Eosin and Methylene blue (Field) stain

The hematological analysis revealed that decrease haemoglobin, packed cell volume and total erythrocyte count in infected buffaloes with haemoglobin less than 8.5. The present findings were in conformity with the finding observed in water buffalo calves and cow infected with *Trypanosoma evansi* [5, 8]. The major cause of erythrocyte injury is releases of hemolysin and Trypanosomal sialidase enzymes through *Trypanosoma Spp*., which results in the loss of red cells in the spleen, lymph node, liver, lung and bone marrow [9, 10].

Table 2. Physicochemical parameters

SN.	Haematological parameters	Mean ± Sd
1	Haemoglobin (gm%)	5.78 ± 1.579
2	Packed Cell Volume (%)	16.29 ± 4.887
3	Total Erythrocyte Count (TEC) (106/ µl)	3.75 ± 1.457
4	Mean Corpuscular Volume (MCV) (fl)	45.42 ± 8.068
5	Mean Corpuscular Hemoglobin (MCH) (pg)	16.23 ± 2.741
6	Mean Corpuscular Hemoglobin Concentration (MCHC) (g/dl)	35.78 ± 2.201
7	Total Leucocyte Count (103/ µl)	7.06 ± 4.522
8	Lymptocyte (103/ µl)	3.38 ± 2.309
9	Monocyte (103/ µl)	0.63 ± 0.429
10	Neutrophil (103/ µl)	2.84 ± 2.929
11	Eosinophil (103/ µl)	0.13± 0.232
12	Basophil (103/ µl)	0.00 ± 0.000

Blood smear examination is a method of choice for detection *T. evansi* blood of infected animals especially in acute cases when heavy parasitaemia found in blood. In this study, *Trypanosoma* organism was found in the blood smear examination. It is an extra cellular motile (rapid twisting motion) organism stained by Eosine Methylene blue (Field) stain [11].

DNA was collected from buffy coat of the anaemic blood samples and PCR were performed and only 20 (35.09 %) samples were found positive for *Trypanosoma evansi* (Figure 2). The amplicons displayed the specific ~205 base pair (bp) band, comparable to the positive control of *T. evansi*.

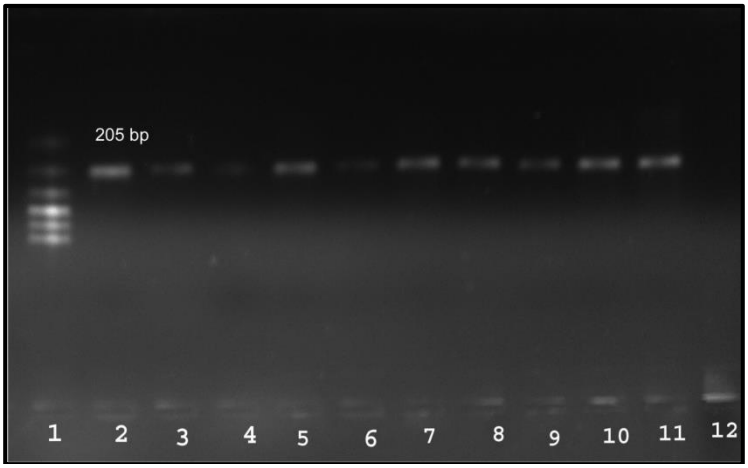

Figure 2. Amplified product of *Trypanosoma evansi* species specific PCR from resolved in 2.0% Gel (In Gel Documentation system): 1- Ladder, 2- Positive control, 3-11 samples, 12- Negative control

PCR is a very specific and sensitive technique for the detection of parasitic DNA in blood specimens [5, 12, 13]. Specific primers for each trypanosomic species have been conceived and used in a wide range of countries [12, 13].

Table 3. Comparative analysis of different techniques in infected animals for detection *T. evansi*

Total number of examined buffaloes	Anaemic buffaloes with Hb<8.5	Microscopic examination		PCR examination	
		No. of +ve samples	Rate samples	No. of +ve	Rate
475	57	5	8.77%	20	35.09 %

Table 4. Comparative evaluation of PCR and blood smear examination for detection of *T. evansi*

Test		Blood smear examination	
		Positive	Negative
PCR	Positive	5	15
	Negative	0	37
Sensitivity (%)		25	
Specificity (%)		100	

In the present study, the microscopic examination of blood smears revealed *Trypanosoma spp*. in 5 (8.77%) out of 57 anaemic animals, while PCR examination recorded the infection in 20 (35.09%) out of 57 anaemic animals positive (Table 3). So PCR is the most confirmatory test for early diagnosis of Trypanosomiasis [5] and consequently controlling programs and considered the confirmatory test.

Considering PCR as the reference test, the relative sensitivity and specificity of blood smear examination were 25 % and 100 %, respectively (Table 4).

Conclusion

There was a significant difference in the effectiveness of the blood smear examination and PCR for detecting *Trypanosoma Spp*. in blood samples suggesting that PCR is a more sensitive and reliable technique to diagnose *T. evansi* infection and ultimately helps in treatment and control strategy that impact on national economy. However, blood smear examination cannot diagnose the earlier stages of the infection

as it is less sensitive. Furthermore, different species of trypanosomes cannot be accurately distinguished on the bases of microscopic examination of blood. Moreover, microscopy is a tedious and laborious technique because each slide needs to be examined. Therefore, to test an entire herd or monitor livestock widely, it is advantageous to use PCR.

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