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Epidemiological And Molecular Study Of Bovine Trypanosomiasis In Diyala Province

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Abstract:
Bovine trypanosomiasis, caused by infection with Trypanosoma species, poses a serious threat to cattle health and results in considerable economic losses in the livestock sector. This study, conducted in Diyala Province, Iraq, aimed to evaluate the prevalence of bovine trypanosomiasis and identify environmental and weather-related factors influencing its spread. It represents the first investigation of Trypanosoma parasites in this region. A total of 5 mL of jugular blood was collected from 550 cattle of varying ages and sexes across different locations in Diyala between July 2024 and May 2025. Giemsa staining of blood showed 7.82% Trypanosoma infection. Thick/thin smear methods found lower rates (1.82-3.45%), with no significant difference between the two techniques ($p > 0.05$). A distinct seasonal pattern was noted, with peak infection rates ranging from while infection rates peaked at 24% during the hot, dry months of July to September and decreased to nearly 0% in December and January. Molecular detection of Trypanosoma species using Kin1 (ITS1) primers identified the parasite in 9 of 36 samples (25.0%). Kin1 The overall infection rate based on Kin1 detection was 25.0%. was determined based on the final reaction volume following parasite amplification, which amounted to 750 bp. Molecular analysis confirmed a 100% sequence identity between the field isolate and a known Trypanosoma congolense type savannah strain, supporting accurate species identification. Comparative BLAST results showed high genetic similarity among isolates from different regions, with minor variations likely linked to geographic factors. Phylogenetic analysis revealed strong monophyly of T. congolense, limited intraspecific diversity, and clear separation from other trypanosomes.

Keywords: *Bovine Trypanosomiasis, Epidemiology, Molecular Detection (PCR), Seasonal Dynamics.*

INTRODUCTION:

Trypanosomiasis, a vector-borne parasitic disease caused by Trypanosoma protozoa, is a major hazard to cattle and human health in tropical and subtropical regions, particularly Africa, Asia, and South America. Key etiological agents in bovine species include T. congolense, T. vivax, and T. evansi. (1,2) While tsetse flies are the primary vectors, additional hematophagous flies and direct injection are also considered (3).The pathology of the disease has a serious negative effect on animal health includes anemia, reduced body mass, reproductive impairment, and mortality, which may be accompanied by hematological abnormalities and organ malfunction. Chronic, untreated African trypanosomiasis (AT) presents a complex immunological challenge due to the intricate interplay between host immune responses and parasite survival strategies (4).Diagnosing chronic infections can be challenging because the low levels of parasites in the blood create persistent reservoirs of the disease. Traditional diagnostic methods that depend on clinical signs and microscopy often lack sensitivity in both early and chronic stages of infection (5). Molecular techniques, such as PCR (polymerase chain reaction), improve diagnostic accuracy by enabling species-specific identification and detection of low-level infections(6). This study aimed to assess the prevalence of bovine trypanosomiasis in Dayla Province, Iraq, using both PCR and light microscopy. The study also sought to evaluate the relationship between PCR results and clinical and epidemiological risk factors. Additionally, the research intended to conduct phylogenetic analysis of PCR-positive samples to understand the genetic relationships among detected Trypanosoma species. This research addresses important gaps in our understanding of trypanosomiasis epidemiology in regions with limited research and emphasizes the importance of molecular tools for accurate disease monitoring and control.

MATERIAL AND METHODS

1.Samples and data collection

The current study conducted from July 2024 to May 2025, a total of 550 cattle were randomly selected from various regions in Diyala Province, Iraq. A 2 ml blood sample was collected from the jugular vein of each animal under aseptic conditions. Both thin and thick blood smears were prepared, and each sample was stored in EDTA for molecular assays. The study also included a direct clinical examination of the animals' mucous membranes, lymph nodes, and any signs of emaciation, as well as reduced milk production. Additionally, case history data were collected based on information provided by the animals' owners.

