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Comparison Between Molecular and Traditional Methods of Diagnosing *Trypanosoma evansi* in Camels

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Abstract | *Trypanosoma evansi* (*T. evansi*) is a significant pathogen affecting camels around the globe. The study aims to evaluate the potential of molecular methods as a reliable alternative to traditional techniques for accurate and rapid diagnosis of *T. evansi* in camel populations. For this purpose, this study was designed to collect seventy-five blood samples (n=75) from camels of different ages of both sexes (male and female) from different areas of northern and southern Babylon. Each blood sample was placed in a special anticoagulant tube (EDTA), with a label containing the number, age, and sex, and processed for analysis by microscopic examination and confirmed by PCR. The data analysis showed that among 75 camels, 17% were females, 73% were adults, 67% from north Babylon. Analysis of microscopic and molecular examination identified positive samples in 9% and 57%, respectively. Comparative analysis between microscopic examination and PCR methods showed had high rate of infection (43/75, 57.33%), while the microscopic examination showed a low rate of infection (9.2%) (7/75), with significant difference of *T. evansi* infection ($P < 0.00001$). Taken together, we noticed that microscopic diagnostic techniques were less sensitive than molecular techniques, indicating that microscopic techniques may provide false negative results. This justify the application of molecular techniques in diagnosis of *T. evansi* for accurate identification and characterization of infection in camels.

Keywords | *Trypanosoma evansi*, PCR, Microscope**Received** | July 12, 2024; **Accepted** | October 01, 2024; **Published** | October 31, 2024***Correspondence** | Wissam Ahmed Sabah Al-Janabi, Department of Parasitology, College of Veterinary Medicine, Al-Qasim Green University, 51013 Babylon, Iraq; **Email:** wsamahmd922@gmail.com**Citation** | Amer QJ, Al-Janabi WAS (2024). Comparison between molecular and traditional methods of diagnosing *Trypanosoma evansi* in camels. J. Anim. Health Prod. 12(s1): 55-60.**DOI** | <http://dx.doi.org/10.17582/journal.jahp/2024/12.s1.55.60>**ISSN (Online)** | 2308-2801**Copyright:** 2024 by the authors. Licensee ResearchersLinks Ltd, England, UK.This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

INTRODUCTION

Trypanosoma evansi (*T. evansi*), posing a significant threat to camels, belong to *Trypanosoma* genus, within the Trypanosomatidae family and Trypanozoon subgenus. This protozoan parasite is notorious for infecting various mammals, including humans (Mossaad et al., 2017). First identified in India in 1880, *T. evansi* is the causative agent of camel trypanosomiasis, commonly known as Surra (Dkhil et al., 2023). Camels, vital for various purposes in desert regions such as meat, wool, milk, transportation, and

racing, are predominantly affected in the Middle East and Africa (Faye et al., 2023; Devaux et al., 2020).

Parasitological techniques involve several methods, including microscopic examination of blood, parasite concentration techniques, and animal inoculation. Among these, direct microscopic examination of blood is the most straightforward and widely used method. This can be conducted through the wet blood film method or stained thick and thin smears (FAO, 2000). In wet film smears, trypanosomes can be observed either directly

between blood cells or indirectly by inducing movement in blood cells. Thin smears, including thin blood and lymph smears, are also employed, particularly for morphologically identifying various trypanosomes under a light microscope (Zhao, 2013).

Serological approaches play a pivotal role in diagnosing trypanosomiasis as they reveal immune interactions between the host and the parasite. These tests are invaluable for estimating the prevalence or incidence studies, as well as for monitoring seasonal or inter-annual variations and assisting in vector control. Commonly used tools include the card agglutination test (CATT/*T. evansi*) for detecting IgM in *T. evansi* infections, and ELISA for *T. evansi*, which also targets IgM. ELISA *T. evansi* demonstrates consistent sensitivity and specificity (ranging from 90% to 95%) across various host species. Notably, CATT exhibits high sensitivity in camels (Desquesnes et al., 2022).

