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PARASITOLOGICAL AND MOLECULAR DETECTION OF TRYPANOSOMA SPECIES IN CATTLE IN NINEVEH GOVERNORATE, IRAQ

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ABSTRACT

Data about bovine trypanosomiasis and its causes in Nineveh Governorate and Iraq are limited. In relation to the origin of cattle, trypanosomes have never been discovered before through molecular study. Blood samples from 221 cattle were examined by microscopy and conventional polymerase chain reaction (PCR) assays in different areas of Nineveh Governorate, Iraq, from October 2023 to August 2024. The Sanger sequencing method was used to sequence the PCR product of the positive samples, which were delivered to Psomagen in the USA. The study detected Trypanosoma in 12.22% by microscopy and 20.81% by using conventional PCR, with significant increases in the PCR assay. Regarding the risk factors for animals (gender, age, and breed) from Positive samples of the PCR assay, significant increases at the level ($P<0.05$) were recorded in female groups 31.34%, age (>2 years) 25.52% and imported breed 26.83%. *Trypanosoma vivax* was the predominant species in the study area. The genetic sequencing findings were saved as text files in the FASTA format. Selected strains were submitted to NCBI's GenBank for registration and the acquisition of unique accession numbers, LC830952.1 for *T. congolense*, LC830948.1 for *T. vivax* and LC830954.1 for *T. brucei*. The percentage of similarity to the nucleotide sequence of the strains that were diagnosed in the study, when BLAST was performed in the database, compared to the nucleotide sequences of the international strains, which were identified during this study and which were used for the purpose of constructing the genetic phylogenetic tree. The results showed that there was a variation in similarity rates between the local isolates and other strains, which was limited to countries on the African continent.

Keywords: Trypanosoma, Cattle, Microscopy, Molecular, Phylogeny, Iraq.

INTRODUCTION

Among the most serious infectious diseases that affect both humans and animals is trypanosomiasis, caused by the protozoan parasite Trypanosoma, and the tsetse fly is the primary biological vector that spreads the disease. One of the most significant global diseases that lowers output, particularly in African animals, is trypanosomosis. As a result, it may reduce the amount of land used to feed the unexpected population growth. (Jaiswa *et al.,* 2015; Giordani *et al.,* 2016). The illness poses a serious risk to both humans and farm animals worldwide, but especially in Africa, South America, and Asia. (Jackson *et al.,* 2009). *Trypanosoma brucei brucei, Trypanosoma vivax,* and *Trypanosoma congolense* in cattle, and *Trypanosoma evansi* in horses, cattle, camels, and water

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buffaloes, are the most significant trypanosomes that impact livestock. (Giordani *et al.,* 2016).
Trypanosomiasis's primary clinical symptoms include weight loss, severe anaemia, decreased appetite, decreased productivity, emaciation, stress, infertility, and abortion. Some animals may also die from acute illness. (Osório *et al.,* 2008; Al-Qayim *et al.,* 2021; Ukwueze *et al.,* 2022). In subclinical or chronic infections, the illness can be fatal if treatment is not received, making diagnosis and management more difficult. (Majekodunmi *et al.,* 2013).

The study's goals were to ascertain the genetic diversity among the trypanosomes found in cattle in the Nineveh Governorate, Iraq, as well as the prevalence and risk factors related to bovine trypanosomiasis.

MATERIALS AND METHODS

Samples and data collection

Two hundred twenty-one cattle were chosen at random from several locations in the Nineveh Governorate, Iraq, from October 2023 to August 2024for this investigation. Instead of 5ml of blood were collected from jugular vein of each animal with different ages, sex, and breed under aseptic conditions, each blood sample was divided into two EDTA tubes, one tube stored in 4º C to be used for microscopic examination and the other tube stored in -20º C for molecular assay, based on the information provided by the animal owners, case history data was gathered.

Microscopic Examination

To detect the parasite, blood samples were analyzed using a light microscope with 1000× power after being routinely examined under a microscope for stained blood smears (wet, thin, thick, and buffy coat layer smears). Microscopically, twenty-seven samples were positive. (Bowman, 2014).