2.Microscopy

Two blood smear slides, both thick and thin, were prepared immediately after blood collection. Each slide was labeled with the corresponding animal number, allowed to dry, and then treated with absolute methyl alcohol(ACS ,chemical,India). After an additional drying period, the slides were stored in a plastic slide box. In the laboratory, the slides were stained according to the Giemsa kit protocol (Apco , Jordan) and subsequently examined with a light microscope (Novel,Korean) at a magnification of 100× to detect parasites(7) .

3.Parasite DNA Extraction Following the manufacturer's instructions, genomic DNA was extracted from bovineblood using the AddPrep genomic DNA extraction kit from tissue mini Kit (Add bio,Korea). Briefly, 200 µl of bovine blood was transferred into a 1.5 ml micro-centrifugetube containing 200 µl of binding solution and mixed well by pulse-vortexing for 15sec. The tube was incubated at 56℃ for 10 min, then, 200 µl of absolute ethanol wasadded and mixed well by pulse-vortexing for 15 sec. The lysate was carefullytransferred into the upper reservoir of the spin column with 2.0ml collection andcentrifuged at 13,000 rpm for 1 min. The flow-through was poured off and the spincolumn was assembled with the 2.0 ml collection tube. The DNA was washed by adding 500 µl of washing 1 solution to the spin column with collection tube andcentrifuged at 13,000 rpm for 1 min. The flow-through was poured off and the spin column was assembled again with the 2.0 ml collection tube. Second wash was performed by adding 500 µl of washing 2 solution to the spin column with a collection tube and centrifuged at 13,000 rpm for 1 min. The flow-through was poured off and the spin column was assembled again with the 2.0 ml collection tube. The spin column was dried by additional centrifugation at 13,000 rpm for 1 min to remove any residual ethanol in the spin column. The spin column was transferred to a new 1.5 ml microcentrifuge tube and 80 µl of elution solution was added to the spin column and let stand for at least 1 min. Finally, the DNA was eluted by centrifugation at 13,000 rpm for 1 min. The obtained DNA was kept at -20 °C until further assay.

4.Polymerase chain reaction (PCR) assay procedure

Polymerase chain reaction (PCR) was performed using specific primers (Table. 1) according to Desquesne, 2001. The primers were obtained from (Macrogen Co, Korea.

Table (3-5) Sequences of primers used for amplification of specific genes of *Trypanosoma*.

Primer Name	Primer Sequence 5’ – 3’	Product size (bp)	Reference
ITS1 F (kin2)	CGCCCGAAAGTTCACC	750	Desquesne, 2001
ITS1 R(Kin1)	GCGTTCAAAGATTGGGCAAT		

RESULTS

1.Microscopic results

The microscopic analysis of blood samples from 550 cows, conducted using Giemsa staining, revealed a *Trypanosoma* infection rate of 7.82%. The *Trypanosoma* infection rates identified through thick smear, thin smear, and combined methods were lower, ranging from 1.82% to 3.45% (refer to Table 1). There was no significant difference in the detection effectiveness of Trypanosoma infection between these techniques ($p > 0.05$)

Table (2) presents the percentage of infections caused by the Trypanosome parasite, using both thin and thick blood smears.

Type smear	N0.animal examination	Positive	%
Thick	550	19	3.45 % a
Thin		14	2.55 % a
Thick and thin		10	1.82 % a
Total	550	43	7.82%

The similarity of the letters(a , a) indicates the absence of a significant difference or a statistically significant difference with. ($P > 0.05$)

