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Phylogenetic analysis of Trypanosoma evansi in cattle in Mosul city, Iraq

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Abstract

Trypanosomiasis is a global disease that infects different types of animals, including cattle. *Trypanosoma evansi* is a zoonotic parasite that mostly affects cattle, and it causes a significant economic loss in the livestock industry in Iraq. This study aimed to determine the infection rate of *Trypanosoma spp.* in cattle in Mosul city, Iraq, using microscopic examination of buffy coat smear and PCR technique, further phylogenetic analysis of the strains detected in this study. One hundred and twenty-five cattle, ages 1-5 years, were investigated for the existence of trypomastigotes. The results presented that 22 (17.6%) and 27 (21.6%) of the study animals were positive for *T. evansi* using microscopic examination and PCR techniques, respectively. PCR technique results are considered the gold standard for comparing infected and non-infected cattle. Demonstrating a significant presence of the disease in this cattle group. The parasitemia of *T. evansi* was determined to be at 2.2%, indicating the parasite's prevalence in the affected cattle population. Furthermore, phylogenetic analysis was performed to assess the genetic relationships of two strains of *Trypanosoma evansi* from the infected cattle. These strains are extremely similar (99.60% - 100%) to those pathogen sequences that are recorded in the NCBI GenBank of different countries, such as Thailand, the Philippines, and Taiwan. In conclusion, according to the results, *T. evansi* is common among cattle in Mosul, Iraq, and these results may be useful for future research and beneficial management of *T. evansi* in the study region.

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Introduction

Trypanosoma, a unicellular protozoan flagellate that causes trypanosomiasis, also known as trypanosomosis, is an important disease transmitted by vectors in tropical and subtropical regions (1). It is a significant disease affecting various domestic animals, including cattle, particularly in Iraq (2-5). The infection can lead to severe economic losses as a result of decreased productivity and increased mortality rates among livestock (6). Among the various species, *Trypanosoma congolense*, *T. vivax,* and *T. evansi* are primary pathogens affecting cattle, while others like T. *theileri, T. simiae, T. godfreyi*, and occasionally *T. suis* are less clearly associated with clinical disease (7,8). The disease poses serious risks to both humans and livestock globally, particularly in South America, Asia, and Africa (9). Trypanosomiasis can infect various domestic animals and wild species, including horses, cattle, buffalo, goats, sheep, dogs, deer, gazelles, pigs, and elephants (10,11). Recent reports indicate that the cause of surra, *Trypanosoma evansi*, poses a considerable threat to animals health in Iraq (12-14). The most common transmission occurs through biological vectors like tsetse flies, but mechanical transmission by biting flies, like tabanids and stomoxyses, is also significant (15,16). Other transmission modes include iatrogenic, direct vertical, oral, or venereal routes (11). The clinical signs in the acute phase may include severe anemia, weight loss, decreased productivity, infertility, abortion, and death (17). While the chronic infections can lead to anemia, leukopenia, and thrombocytopenia, alongside inflammatory diseases affecting vital organs (17,18). According to Pereira *et al.* (19), subclinical or chronic trypanosomiasis can be lethal if

left untreated, which makes diagnosis and treatment difficult. Trypanosomiasis has a great effect on cattle in Iraq, with *Trypanosoma evansi* producing a special harm to the health of cattle. This parasite can live in regions without tsetse flies since it is commonly transmitted via biting flies like house flies (*Musca domestica)* and stable flies (*Stomoxys calcitrans*) (16,17). Current research has confirmed how significant it is to comprehend the whole thing possible vectors sharing to parasite transmission, as this information is important for the management of disease (20,21). Current research points to *Trypanosoma evansi* as a main danger to cattle health in the area (22). This biting flies transmission mechanism warrants *T. evansi* to remain alive in districts unavailable to tsetse flies, which leads to the control of disease is complicated (23). In cattle, clinical signs of *Trypanosoma spp*. are rarely beneficial in detecting the disease because it is clinically confused with other hemoparasites in cattle, like *Theileria annulata* (24,25), *Anaplasma phagocytophilum* (26) and *Babesia spp*. (27). Thus, microscopic investigation of blood smears is one of the laboratory tests that must be done to confirm the *Trypanosoma spp*. diagnosis in cattle, this test is considered a rapid and low-cost method (28,29). Furthermore, polymerase chain reaction (PCR) is one of the molecular assays that promoted the accuracy of demonstrating and distinguishing different *Trypanosoma species* in cattle, indicating the disease's outstanding prevalence in Iraq (30). Phylogenetic analyses of these strains supplied facts about the epidemiological patterns and genetic diversity of *Trypanosoma spp*. in cattle of Iraq (20,31). These researches show the importance of using molecular assays for better diagnosis and management of trypanosomiasis in Iraqi domestic animals (32). The molecular study has significantly enhanced our information on Trypanosoma spp., making it possible to accurately detect and differentiate diagnoses of trypanosomiasis in domestic animals (30).

