

Comparative Studies on Diagnosis of *Trypanosoma evansi* in Experimentally Infected Goats

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Abstract: The present study was conducted to evaluate the use of polymerase chain reaction (PCR) for detection of *Trypanosoma evansi* in experimentally infected goats. Special emphasis was paid to comparison with the other methodologies such as: parasitological techniques wet blood film (WBF), buffy coat technique (BCT), mouse inoculation (MI) and the diagnostic serological test (Card agglutination trypanosoma test (CATT)). The diagnostic sensitivity of each test was also estimated. To achieve these purposes, ten adult female dry goats, 18-24 months old were used. The animals were verified to be free from *T. evansi* infection by mouse inoculation test. After the infection of the animals, blood samples were taken and used for performing the different analyses. This study revealed that application of PCR using *T. evansi* specific primers was the highest method of *T. evansi* detection (93.8%) followed by the CATT(60%), MI(55.4%), BCT(18.5%) and WBF(13.8%).

Key words: *Trypanosoma evansi* Diagnosis % Goat % PCR % Serodignosis

INTRODUCTION

Trypanosomosis is a vector-borne disease of humans and livestock, caused by unicellular, flagellated protozoa of the genus *Trypanosoma*. It is a major constraint for ruminant production in Africa, Asia and South America [1, 2]. Trypanosomosis in goats produces acute, subacute and chronic or subclinical forms. The most invasive trypanosomes for goats are *Trypanosoma vivax*, *T. congolense* and *T. evansi*. Moreover, it has been reported that goats may serve as a reservoir host of trypanosome infection for the other species [3].

Early detection of *T. evansi* plays an important role in the epidemiology and animal health [4]. Despite the application of variety of diagnostic tests, the diagnosis of trypanosomosis remains problematic and the commonly used tests have important limitations [5]. *T. evansi* is usually detected by the microscopical examination of infected blood (wet blood film, stained blood smears and buffy coat examination), mouse inoculation and immunological methods. However, microscopical observation requires skilled technicians

and has poor sensitivity. Mouse inoculation is impractical for a large-scale epidemiological study. The immunological methods yield false negatives and positives due to *T. evansi*'s antigenic variation and it can not differentiate between infected and treated cases [4].

The conventional parasitological methods lack sensitivity and serological techniques, which detect antibodies or antigens lack specificity or sensitivity, respectively. Therefore a molecular technique, especially polymerase chain reaction (PCR) has been developed in order to overcome the problems faced with conventional and serological techniques. In addition, it was reported that PCR is a reliable method for diagnosis and epidemiological studies [6, 7].

The present study was conducted to determine the diagnostic sensitivity of the current parasitological techniques used for the detection of *T. evansi* in experimentally infected goats; [wet blood film (WBF), buffy-coat technique (BCT), mouse inoculation (MI)] and antibody detection test (card agglutination trypanosoma test (CATT)] in comparison with the diagnostic sensitivity of PCR.

MATERIALS AND METHODS

Strain: Camels (*Camelus dromedaries*) blood (0.3 ml) was intraperitoneally inoculated in White Swiss mice, 3 days post injection blood samples were collected from the tail vein of mice and examined for positive sample (MI). Positive samples were cryopreserved [8].

Experimental Animals: Ten adult Baladi dry female goats; 18-24 months old were used in the present experiment that was carried out at Abu-Rawash Research Station of National Research Centre, Giza governorate. Animals were kept under routine management system and protected from insects.

The animals were verified for the absence of *T. evansi* infection by mouse inoculation (MI). Before start the experiment, all animals were treated with trypanocidal drug (Barenil®-Intervet Egypt for Animal Health - Cairo; 1 ml / 10 Kg body weight) and a broad spectrum anti-helminthic and ectoparasitic drug (AVIMEC-Inj®-Arab Veterinary Industrial Co. (AVICO) - Jordan; 0.5 ml/25 Kg body weight).

After 30 days post treatment, goats were randomly assigned into two equal groups of similar age and weight. Each group was placed in a separate pen. The first group was kept as the control group and the second was the infected group.

Experimental Infection: The proposed Infected group was subcutaneously inoculated with 0.5×10^6 trypanosomes/ml. The control group was injected with saline. 8 ml of blood was collected by jugular venipuncture during the early morning before ration was offered. Two blood samples were collected from each animal. The first one was anticoagulated by disodium ethylene diamine tetra acetic acid (EDTA) and was used for detection of trypanosomes using three traditional diagnostic tests (wet blood film, buffy coat technique and mouse inoculation test) and isolation of DNA. The second sample was placed in a plain centrifuge tube for serum separation.