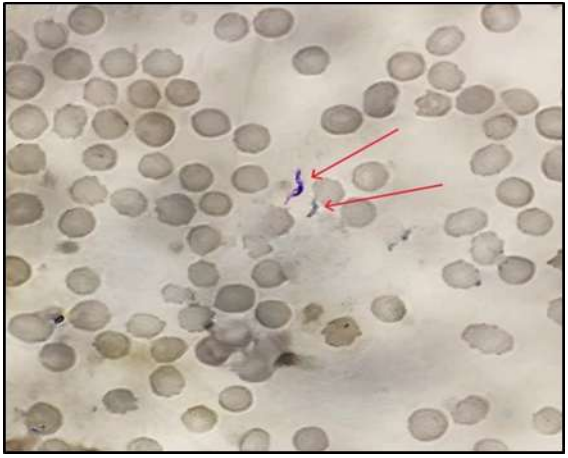
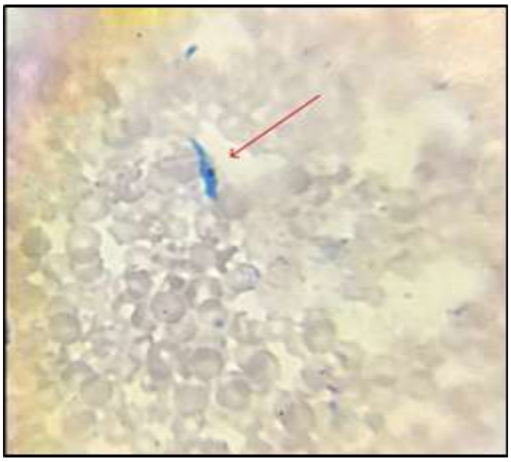


Figure (1) Trypanosome congolense in thin blood smaer with Giemsa stain at 100X



firure (2) Trypanosome congolense in thick blood smaer with Giemsa stain at 100X

3.Prevalence of Trypanosomiasis Acording the Seasonal Variations

A seasonal study investigating bovine trypanosomiasis infections in the Diyala Governorate from July 2024 to May 2025 revealed significant monthly variations in infection rates. The highest infection rates were recorded during the hot and dry summer months from July to September, ranging from 16% to 24%. In contrast, infections were nearly absent in December and January, with a rate of 0%.

Statistical analysis indicated a strong positive correlation between infection rates and ambient temperature ($p < 0.001$), along with a strong negative correlation with relative humidity. These findings suggest that the hot and dry conditions in Diyala enhance the mechanical transmission of the disease through vectors such as Tabanus and Stomoxys flies, which thrive in temperature ranges of 25–41°C and humidity levels of 30-50%..

Table(3) Prevalence of Trypanosomiasis Infection Rates in Relation to Environ mental Factors and Seasonal Variations .

Month	Total number	Positive Cases(%)	Temp. (°C) Average	Humidity % Average	Seasonal Phase

July (2024)	50	8(16%)	38 °C	24.5 %	Peak Summer a
August	50	10 (20%)	38.5°C	31%	Peak Summer a
September	50	12 (24%)	33.5 °C	30 %	Late Summer a
October	50	3 (6%)	24 °C	33.5%	Early Autumn b
November	50	2(4.0%)	16.5 °C	61%	Late Autumn b
December	50	0 (0%)	13.5 °C	55.5%	Early Winter c
January(2025)	50	0 (0%)	12.5 °C	60%	Peak Winter c
February	50	1 (2%)	10.5 °C	55.5%	Late Winter b
March	50	1 (2%)	19 °C	51%	Early Spring b
April	50	4 (8%)	26 °C	34%	Mid-Spring d
May	50	4 (8%)	31.5 °C	33%	Late Spring d
Total	550	43(7.82)	.....	.....	....

Different letters (a, b ,c) indicate that the values differ significantly at the probability level (p<0.001)