This study focuses on the significance of including molecular methods to promote trypanosomiasis diagnosis and control in domestic animals of Iraq. The purpose of this work was to detect *Trypanosoma species* in cattle of Mosul city by using the PCR technique and phylogenetic analysis.

Materials and methods

Ethical approval

Permission for this research was granted by the Animal Ethics Committee in the College of Veterinary Medicine at the University of Mosul under No. UM.VET.2023.140, dated June 1, 2023.

Samples and data collection

The study included one hundred and twenty-five cattle, ages 1-5 years, from various regions in Mosul city, Iraq, and was conducted during the period from June 2023 to August 2024. The animals displayed symptoms of pale mucous membranes, emaciation, anemia, and lower body edema. Additionally, tick infestations were discovered in various parts of the animal's body. Thin and thick Giemsa-stained buffy coat smears were used to initially identify and diagnose the *Trypanosome evansi* infection, which was then confirmed by PCR assay. Five milliliters of jugular blood were obtained from each animal under aseptic conditions; each blood sample was split into EDTA tubes used for buffy coat smear to diagnose and molecular analysis.

Microscopy examination

To check for Trypomastigotes, a clinical pathologist examined Giemsa-stained buffy coat smears, and blood samples of 2.5 mL were drawn from each animal via venipuncture of the jugular vein into specially prepared test tubes (ALFA-Med, China) containing the anticoagulant ethylenediaminetetraacetic acid (EDTA). The samples were kept in a refrigerator at 4°C until transported to the laboratory. Buffy coat smears were prepared immediately after blood collection. Approximately 75 microliters of blood were drawn into a capillary tube (Vitrex Medical, Germany) and centrifuged for five minutes at 12,000 rpm. The resulting buffy coat was then transferred onto a glass slide to create a smear. Following drying with air, the slides were methanol-fixed and stained with Giemsa stain; a microscope examination under an oil immersion lens (100X) was done (33). The percentage of parasitemia was calculated as the total number of trypomastigotes in 100 microscopic fields using the following formulas by Albadrani and Hammed parasitemia (%)= (number of positive cattle* number trypomastigotes/field)/(100 field* WBCs/field)*100 (34).

DNA Extraction and PCR Technique

According to the manufacturer's instructions, the Add Prep genomic DNA extraction kit from blood and tissue Kit (Add bio, Korea) was used to extract genomic DNA from all cattle blood. The extracted DNA's concentration and purity were determined, and the Polymerase chain reaction technique was carried out by specific primers used for the amplification of specific genes of *Trypanosoma evansi*, according to Desquesne *et al.* (35).

The source of primers was Macrogen Company in Korea, which includes the ITS1 forward primer Kin2 CGCCCGAAAGTTCACC and the ITS1 reverse primer Kin1 GCGTTCAAAGATTGGGCAAT. The cattle that confirmed positive for *T. evansi* were detected in a band size of around 540 bp. The PCR reaction and program were performed without any change, according to Desquesne *et al.* (35). The amplified products were separated by gel electrophoresis using 1.5% agarose (AddBio, Korea) combined with 3 µl of Gel Red dye (AddBio, Korea) after the final PCR results were loaded onto an agarose gel. To document and identify predicted bands, the gel was observed under UV light utilizing a gel documentation system (Gel Doc EZ Image, Bio-Rad, USA).

