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Original Research Article

Comparison between Molecular and Conventional Methods for the Diagnosis of *T. evansi* in Humans at Babylon, Iraq

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Abstract: In this study, the presence of Trypanosoma evansi was detected and identified humans in Babylon province in Iraq by blood smears and confirmed by polymerase chain reaction (PCR). Try this study by taking a sample blood from a camel breeders (humans) by Venipuncture from north and south of Babylon. 75 blood samples were collected from humans, adult and young of all sexes. The blood smears result was in humans 0% (0/75). The infection rate using PCR technology was 12% (9/75). This was the first study in Iraq to reveal the presence of the disease. Trypanosoma evansi in camel breeders (humans).

Keywords: Human, *T. evansi*, PCR, microscope.

INTRODUCTION

Trypanosoma evansi is a significant pathogen affecting camels within the genus Trypanosoma, belonging to the family Trypanosomatidae, and the subgenus Trypanozoon. This protozoan parasite is known to infect various mammals, including humans (Mossaad *et al*., 2017; Kamidi *et al*., 2018). Despite being primarily recognized as an animal parasite, there has been a growing number of reports in recent years highlighting cases of human illnesses linked to *Trypanosoma evansi* infection, particularly from India and other regions. Some of these cases have been extensively studied and well-documented. Consequently, the emergence of atypical human trypanosomosis (aHT) caused by *Trypanosoma evansi*, a disease relatively unfamiliar to human medical practitioners, has become a cause for concern in recent times. While timely diagnosis and treatment have led to the preservation of patients' lives in certain instances, the lack of accurate diagnosis or appropriate treatment has unfortunately resulted in fatalities in other cases (Truc *et al*., 2013). Some of them are well demonstrated and documented. Thus, atypical human trypanosomosis (aHT) due to *T. evansi*, a newly emerging disease which is little known among human physicians, is a matter of concern since recent past. In some cases, though patient's life could be saved by timely diagnosis and treatment, in others, the patients succumbed due to paucity of actual diagnosis or treatment (Truc *et al*., 2013). In 1977, a noteworthy incident occurred when an individual was inadvertently infected by an infected syringe containing *T. evansi*. Subsequently, successful treatment was administered using an arsenic compound called atoxyl (Gill in 1977). This case stands as a confirmed instance of *T. evansi* infection due to the known transmission. In 1999, another significant occurrence was reported from Sri Lanka, involving a human suspected of being infected with *T. evansi*. The individual presented symptoms such as headaches and episodes of hyperthermia concurrent with high parasitemia, as detailed by (Truc *et al*., in 2013). In 2004, a well-documented case was described from Maharashtra (India) in which a farmer exhibited proteoplasmic trypanosome parasitemia with febrile seizures for more than 5 months. Infection with *Trypanosoma evansi* was confirmed by molecular and microscopic methods. Later the plantations were cured by treatment with trypanocide-suramin (Joshi *et al*., 2005). Reported a case of a 40-year-old woman, resident of Canning district of South 24 Parajanas district in West Bengal. The patient showed blood parasitaemia on blood smear examination by microscopy. However, no serological or molecular testing was performed. The patient died due to delayed diagnosis and necessary treatment. In 2010, a livestock farmer became infected with *Trypanosoma evansi* in Egypt (Sengupta *et al*., 2022).

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MATERIALS AND METHODS

Design of the study

This study was designed to collect seventy-five blood samples 75 human samples, of different ages of both sexes (male and female) 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.

Thin Blood Film by Giemsa stain

Blood collection for the examination of blood parasites in humans typically involves drawing blood from the Venipuncture. Sterile needles and syringes are commonly utilized, although sterilized disposable products offer convenience. Direct smear examination is preferred over blood obtained from the Venipuncture 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, apply 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. Use 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 label it with either a pencil or a diamond-tipped pencil. Submerge the thin smear in methyl alcohol for one minute to fix it. Then, invert the slide and immerse it in Giemsa stain solution (1 ml in 9 ml distilled water buffered to pH 7.2) for 30 minutes. After staining, rinse the slide with water and allow it to drain in an upright position until completely dry. Examine the prepared slide under a microscope at magnifications of 100x and 1000x, using immersion for optimal visualization.

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).

Table 1: Primers 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			

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: 1. Power on the Nanodrop machine and select the appropriate application for nucleic acid analysis, specifically DNA. 2. Clean the measurement pedestals thoroughly with a dry chem-wipe multiple times. Carefully pipette 1µl of Elution buffer onto the surface of the lower measurement pedestals to blank the system. 3. Lower the sampling arm and initialize the Nanodrop by clicking OK. Once initialized, clean off the pedestals again and pipette 1µl of genomic DNA onto the measurement area. 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 table (2, 3). Subsequently, all samples were prepared in a PCR machine plate and set up for running using the appropriate settings as detailed in table (4).

Table 2: The content of Promega Master mix

No	PCR Master mix components	Company	Origin
1	Tap DNA polymerase		
2	MgCl2		
3	dNTPs (dATP, dCTP, dGTP, dTTP)	Promega	SA

No	PCR Master mix components	Company	Origin
4	Tris-HCl pH 9.0,		
5	KCl,		
6	Stabilizer and Tracking dye		

Table 3: The contents of PCR Reaction mixture

No	Components	Volume
1	DNA	5 µl
2	Forward primer	1.5µl
3	Revers primer	1.5µl
4	Master mix	12.5µl
5	Free nuclease water	4.5µl
6	Total volume	25µl

Table 4: 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	1min	
Final extension	72 ˚C	5min	

Agarose Gel Electrophoresis Technique (Tayyeb *et al.*, 2023)

Two concentrations of agarose gel were prepared, 1% and 1.5%. 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

Demographic Characteristics

Among 75 camels, 40% were females, 73% were adults, 67% from north Babylon and about the result of microscopic and molecular examination were positive in 0% and 12% respectively.