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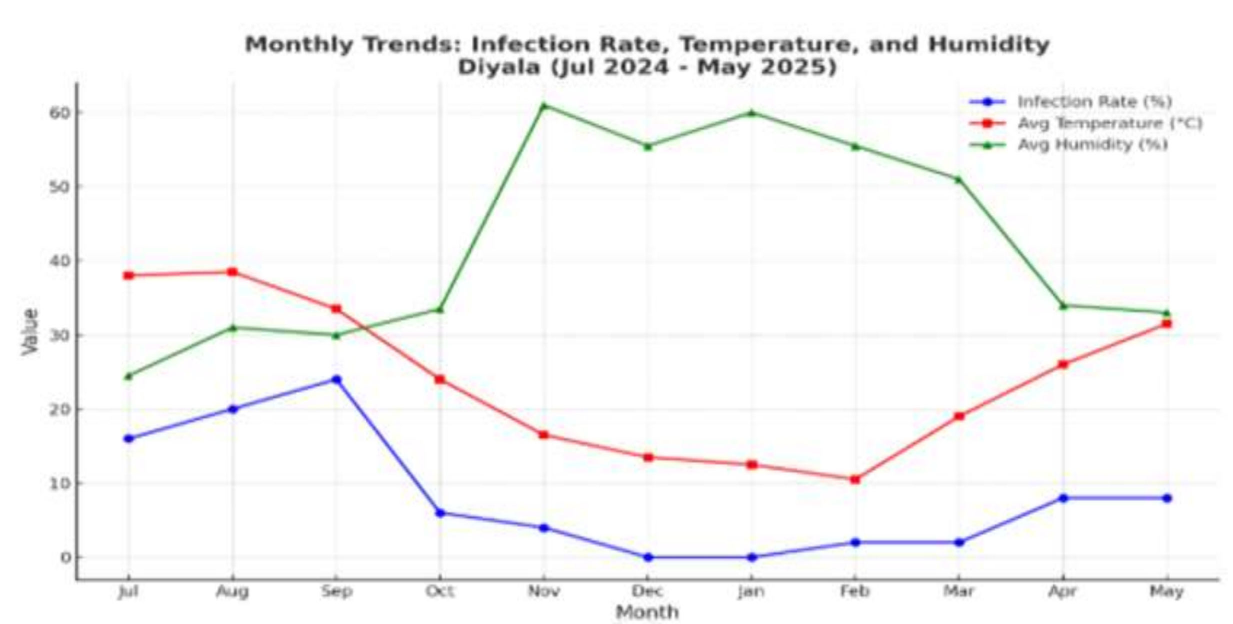


Figure (3) Monthly chart relation between infection rat , temperature and humidity

The chart shows how infection rates (blue, in percent), average temperature (red, in degrees Celsius), and average humidity (green, in percent) changed each month from July 2024 to May 2025. The infection rate and temperature were highest in the summer, when humidity was at its lowest. The chart also shows other changes related to the spring and winter seasons.

4.Molcular examination : This study was conducted in the Diyala Governorate to detect trypanosome parasites using the ITS1 F (Kin1) and ITS2 R (Kin2) primers.The ITS1/2 regions, located between ribosomal RNA genes, enable accurate parasite detection. Conserved flanking regions allow for species identification via fragment length analysis or sequencing, enhanced by simple or nested PCR. Thus, ITS1/2 is a reliable method for trypanosome study in both lab and field environments(8) .

Molecular detection of Trypanosoma species using Kin1 (ITS1) primers identified the parasite in 9 of 36 samples (25.0%). Kin2 (ITS2) primers failed to detect any positive samples, suggesting lower sensitivity or target sequence incompatibility. The overall infection rate based on Kin1 detection was 25.0%. was determined based on the final reaction volume following parasite amplification, which amounted to 750 bp , Figure(4).