DNA sequencing and phylogenetic analysis

The Sanger sequencing method was used to sequence the PCR product of positive samples, which was delivered to Macrogen in Korea. In brief, the matching primer was sent with 25 µl of the target gene's PCR product. After receiving them as text files in the FASTA format, the sequencing results were subsequently submitted to GenBank of NCBI for registration and the getting of special accession numbers. Applying phylogenetic analysis to compare the evolutionary relationship between the Trypanozoon strains, the chosen sequences were analyzed by Mega 11 software.

Results

The present study indicated that the infection rate of *Trypanosoma species* in cattle in Mosul city, Iraq, was 17.6% and 21.6% using microscopic examination of buffy coat smears and PCR technique, respectively (Table 1). PCR technique results are considered the gold standard for comparing infected and non-infected cattle. The examination of a cattle buffy coat smear stained with Giemsa stain revealed the presence of *Trypanosoma spp*., a flagellated protozoan parasite. Within the microscopic field, at least two parasites were observed, indicating a high level of parasitemia. The *T. evansi* organisms displayed their characteristic elongated shapes, with a prominent undulating membrane and a central nucleus that stained more intensely (Figure 1). The active motility of these parasites highlights their infectious nature. The detection of *T.evansi* is critical, as elevated parasitemia of 2.2% can lead to significant clinical symptoms in cattle, including fever, anemia, and lethargy. The parasitemia in cattle infected with *Trypanosoma evansi*, given that there are 2 trypomastigotes observed in each microscopic field and 22 cattle were found positive for the parasite, and the parasitemia was 2.2%.

Table 1: The infection rate of Trypanosoma species in cattle in Mosul city, Iraq, using different diagnostic methods

Number of tested animals	Type of test	Number of positive animals	Percentage %
125	Microscopic examination	22	17.6
	PCR technique	27	21.6

The gel electrophoresis results for the DNA extracted from bovine blood samples reveal important insights into the presence of specific pathogens (Figure 2). The gel electrophoresis results for the ITS1 gene of *Trypanosoma evansi* demonstrate significant findings regarding the presence of this parasite in the analyzed samples. In the gel, lane 1 shows a 100 bp DNA marker, which serves as a size reference for the subsequent samples. Lanes 1 to 5 contain positive samples that distinctly exhibit bands at the expected size corresponding to the ITS1 gene of *Trypanosoma evansi*, confirming the presence of the parasite's genetic material in these tested samples. In contrast, lane 6, which serves as the negative control (Figure 3).

The analysis of the sample accession numbers PQ800271 [available at] and PQ800272 [available at] reveals the identification of two strains of *Trypanosoma evansi* strain T-HKB1-24 and strain T-HKB2- 24, with a remarkable query cover percentage of 100% and an identical number percentage consistently near 100% across the sequences compared. The GenBank accession numbers associated with the identified strains originate primarily from Thailand and the Philippines, suggesting a high degree of genetic similarity between the Iraqi isolates and those from these countries. Notably, all specimens displayed minimal variation, with the lowest identity percentage recorded at 99.60% (Table 2). This high level of conservation among *T. evansi* sequences underscores the potential for widespread distribution of this parasite in Southeast Asia, emphasizing the importance of continuous monitoring and research into its epidemiology and impact on livestock health in Iraq and surrounding regions.

The phylogenetic tree of *Trypanosoma evansi* from Iraq was created utilizing the Maximum Likelihood method, specifically applying the Tamura-Nei model in MEGA11 software. This analysis combined bootstrap resampling with 1000 iterations to improve the robustness of the phylogenetic inference. The work utilized concatenated partial DNA sequences of the 5.8 rRNA gene as input data, supplying significant insight into the genetic relationships and variety of *T. evansi* in the region. The phylogenetic tree result clarifies the evolutionary links between various strains of *T. evansi,* assisting in perfectly understanding the genetic diversity and epidemiology of this important parasite in Iraq (Figure 4).