Furthermore, enzyme immunoassays have been developed to detect antigens instead of antibodies, offering an alternative diagnostic approach (Galal et al., 2014). Specific serological tests like the capillary agglutination tests and the passive hemagglutination test have also been devised for this purpose (Zewdu et al., 2016). The immunofluorescent antibody assays have been utilized for the serodiagnosis of *T. evansi* infection in camels (Salwa and Shams, 2012). Antibody techniques include the complement fixation test (CFT), which has been employed in diagnosing *T. evansi* (Anitoín, 2004). The latex agglutination test (LAT) has also been specifically used for *T. evansi* (Desquesnes et al., 2007).

Molecular techniques provide a robust means of detecting parasites in both mammalian hosts and insect vectors, serving as primary research tools in this field. Polymerase Chain Reaction (PCR), a fundamental method in molecular diagnostics, relies on DNA polymerase enzymes to amplify specific DNA sequences (Desquesnes et al., 2007). A range of different modifications in PCR have utilized to diagnose trypanosomiasis in camels, achieving diagnostic accuracies ranging from 97% to 100% in naturally infected camels (Ali et al., 2011; Njiru et al., 2004; Singh et al., 2004). The sensitivity of PCR diagnosis varies based on the types of primers used, with primers targeting multiple-copy genes generally exhibiting higher sensitivity compared to those targeting genes with fewer copies (Holland et al., 2001a; Zewdu et al., 2016).

The study aims to evaluate the potential of molecular methods as a reliable alternative to traditional techniques for accurate and rapid diagnosis of *T. evansi* in camel populations.

DESIGN OF THE STUDY

This study was designed to collect seventy-five blood samples from camels of different ages of both sexes (male and female) from different areas of northern and southern Babylon (hence there were no specific habitant for camel shepherd). Each blood sample was placed in a special anticoagulant tube (EDTA), with a label containing the number, age, and sex, and transferred to the Parasitology Laboratory at Al-Qasim Green University.

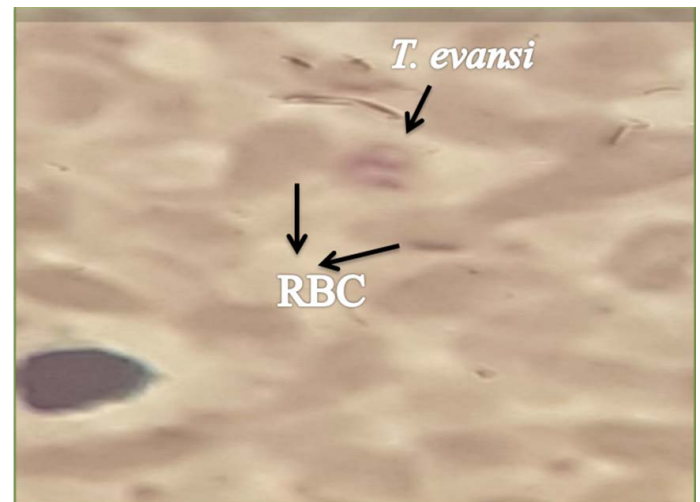


Figure 1: Thin blood smear stained with Giemsa stain (10%) show *T. evansi* between red blood cells of camel (X 100).