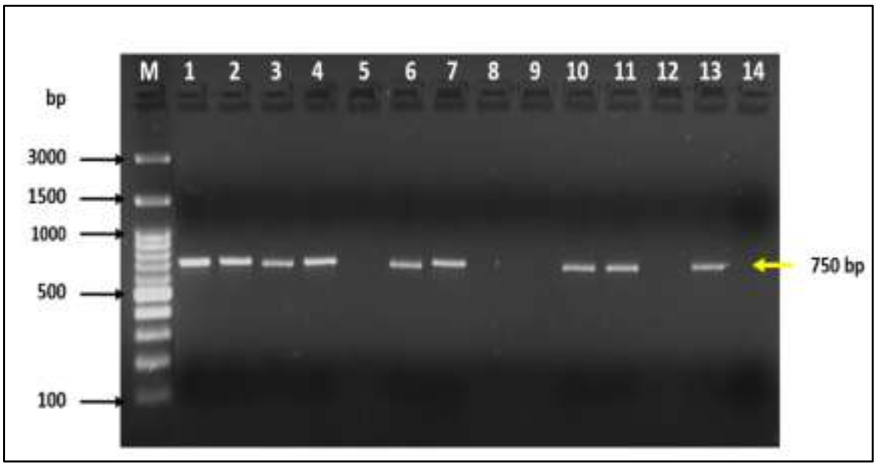
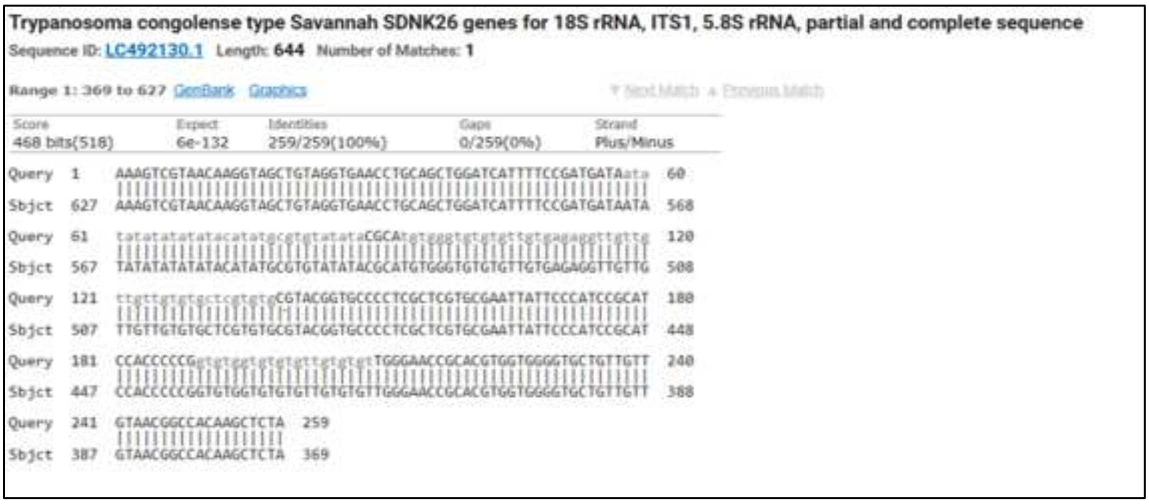


Figure (4). Gel electrophoresis for ITS1 gene of Trypanosoma spp. lane 1 represent 100 bp DNA marker. Lanes 1-4, 6,7, 10,11 and 13 are positive samples, lane 5, 8, 9, 12 are negative samples. lane 14 is negative control.

5.Sequence analysis results :

The results of the molecular sequence analysis using the NCBI-BLAST tool showed that the studied sample achieved a complete match of 100% with the sequence of *Trypanosoma congolense* type Savannah SDNK26 (GenBank accession number: LC492130.1). The number of matching bases reached 259 out of 259 with no gaps (0% gaps), and a high statistical value (E-value = 6e-132) indicating the reliability of the match and that it did not occur by chance. This result reflects the accuracy of the molecular diagnosis of the parasite and confirms the sample's belonging to the same known strain, thereby enhancing the credibility of the results relied upon in this study. Figure (5).