Procedures for diagnosis of *Trypanosoma evansi*

Standard Trypanosome Detection Methods (STDM), [9]

Wet Blood Film (WBF): Ten microliters of blood on EDTA were put on a clean slide, mixed gently with 10 μ l of phosphate buffered saline (BPS), covered with coverslip (18 \times 18 mm²) and the entire preparation was examined at high magnification (400 X) for 15-20 min.

Mouse Inoculation (MI): Two white Swiss mice were intra-peritoneally inoculated with 0.2-0.3 ml camel's blood. Three days post injection, blood samples were collected from the tail vein. Blood films were prepared and were examined by light microscope (400 X) for detection of trypanosomes.

Buffy-Coat Technique (BCT). [10]: Microhaematocrite centrifuge tubes (75 \times 1.5 mm) were filled with blood containing anticoagulant and sealed with clay and centrifuged in microhaematocrite centrifuge at 12,000 rpm for 5 minutes. A smear was prepared by scratching and breaking the capillary tube 1mm below the surface of the buffy-coat and one drop of the buffy coat was expelled onto microscope slide, smeared and covered with a coverslip (18 \times 18 mm) and examined with 400 X magnification. The slide was examined for 15-20 min with 400 X magnification.

Serological Technique

Card Agglutination Trypanosoma Test (CATT): CATT/*T. evansi* [11] was donated by the Institute of Tropical Medicine, laboratory of Serology, Antwerp, Belgium. The test was performed as described by the authors.

DNA-based Technique, Polymerase Chain Reaction (PCR)

DNA Isolation and Quantification: Isolation of DNA was performed using salting out procedure as described by Miller *et al.* [12]. The DNA concentration was measured at 260 nm using UV-Spectrophotometer and then diluted to 50 ng/ μ l, kept until assayed for PCR.

PCR Amplification: Trypanosomal DNA (Trypanozoon) was detected using nuclear primer set TBR1/TBR2 [13]. The forward and reverse primer set sequence was: GAATATTAA ACAATGCGCAG and CCATTTATTAGCTTTGTTGC.

PCR Reaction Cocktail and Cycle: PCR was carried out on 50 ng of genomic DNA in a 10 μ l reaction of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μ M dNTP, 1.5 mM MgCl₂, 1 mM tetra-ammonium-chloride, 0.1% Triton X-100, 0.01% gelatin, 4.5 pmol of each primer and 0.25 U Taq DNA polymerase. The standard PCR run cycle was usually as: Primary denaturation: 95 °C for 3 min. then: 35 cycles as: 95 °C for 15 sec.; 55°C for 60 sec.; 72°C for 30 sec. Final extension: 72 °C for 5 min., storage:

4 °C forever. The success of the PCR was detected on 2% agarose which is supplemented with ethidium bromide (0.5 µg/ml buffer), the horizontal electrophoresis run usually lasts 30 min. at 110 volt in a TAE buffer. After the end of the run the image was captured electronically using Biometra gel documentation system. The band sizes were determined using free software named Lab. image V2.7. It is dispersed free from Proland company (Germany), from the internet through the web page: <http://www.labimaging.com/servlet/engine/home/start.html>

Diagnostic Sensitivity: Diagnostic sensitivity of each test was first calculated for each infected animal as the percentage of positive samples over 13 sampling rounds. Combined estimates of sensitivity (CE_{se}) for each technique were obtained as the mean of these individual values. The values of CE_{se} obtained with this study should not be considered as point estimates for accuracy of the used tests, but should be interpreted as a measure for qualitative comparison of the tests included within this study.

The diagnostic sensitivity of a test was also calculated for each week post-infection (PI) using $Se_s = (n_{sp} / n_s) \times 100$ where n_{sp} is the number of samples from sth PI having a positive result from a total of n_s samples collected in that week. The value of Se_s which is a measure for the accuracy of a test at given time PI can be used to compare the different tests system at that given time.

RESULTS

The data presented in Table 1 showed that the combined estimate of sensitivity (Ce_{se}) of the PCR prove to be the highest (93.8%), followed by CATT(60%), MI(55.4%), BCT(18.5%) and WBF(13.8%).

The data presented in Table 2 declared that Se_s of the PCR increased to 100% at the 2nd week post infection (PI) and remained at this level until the end of experiment. MI reached 100% only at the 4th and 6th weeks while for CATT these high levels were reached only at the 3rd and 8th weeks PI. The Se_s of WBF and BCT fluctuated with maxima of 40%.