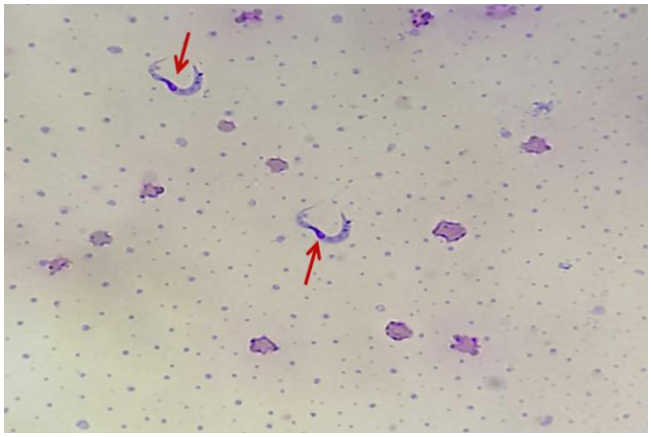

Figure 1: Buffy coat smear from cattle blood, Giemsa stain reveals high parasitemia of *Trypanosoma evansi.*

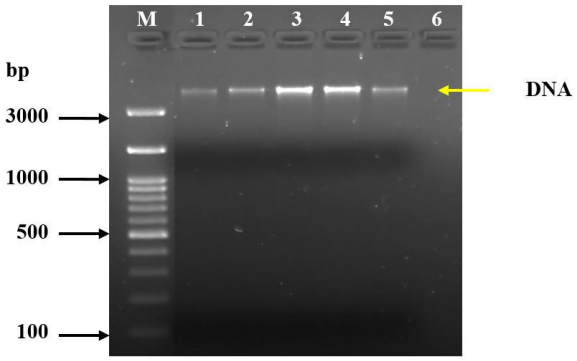

Figure 2: Gel electrophoresis for DNA extracted from bovine blood samples.

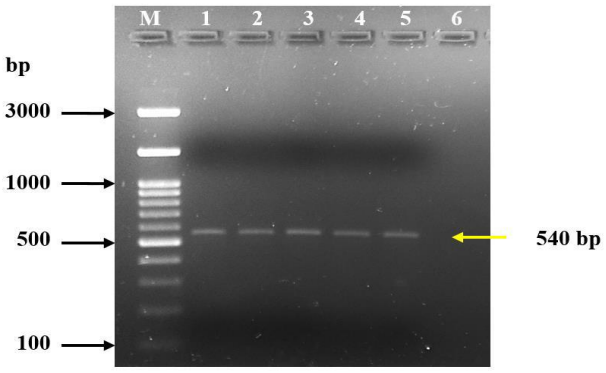

Figure 3: The gel electrophoresis of *Trypanosoma evansi* ITS1 gene. Lane 1 represents a 100 bp DNA marker. Positive samples are found in Lanes 1-5, while negative controls are found in lane 6.

Table 2: Similarity between sequences that were gotten of *Trypanosoma evansi* and sequences of the same parasite in the NCBI GenBank by utilizing the BLASTn program

Sample Accession No.	Query cover (%)	Similar number (%)	GenBank Accession No	Country
	100	100	EF546000	Thailand
	100	99.98	HQ593643	Philippines
	100	99.98	DQ472707	Thailand
	100	99.98	AY912273	Thailand
	100	99.98	EF546011	Thailand
PQ800271.1	100	99.98	DQ472679	Thailand
PQ800272.1	100	99.98	DQ472692	Thailand
	100	99.98	EF546002	Thailand
	100	99.98	HQ593642	Philippines
	100	99.98	D89527	Taiwan
	100	99.98	DQ472681	Thailand
	100	99.60	DQ472684	Thailand