Trypanosoma congolense type Savannah SDNK26 genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence

Sequence ID: LC492130.1 Length: 644 Number of Matches: 1

Range 1: 369 to 627

Score	Expect	Identities	Gaps	Strand
468 bits(518)	6e-132	259/259(100%)	0/259(0%)	Plus/Minus

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Query  1    AAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATAata  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  627  AAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATAATA  568

Query  61   tatatatatatacatatgcgtgtatataCGCAtgtgggtgtgtgttgtgagaggttgttg  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  567  TATATATATATACATATGCGTGTATATACGCATGTGGGTGTGTGTTGTGAGAGGTTGTTG  508

Query  121  ttgttgtgtgctcgtgtgCGTACGGTGCCCCTCGCTCGTGCGAATTATTCCCATCCGCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  507  TTGTTGTGTGCTCGTGTGCGTACGGTGCCCCTCGCTCGTGCGAATTATTCCCATCCGCAT  448

Query  181  CCACCCCGgtgtggtgtgtgttgtgtgtTGGGAACCGCACGTGGTGGGGTGCTGTTGTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  447  CCACCCCGGTGTGGTGTGTGTTGTGTGTTGGGAACCGCACGTGGTGGGGTGCTGTTGTT  388

Query  241  GTAACGGCCACAAGCTCTA  259
            |||||||||||||||||||
Sbjct  387  GTAACGGCCACAAGCTCTA  369
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The figure. (5) indicate identification of query sample Trypanosoma congolense, alignment with NCBI gene bank.

Phylogenic tree :

This phylogenetic tree illustrates the genetic relationships among T. congolense strains, with T. theileri acting as the outgroup. Robust branch support (bootstrap ≥95%) indicates a high level of statistical confidence in the inferred topology. Horizontal branch lengths reflect genetic distance, indicating that shorter branches suggest greater genetic similarity. Field isolates PV537394-PV537397 demonstrate a close phylogenetic affinity to established reference strains (bootstrap = 98%). The T. congolense clade is supported by a high bootstrap value (100%), confirming its monophyly and clear divergence from T. theileri. The analysis reveals limited genetic diversity within T. congolense, which may influence disease management strategies, figure(6

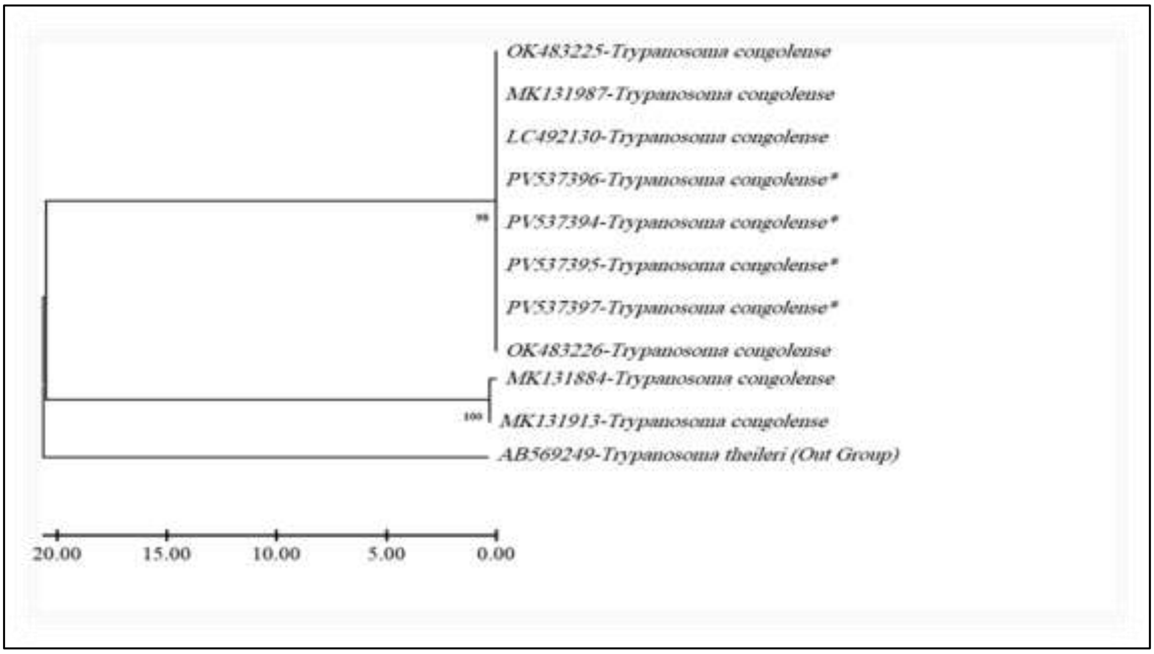

Figure (6) Phylogenic tree of Trypanosoma congolense from Iraq. The phylogenic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings. Partial DNA sequences of concatenated partial fusion protein gene were used as input dat.