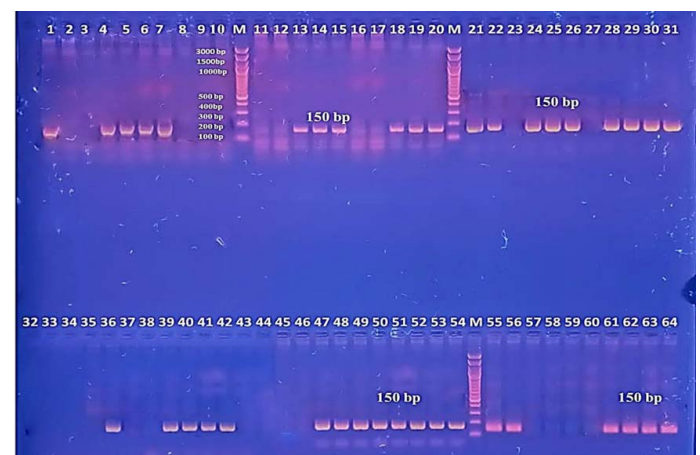


Figure 2: It shows the PCR amplification results of internal transcribed spacer1 gene (ITS1) of *Trypanosoma Evansi* isolated from Camel blood. Agarose gel picture appears the PCR product bands with molecular weight of 150 bp. (M) refers to (3000 bp) DNA ladder, (1) positive control. (2) Negative control. (3-64) some of PCR results of blood samples.

THIN BLOOD FILM BY GIEMSA STAIN

Blood collection for the examination of blood parasites in camels typically involves drawing blood from the jugular vein.

Table 1: Primers targeting 18S rRNA used for the detection of *Trypanosoma evansi*.

Primers sequence 5'- 3'	Amplicon size	Annealing temperature	Gene bank
F CTGAAGAGGTTGGAAATGGAGAAG	150 bp	53 °C	OQ674233.1
R GTTTCGGTGGGTCTGTTGTTGTTA			

Sterile needles and syringes are commonly utilized, although sterilized disposable products offer convenience. Direct smear examination is preferred over blood obtained from the jugular vein for better parasite detection. The procedure entails making blood smears using fresh blood immediately after collection, which is ideal for morphological examination. If fresh blood smears cannot be made, anticoagulants such as EDTA tubes are necessary. It's crucial to minimize the storage period after blood collection. When mixing an anticoagulant with blood, avoid vigorous shaking. High-quality, defatted, and washed slides are essential for sample preparation, as slide quality significantly impacts examination results. Normal glass slides washed and defatted in absolute ethanol are commonly used. If slides have been stored for more than six months, we applied a small drop of blood from a micro hematocrit capillary tube or pipette onto a glass slide, positioning it about 20 mm from one end. Using a spreader angled at 20-30 degrees to evenly spread the blood along the slide in a smooth, rapid motion, reaching each end of the spreader. Promptly air-dry the slide and labelled it with either a pencil or a diamond-tipped pencil. Submerge the thin smear in methyl alcohol for one minute to fix it. Then, inverted the slide and immersed it in Giemsa stain solution (1 ml in 9 ml distilled water buffered to pH 7.2) for 30 minutes. After staining, we rinsed the slide with water and allowed it to drain in an upright position until completely dry. Finally, we examined the prepared slide under a microscope at magnifications of 100x and 1000x, using immersion for optimal visualization.

CHARACTERIZATION PROPERTIES AND MEASURING OF THE TRYPOMASTIGOTE

In Giemsa-stained blood smears, typical morphological characteristics of *T. evansi* include a large size ranging from 25 to 35 micrometers, a small and sub-terminal kinetoplast, a thin posterior extremity, a prominent undulating membrane, a central nucleus, and a distinct free flagellum (Desquesnes et al., 2022).

MOLECULAR DETECTION PRIMERS

In this study, the primers were designed based on the sequence of 18S rRNA (ITS1) of *T. evansi* as shown in Table 1.

GENOMIC DNA EXTRACTION

All blood samples underwent genomic DNA extraction using the Genaid DNA purification kit according to the

manufacturer's protocol. Initially, 0.5 ml of blood was collected in a 1.5 ml centrifuge tube, followed by the addition of 500 µl of RBC-lysis buffer and a 10-minute incubation period. After centrifugation at 12,000 rpm for 2 minutes, the supernatant was carefully transferred to another 1.5 ml centrifuge tube.