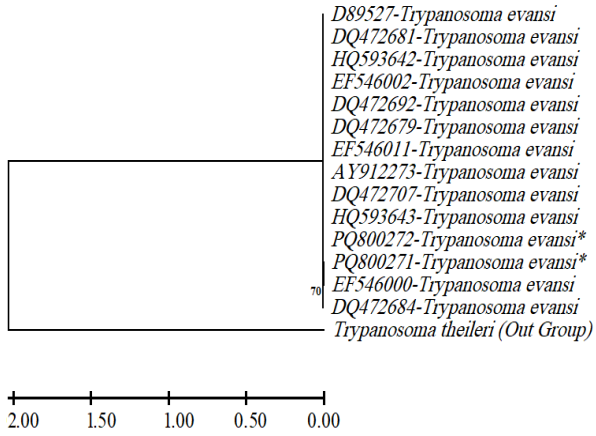

Figure 4: Phylogenetic tree of gotten sequences of *Trypanosoma evansi* in cattle in Mosul city, Iraq (*), and *Trypanosoma theileri* used as an Out Group.

Discussion

The buffy coat smears investigation and molecular phylogenetic analysis both help to gain deep insight and understanding of the epidemiology and the genetic variety of *Trypanosoma evansi*, mainly in Iraq (11,34). The examination of the buffy coat makes it probable to have a competent concentration of parasite DNA from blood samples, which makes it simple to detect *T. evansi* with major sensitivity (17). This technique validates the presence of parasites and supports the estimate of parasitemia, which is crucial for deciding how cattle are severely infected (9).

Moreover, by microscopic examination, Alimam *et al.* (29) recorded the existence of *Trypanosoma spp*. in the blood of numerous animals in the Ninva governorate. The phylogenetic analysis, mainly by the building of the maximum odds phylogenetic tree dependent on the Tamura-Nei model, gives important details about the genetic connections between *T. evansi* strain (36). Percentage

identity is how much of that match is identical present during the analysis. It can be seen that Iraqi strains have a strong match with strains from Thailand, the Philippines, and Taiwan, signifying a possible route of transmission via the introduction of cattle from these countries (30). Also, the strain has minimum genetic diversity, a hint of modern entrance, or a demographic equilibrium; both of them are the main details for controlling disease programs (7). The complete strategy focuses attention on the feasible risk that *T. evansi* creates to the health of animals in Iraq and identifies the high degree of genetic analogy with other local strains that may have an effect on the disease dynamic (6).

Understanding these connections is crucial for generating logical diagnostic tools and therapeutic advances as well as for setting ready concentrated control measures to avoid trypanosomiasis from layout (8). Additional research is required to understand the influence of the ecological factors on the *T. evansi* prevalence and the likelihood of zoonotic transmission to livestock or Human beings (11,37,38). Finally, the results highlight the necessity of monitoring the area and devising a varied strategy to address the complicated trypanosomiasis problem (39). Phylogenetic analysis of the Trypanosoma parasite is crucial for understanding the development connection and genetic diversity for this variant group of kinetoplastid parasites. These studies improve our probability of recognizing, treating, and controlling trypanosomiasis in various animal hosts, as well as clarifying the complex developmental history of these parasites (40). The study of phylogenetic analysis of *T. evansi* identified different clades, which are significant domestic animal parasites that are mainly prevalent in Iraq. *Trypanosoma evansi* is very often combined with cattle and camels and has an adjacent developmental relationship with *T. brucei*, the causative agent of African sleeping sickness, more than with other cattle infections according to the genetic sequences of definite markets. This finding emphasizes the necessity for more investigation into the zoonotic potentials of *T. evansi* and its complications for animals and public health (41).

Moreover, this study showed a great genetic variety within *T. evansi* populations, which may be an important indicator of adaptation to different host interactions and environmental situations. Genetic variety can play a main role in the discovery of vaccines and treatments to effectively eliminate trypanosomiasis in livestock (8). Furthermore, environmental factors and host immune response may influence *T. evansi* population traits, which may be significant for their evolutionary trajectories (32).