DISCUSSION:

Bovine trypanosomiasis remains a critical impediment to livestock productivity in tropical and subtropical regions, exacting substantial economic losses through decreased milk yield, weight loss and, in severe cases, mortality. In our study of 550 cattle in Diyala Governorate (July 2024–May 2025), Giemsa-stained blood smears revealed an overall Trypanosoma infection rate of 7.82%, whereas thick and thin smears detected between 1.82% and 3.45% of cases ($p > 0.05$). These findings are broadly consistent with other Iraqi reports: Shaeel and Al-Sadoon (9) found 3.33% positivity in Wasit Province by microscopy, while Albadrani and Alabadi (10) and Abdulazeez and Esmaeel (2024) reported higher rates of 18.3% and 8.7% in Mosul, respectively. The observed regional variation likely reflects differences in vector density, environmental conditions, host immunity and sampling methodologies (11,12).

Seasonal analysis demonstrated a marked peak in infection rates during the hot, dry months of July to September (16–24%), with infections virtually absent in December and January (0%). Statistical modelling revealed a strong positive correlation with ambient temperature ($p < 0.001$) and a strong negative correlation with relative humidity, suggesting that mechanical vectors such as Tabanus and Stomoxys spp. are most active under warm, arid conditions (25–41 °C; 30–50% RH). These results align with regional studies in Mosul (13,14) and in Saudi Arabia (15), but contrast with the humid-climate preference of tsetse-transmitted African trypanosomiasis (23–25 °C; 75–90% RH) (16), Beyond vector ecology, unhygienic veterinary practices (e.g. needle sharing) and vertical (transplacental) transmission also contribute to year-round parasite maintenance (17,18).

Molecular diagnostics revealed a higher infection rate: ITS1-PCR detected Trypanosoma DNA in 25.0% (9/36) of samples, while ITS2-PCR yielded no positives, possibly due to primer mismatches or lower sensitivity. ITS1-PCR (25.0%) was more effective than microscopy (7.8%), consistent with findings from in Nigeria, ITS1-PCR detected 3.0% positivity versus 0.8% with microscopy, T. congolense being the most prevalent (50%) (19). Ugandan studies showed 4.3% versus 2.0% positivity, respectively, with T. congolense at 81.8% (20) and In Somalia ITS1-PCR found 16.4% positivity against 3.4% by microscope (23.8% in cattle via ITS1-PCR) ,(21).

In Iraq, PCR-based studies remain scarce: Shaeel and Al-Sadoon (2020) reported 9.3%(9)PCR positivity in Wasit cattle, while Khalaf and Alobaidii's 2024 Mosul survey lacked molecular confirmation(22).

Phylogenetic analysis confirms that Iraqi Trypanosoma congolense isolates exhibit high genetic similarity to global reference strains. Our phylogenetic tree, built using advanced statistical methods and strongly supported (100%), shows that all T. congolense are closely related, confirming previous research. This supports the findings of those who also observed genetic similarity among isolates from different regions(23).Although T. congolense infections in Iraq are uncommon, our analysis of field isolates (e.g., PV537394–PV537397) shows they are genetically similar to known reference strains like IL3000 from GenBank. We did not find any new evolutionary branches, and all sequences grouped in a well-supported single evolutionary group (bootstrap value approximately 100%) (24).

These results support the idea that T. congolense is a genetically similar species. The close genetic relationship between Iraqi isolates and reference strains, shown by their grouping in a single, well-supported branch, highlights the species' evolutionary stability. This genetic consistency could impact how we diagnose and control the disease.

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