The data presented in Table 3 showed that 9 negative samples (by mouse inoculation,MI) produced positive reaction by card agglutination trypanosoma test (CATT) and 6 negative samples by CATT developed positive

Table 1: Comparative study of five diagnostic techniques in blood samples from five experimentally infected goats with *T. evansi*

Test	No. of Positive Tests	
	(Total No. of Tests)	CE _{se} ±95% CI
WBF	9.0 (65)	13.8±0.35
BCT	12 (65)	18.5±0.39
MI	36 (65)	55.4±0.50
CATT	39 (65)	60.0±0.49
PCR	61 (65)	93.8±0.24

WBF: Wet blood film; BCT: Buffy-coat technique; MI: Mouse inoculation; CATT: Card agglutination trypanosoma test; PCR: Polymerase chain reaction; CE_{se}: Combined estimate of sensitivity; 95% CI: 95% Confidence interval; No.: Number.

Table 2: Sensitivity (%) of five diagnostic methods, calculated per week (Se_s). In detecting *T. evansi* in experimentally infected goats in different periods for 8 weeks

Week (PI)	WBF	BCT	MI	CATT	PCR
1	5.0	10	10.0	5.00	80.0
2	0.0	0.0	46.7	80.0	100
3	20	20	80.0	100	100
4	20	40	100	80.0	100
5	40	40	80.0	80.0	100
6	40	40	100	80.0	100
7	20	20	80.0	80.0	100
8	20	20	80.0	100	100

Week PI: week post infection; WBF: Wet blood film; BCT: Buffy-coat technique; MI: Mouse inoculation; CATT: Card agglutination trypanosoma test; PCR: Polymerase chain reaction.

Table 3: Relationship between CATT / *T. evansi* and MI in detecting *T. evansi* in experimentally infected goats

Degree	Mouse Inoculation Test (MI)		
	Positive	Negative	Total
CATT Positive	30	9.0	39
CATT Negative	6.0	20	26
Total	36	29	65

Table 4: Relationship between PCR and MI in detecting *T. evansi* in experimentally infected goats

Degree	Mouse Inoculation Test (MI)		
	Positive	Negative	Total
PCR Positive	36	25	61
PCR Negative	0.0	4.0	4.0
Total	36	29	65

results by MI while data in Table 4 declared that 25 MI negative samples produced specific fragment for *T. evansi* by polymerase chain reaction (PCR).

Table 5: Detection of Trypanosomes, DNA and antibodies in blood of five goats following experimental infection with *T. evansi*

Day Post Infection	Goat No. (1)					Goat No. (2)					Goat No. (3)					Goat No. (4)					Goat No. (5)				
	WBF	BCT	MI	CATT	PCR	WBF	BCT	MI	CATT	PCR	WBF	BCT	MI	CATT	PCR	WBF	BCT	MI	CATT	PCR	WBF	BCT	MI	CATT	PCR
2	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
4	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-
6	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+
8	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+
10	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+
12	-	-	+	+	+	-	-	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-	-	+
14	-	-	+	+	+	-	-	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	+	+	+
21	-	-	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+
28	-	-	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+
35	-	-	-	+	+	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+
42	-	-	+	+	+	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+
49	-	-	+	+	+	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
56	-	-	+	+	+	-	-	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+

WBF: Wet blood film; BCT: Buffy-coat technique; MI: Mouse inoculation; CATT: Card agglutination trypanosoma test; PCR: Polymerase chain reaction

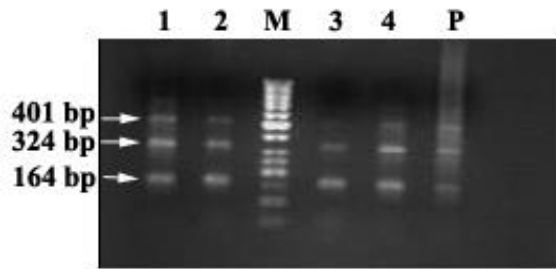


Fig. 1: Showing 4 samples proved to be a positive due to the presence of specific bands (lanes 1, 2, 3 & 4). M: is a DNA size marker 50 bp ladder P: is a positive control sample

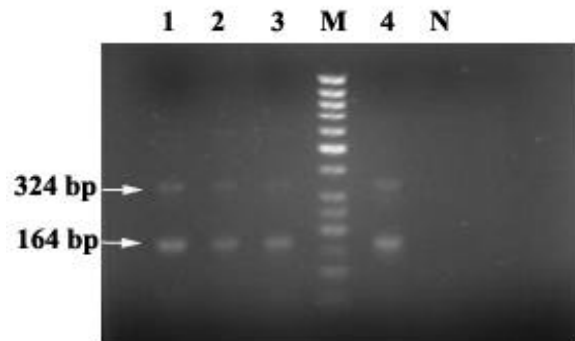


Fig. 2: Showing 4 samples proved to be positive due to the presence of 164 bp specific band. N: is a negative control sample did not show any specific band. M: is a DNA size marker 50 bp ladder

The data presented in Table 5 illustrated that wet blood film failed to detect trypanosomes in goats number No. 1 and 3 overall the experiment, while the trypanosomes were only detected at 4 day post infection

in goat No. 2 by BCT and the test remain negative till the end of experiment. In addition, after 2 day post infection, PCR with TBR1/TBR2 primer set was positive in two out of the five goats, while trypanosomes were not detected at that time on the other experimental goats.