Understanding these facts is important for specific interventions to enhance animals' management strategies in regions where *T. evansi* exists. The utilization of phylogenetic analysis to differentiate between trypanosome species enhances our diagnostic capacities. Molecular tests such as PCR and sequencing aid in identifying the particular trypanosome species that impact animal health in Iraq and emphasize genetic relationships (30,42). According to Adham and Abdul-Zahra (43), relying on the conventional diagnosis method may lead to inaccurate diagnosis, especially in areas where different animal hosts live. So, the use of molecular methods in routine laboratory diagnosis can importantly improve the accuracy of diagnosis of different species of trypanosome parasites and ease disease control (28,39).

The molecular analysis of *Trypanosoma species* needs additional investigation due to the evolutionary visions given by this study. Coming research should focus on including a broader geographic coverage and various hosts in order to find complete evolutionary outlines (44,45). Conjoining genetic analyses with ecological data may possibly also assist us in understanding the variables that influence the distribution and diversity of trypanosomes (9,46). These results confirm the competence of *T. evansi* in the region and highlight the importance of using molecular techniques like PCR to diagnose and control trypanosomiasis in Mosul, Iraq's livestock.

Conclusion

The evolutionary analysis of *Trypanosoma evansi* in this study highlighted the importance of knowing the evolutionary dynamics of these parasites. Together, these results confirmed the successful amplification of the ITS1 gene from positive animal samples, supporting the advantage of molecular methods in diagnosing Trypanozoon infection and revealing the prevalence of trypanosomes in the examined animals. In veterinary practice, these molecular diagnostics are important for successful control of the disease, its management and prevention, and for protecting animal health and public health in Iraq and other affected regions.

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Conflict of Interest

The authors have disclosed no potential conflicts of interest.

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تحليل شجرة النشوء للمثقبية الإيڤانسية في الماشية في مدينة الموصل، العراق

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الخلاصة

داء المثقبيات هو مرض عالمي يصيب أنواعًا مختلفة من الحيوانات، بما في ذلك الماشية. المثقبية الايڤانسية هي طفيليات حيوانية المصدر تصيب الماشية في الغالب، وتتسبب في خسارة اقتصادية كبيرة في قطاع الثروة الحيوانية في العراق. هدفت هذه الدراسة إلى تحديد معدل الإصابة بداء المثقبيات في الأبقار في مدينة الموصل، العراق، باستخدام الفحص المجهري لمسحات الغلالة البيضاء وتقنية تفاعل البلمرة المتسلسل، مزيد من التحليل الوراثي للسلالات المكتشفة في هذه الدراسة. تم فحص مائة وخمسة وعشرين رأساً من الماشية، تتراوح أعمارها بين سنة وخمس سنوات، للتأكد من وجود طفيلي المثقبيات. أظهرت النتائج أن ٢٢ (١٧,٦%) و ٢٧ (٢١,٦%) من حيوانات الدراسة كانت إيجابية بالنسبة لطفيلي المثقبية الايڤانسية باستخدام الفحص المجهري وتقنية تفاعل البلمرة المتسلسل على التوالي. تعتبر نتائج تقنية تفاعل البلمرة المتسلسل المعيار الذهبي لمقارنة الماشية المصابة وغير المصابة. مما يدل على وجود كبير للمرض في هذه المجموعة من الماشية. تم تحديد نسبة تطفل طفيلي المثقبية الايڤانسية بنسبة ٢,٧%، مما يشير إلى انتشار الطفيلي في قطعان الماشية المصابة. علاوة على ذلك، تم إجراء تحليل النشوء الجيني والتطور لتقييم العلاقات الوراثية لسلالتين من طفيلي المثقبية الايڤانسية من الماشية المصابة. هذه السلالات متشابهة للغاية ٩٩,٦٠ - ١٠٠% مع تسلسلات نفس العامل الممرض المسجل في بنك الجينات في بلدان مختلفة، مثل تايلاند والفلبين وتايوان. في الختام، وفقا للنتائج، فإن طفيلي المثقبية الايڤانسية شائع بين الأبقار في الموصل، العراق، وقد تكون هذه النتائج مفيدة للبحث المستقبلي والإدارة المفيدة لطفيلي المثقبية الايڤانسية في منطقة الدراسة.

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