Table 5: Demographic characteristics

Variables		Count	Percent
Sex	Male	45	60%
	Female	30	40%
Age group	Young	20	27%
	Adults	55	73%
Area	North Babylon	50	67%
	South Babylon	25	33%

Variables		Count	Percent
Microscope	Positive	0	0%
	Negative	75	100%
CR	Positive	9	12%
	Negative	66	88%

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 rate infection (12%) (9/75), while the microscopic examination which has infection (0%) (0/75), with significant difference of *Trypanosoma evansi* infection (P< 0.003) Table 6.

Table 6: Showed comparison of *Trypanosoma evansi* infection between the results of thin blood stain and molecular methods

Test	Total examined samples	Positive	Percent
Conventional	75	0	0%
Molecular	75	9	12%
Chi-square Test	32.3		
P value	0.003		
Significant	Significant		

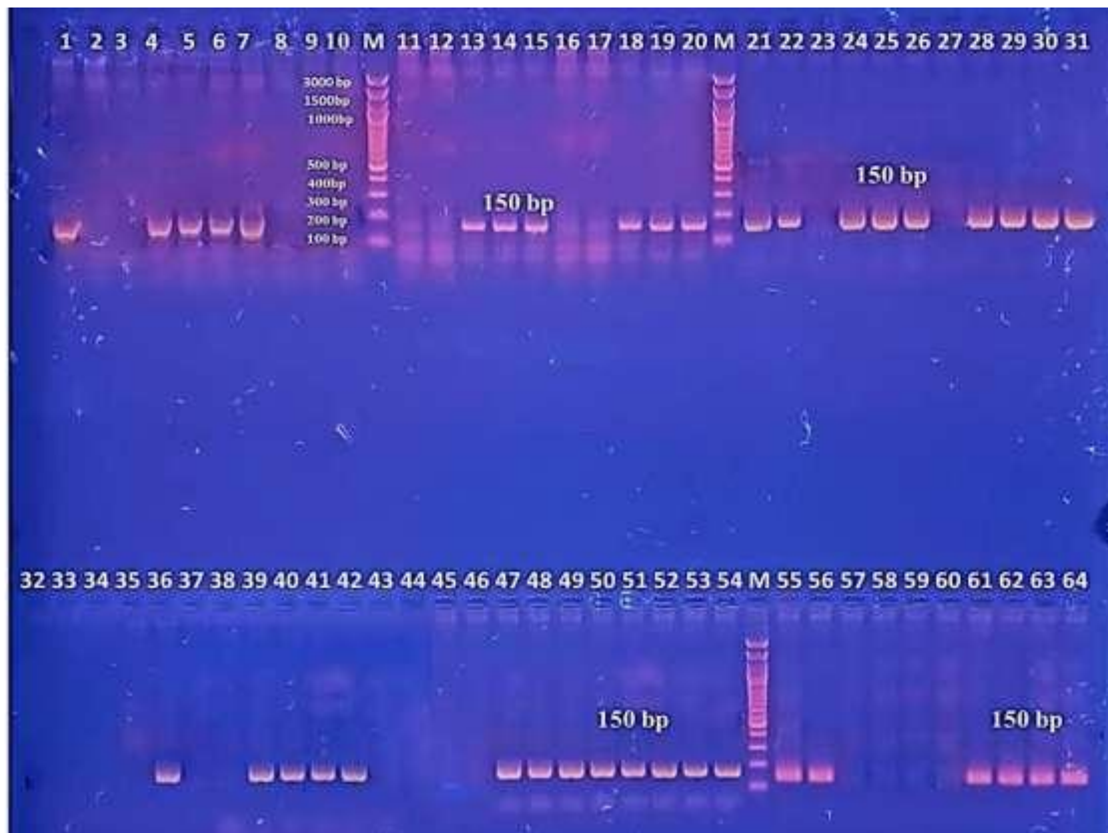

Figure 1: It shows the PCR amplification results of internal transcribed spacer1 gene

Figure 1 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.

DISCUSSION

The current study did not find any case of T. evansi in humans using traditional microscopy (direct blood smears). This implies that traditional methods used locally in the current study are not effective or that in general, hence there were 9 false negative cases in comparing with PCR methods. While in another study by (Sawitri *et al.*, 2019). In Indonesia used the serological methods, of the 24 serum samples, 4 (16.7%) samples were seropositive for the antigen T. evansi using. This indicate that the serological methods that used in the study of Indonesia were more sensitive in detection of *T. evansi* in humans. The current study using PCR method found 12% (9/75) of cases positive, PCR methods was more sensitive in

diagnosing T. evansi in humans. There were no statistically significant association between infection rate and sex, age group and area. In comparison of the molecular and traditional methods used in the current study, the molecular methods were more effective and no false negative results. Another study by (Sengupta *et al.*, 2022). Used the molecular (PCR) methods, found 2.89% (5/173) positive case, this indicate that the molecular methods used in the current study were more sensitive in detection *T. evansi* infections in human.

CONCLUSION

The results demonstrated a stark contrast between the two diagnostic methods employed. Microscopic examination failed to detect any infections, with 0% of the samples testing positive. In contrast, the PCR method revealed a 12% infection rate, detecting *Trypanosoma evansi* in 9 out of 75 camels. This significant discrepancy underscores the limitations of conventional microscopic techniques in diagnosing *Trypanosoma evansi* compared to molecular methods.

Conflict of Interest: None

Funding: None

Ethical Approval: Was obtained from the ethical committee in the Al-Qasim Green University, College of Veterinary Medicine.

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