Subsequently, 100 µl of binding buffer (GT) and 20 µl of Proteinase K were added to the supernatant, thoroughly mixed by vortexing, and then 500 µl of GB buffer was added and mixed well by inversion. The mixture was then incubated at room temperature for 10 minutes. The entire solution was transferred into a GD column and centrifuged for 30 seconds at 12,000 rpm. The flow-through was discarded, and 500 µl of wash buffer was added to the column, followed by another centrifugation step for 30 seconds at 12,000 rpm. This washing step was repeated twice.

Finally, the GD column was transferred to a 1.5 ml collection tube, and 50 µl of elution buffer was added. The eluted solution was stored at -20°C until further use.

DETERMINING THE CONCENTRATION OF DNA EXTRACTS

The extracted DNA was assessed using a Nanodrop spectrophotometer (Thermo.USA) following the manufacturer's instructions. DNA concentration (in ng/µL) was quantified by measuring the absorbance at 260/280 nm with the following steps:

PCR SAMPLE PREPARATION

The amplification of 18S rRNA was conducted in a final volume of 25 µl reaction mixture following the manufacturer's instructions as outlined in Tables 2 and 3. Subsequently, all samples were prepared in a PCR machine plate and set up for running using the appropriate settings as detailed in Table 3.

Table 2: The content of promega mastermix.

No	PCR Master mix components	Company Origin
1	Tap DNA polymerase	Promega USA
2	MgCl2	
3	dNTPs (dATP, dCTP, dGTP, dTTP)	
4	Tris-HCl pH 9.0	
5	KCl	
6	Stabilizer and tracking dye	

Table 3: PCR program for 18sr RNA gene amplification.

PCR step	Temp.	Time	Repeat
Initial denaturation	94 °C	5min.	35 cycle
Denaturation	94 °C	45sec.	
Annealing	53 °C	35sec.	
Extension	72 °C	1 min	
Final extension	72 °C	5min	

Table 4: Demographic characteristics.

Variables		Count	Percent
Sex	Male	13	17%
	Female	62	83%
Age group	Young	20	27%
	Adults	55	73%
Area	North Babylon	50	67%
	South Babylon	25	33%
Microscope	Positive	7	9%
	Negative	68	91%
PCR	Positive	43	57%
	Negative	32	43%

AGAROSE GEL ELECTROPHORESIS TECHNIQUE

Two concentrations of agarose gel (1% and 1.5%) were prepared. The 1% agarose concentration was utilized in the electrophoresis following the DNA extraction process, whereas the 1.5% agarose concentration was employed after PCR detection. Subsequently, the subsequent steps were carried out accordingly.

CASTING OF THE AGAROSE GEL

The gel was set up by assembling it into a casting tray with the comb positioned at one end. Approximately 1-1.5 g of agarose powder was dissolved in 100 ml of TBE buffer using a microwave. Ethidium bromide (3 µl) was added to the agarose solution, and the mixture was poured into the gel tray. It was then allowed to cool at room temperature for 30 minutes. Afterward, the comb was carefully removed, and the gel was placed in an electrophoresis chamber. The chamber was filled with TBE electrophoresis buffer until the buffer reached 3-5 mm above the surface of the gel.

LOADING AND RUNNING AGAROSE GEL

Ten microliters of the amplification sample were directly loaded onto a 1.5% agarose gel containing 3 µl of ethidium bromide per 100 ml. Loading buffer was added, and a DNA marker was used as a standard for amplicon size in electrophoresis. The gel was run at 80 V for 1 hour. Positive results were identified when the DNA band base pairs of the sample matched the target product size. The products were visualized using a UV transilluminator, and a digital camera was used to photograph the results.

STATISTICAL ANALYSIS

Descriptive results were viewed as count and percent, were done using SPSS v.26 and Microsoft Excel 2016. Associations between qualitative variables were tested using Chi-square test, significance level sut-off was less than 0.05.