A single *T. evansi* specific band (~164-bp) was only detected in lane 1 (Figure 1E) and lane 3 (Figure 1A). It is of interest that other lanes, ~ 324- and 401-bp bands are present, in addition to the 164-bp except in Fig. 1B, ~ 324-bp was only detected. The lack of 164-bp bands in lanes N₁ (Figure 1B) and N₂ (Figure 1E) also indicates that the positive results obtained with the *T. evansi* control and infected goats are not false-positive results resulting from contamination of the reaction mixture with *T. evansi* DNA or with 164-bp amplicons produced in previous PCR runs in which the TBR1/TBR2 primer set was used.

DISCUSSION

Experimental trypanosomal infection in goats had been discussed by a few workers [14,15]. It has been suggested that small ruminants may be important as reservoir of infection [16].

The present study was designed to compare the PCR using TBR1/TBR2 primer set specific for subgenus *Trypanozoon* with a traditional parasitological methods [WBF,BCT and MI] and serological method [CATT] in five goats experimentally infected with *T. evansi* strain collected from camels in Egypt.

The present finding showed that the diagnostic sensitivity of WBF detect *T. evansi* in experimentally infected goats was 13.8% (9/65). These finding support the report of Boid *et al.*[17] who found detected trypanosomes in 6% of *T. evansi* experimentally infected

sheep and goats when wet blood films were examined. The more acceptable explanation is that the experimental goats used by de, Almeida *et al.*[18] and infected with *T. brucei* developed a severe illness within 4 weeks of infection which indicated the occurrence of acute parasitaemia. This acute parasitaemia may be led to increase trypanosomes concentration in peripheral blood above the detection limit of WBF (> 10.000 trypanosomes / ml), [19] and consequently increased the test sensitivity.

The present result declared that the diagnostic sensitivity of BCT was 18.5% (12/65). This was in accordance with Masake *et al.* [20]. However, this result disagree with de, Almeida *et al.* [18] and Monzon, *et al.* [21]. A more conceivable explanation is that the sensitivity of parasitological tests used depends on the number and virulence of the inoculated trypanosomes [22].

The present finding showed that MI possessed the highest combined estimate of sensitivity CE_{sc} (55.4%) and was in line with the observations by Monzon *et al.*[21] in horses, Wuyts *et al.* [23] in cattle, Pathak *et al.*[24] in camels and Holland *et al* [22] in buffaloes. The CE_{sc} of MI (55.4%) observed in this study was considerably lower than the reported values [21, 22, 24] in buffaloes, camels and horses, respectively. This may be related to the number and virulence of the trypanosomes and experimental animals used.

The present data showed that the diagnostic sensitivity of CATT in goats experimentally infected with *T. evansi* was 60% (39/65). This is in agreement with Davison *et al.*[5], Pathak *et al.* [24] and Ngaira *et al.* [25]. However, this result was different from the reports of Dia *et al.* [26] and Verloo *et al.* [27]. The results revealed that six blood samples were found to be positive for *T. evansi* infection by MI technique but proved to be negative as tested by the CATT. Two out of these six samples were collected at 4 day post infection, while the other four samples were collected from goats at 28, 35, 42 and 49 days post infection. In the first two samples, the negative CATT result may be attributed to low antibodies titre. This explanation is correlated well with Verloo *et al.* [28] who proved that RoTat 1.2 specific antibodies appeared at 6-32 days post infection. While in the other samples, showing negative reaction of CATT could be as a result of antigenic variation which is considered as common feature in trypanosomes [29].

The present result showed that the high diagnostic sensitivity of PCR in goats experimentally infected with *T. evansi* was 93.8% (61/65) and *T. evansi* specific PCR products were detected 2 day post infection. This was in

contrary with de, Almeida *et al.* [18] who detect *T. brucei* by PCR one week post infection. Finally, in addition to the expected ~ 164 bp band, further bands (~ 324 and 401 bp) were also seen. This was also obtained in goats experimentally infected with *T. vivax* by de, Almeida *et al.* [30]. This could be due to the repetitive character of the target satellite sequences; a ladder pattern was frequently obtained, corresponding to the varying number of repeat units present in the amplification products[30].

In conclusion, the PCR represents a viable tool for wide-scale epidemiological studies, which will be used to report the true prevalence of the trypanosome infection and to allow the implementation strategies to control the disease

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