RESULTS AND DISCUSSION

DEMOGRAPHIC CHARACTERISTICS

Among 75 camels, 17% were females, 73% were adults, 67% from north Babylon and about the result of microscopic and molecular examination were positive in 9% and 57%, respectively.

COMPARISON BETWEEN THE RESULTS OF THIN BLOOD STAIN AND MOLECULAR METHODS IN CAMELS

The result of comparison between microscopic examination and PCR methods. PCR method showed had high rate of infection (43/75, 57.33%), while the microscopic examination showed a low rate of infection (9.2%) (7/75), with significant difference of *T. evansi* infection (P< 0.00001) (Table 5).

Table 5: Comparison of *Trypanosoma evansi* infection between the results of thin blood stain and molecular methods.

Test	Total examined samples	Positive	Percent
Conventional	75	7	9.2%
Molecular	75	43	57.33%
Chi-square test	38.8		
P value	0.00001		
Significant	Significant		

The current investigation documented a total infection rate of 9.3% with *T. evansi* in camels observed through blood smear Giemsa staining, comprising 7 out of 75 samples. This finding closely mirrors a previous study conducted in Iraq previously (Awkati and Al-Katib, 1972) where authors have reported an infection rate of 10%. However, it exceeds the infection rate of 6% reported by Al-Eumashi (2004) in camels of the Al-Qadisiyha province. Studies conducted in neighboring countries have yielded diverse results. In Kingdrom of Saudi Arabia (KSA), El-Wathig and Faye (2013) have reported an infection rate of 43.8% using CATT and 3% using ELISA, whereas El-Wathig et al. (2016) have recorded a rate of 33% via direct smear stain technique in Jordan. In Iran, Khosravi et al. (2015) havereported infection rates of 10.57% using mercuric chloride and 2.1% using microscopic examination, respectively. Similarly, in other Arabian countries, infection rates of *T. evansi* ranged from 4.1% to 20.63% as detected through stained blood smears conducted in Egypt by

various researchers. In Sudan, Ali et al. (2011) have reported a 6% infection rate using microscopic methods, while Tehseen et al. (2015) have reported a rate of 0.7% in Pakistan using stained blood smears. The disparities in these findings may be attributed to differences in environmental and climatic conditions, sample sizes, diagnostic methods employed, vector densities, and overall animal health. Molecular methods, known for their heightened sensitivity and specificity, have increasingly been utilized for *T. evansi* detection. In the present study, PCR revealed a high infection rate of 57.3%. This result echoes findings from studies in Sudan and Egypt, where infection rates of 60% were recorded. However, discrepancies exist with studies from Pakistan, Saudi Arabia, and Kenya, where lower rates using PCR were reported. The higher infection rate observed in the present study may be linked to the parasite's ability to undergo antigenic variation, aiding in evading host immune responses. This attribute may contribute to the chronic nature of the disease in camels and underscores the parasite's adaptability to infect various host species which warrant future studies.

CONCLUSIONS AND RECOMMENDATIONS

Traditionally applied microscopic diagnostic techniques appeared less sensitive than molecular techniques, indicating that microscopic techniques could provide false negative results. This highlights the importance of molecular techniques in the diagnosis of *T. evansi* and should pay attention to the quality of microscopic devices used in this context.

NOVELTY STATEMENT

Evaluate the potential of molecular methods as a reliable alternative to traditional techniques for accurate and rapid diagnosis of *T. evansi* in camel populations was reported as a first time atleast in the region, as a novel biotechnique for diagnostic veterinary in camelid.

AUTHOR'S CONTRIBUTION

Qasim Jawad Amer and Wissam Ahmed Sabah Al-Janabi are participated in research desing, proposal writing, experimental and clinical works and also drafting the manuscript.

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ETHICAL APPROVAL

Ethical approval was obtained from the ethical committee (project ID: 124/2023) in the Al-Qasim Green University,

College of Veterinary Medicine.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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