DNA extraction and PCR protocol

A Qiagen DNA blood extraction kit was used to extract DNA from the entire blood sample; the procedure was carried out according to the manufacturer's instructions. In this investigation, trypanosome DNA was detected using primers based on internal transcribed spacers (ITS). To amplify PCR products at 518 bp, Bioneer, South Korea, supplied a set of primers consisting of ITS1- CF (5'CCGGAAGTTCACCGATATTG-3') and ITS1- BR (5'TTGCTGCGTTCTTCAA-CGAA-3'). Table1.A ready-to-use PCR kit (Bioneer, South Korea) was used to prepare the PCR master mix, which had a final volume of 20 µL. An initial denaturation stage of 95ºC for 6 minutes, 35 cycles of 95ºC for 90 seconds, 58ºC for 90 seconds, 72ºC for 120 seconds, and a final extension step of 72ºC for 5 minutes were all part of the PCR reaction that was carried out in a thermal cycler (Bio-Rad, USA). The final PCR products were separated by electrophoresis, stained with ethidium bromide, and viewed under Gel Documentation after being examined in a 2% agarose gel using a 100–1200 bp DNA ladder (New England Biolabs Inc., NEB, England). (Njiru *et al.,* 2005).

Table 1: The amplification conditions and PCR primers utilized in this investigation

Taxa	Band size	Target gene	Specific primers	Primer sequence (5′ -3′)	Reference
T. congolense savannah	700	ITS1	ITS1 CF ITS1 BR	CCGGAAGTTCACCGATATTG TTGCTGCGTTCTTCAACGAA	(Njiru *et al*., 2005)
T. congolense forest	710				
T. congolense kilifi	620				
T. brucei	480				
T. vivax	250				

Sequencing DNA and adding it to GenBank:

The Sanger sequencing method was used to sequence the PCR product of the positive samples, which was delivered to Psomagen in the USA. In short, the matching primer was sent with 25 µl of the target gene's PCR product. Sequencing results were saved as text files in the FASTA format. A particular accession number was obtained by registering the chosen strains with GenBank, NCBI.

Phylogenetic analysis: For sequencing, pure PCR products were shipped to the USA. The UPGMA method was used to perform a phylogenetic tree analysis between local trypanosome isolates and NCBI Blast-provided trypanosome species, and the isolates' species identification was reported to NCBI-GenBank.

Statistical analysis: All of the data were tabulated and analyzed using SPSS (version 23). The study's findings were statistically analyzed using the Chi-Square test, which was utilized to find significant differences at a P<0.05 level. (Petrie and Watson, 2006).

RESULTS

In total, 221 blood samples of cattle were examined by microscopy and conventional PCR assays in different areas of Nineveh Governorate. Trypanosoma prevalence obtained by using microscopy was 27/221 (12.22%), while the results were 46/221 (20.81%) by using conventional PCR, with significant increases in PCR assay (Table 2, Figure 1).

Table 2: Comparison between Microscopic and PCR results of *Trypanosoma* infection in cattle

Test	No. of samples	Positive samples	Prevalence (%)
Microscopic results	221	27	12.22
PCR results	221	46	20.81 *

* Denotes statistical significance at level (P<0.05).

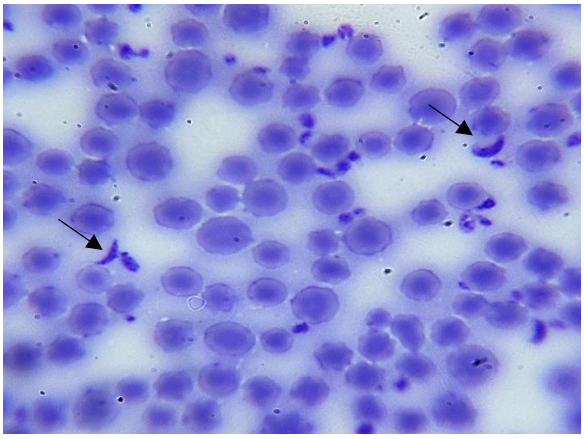

Figure 1: Positive blood film stained with Giemsa and examined by microscopy under oil immersion (1000×)

The relationship between positive PCR results and animal risk factors (gender, age and breed) showed significant increases at the level (P<0.05) in female groups 21/67 (31.34%), age (>2 years) 37/145 (25.52%) and imported breed 33/123 (26.83%), Table (3).

Table 3: The relationship between positive PCR results and animal risk factors

Risk factor	Group	No. of samples	Positive samples	Prevalence (%)
Gender	Male	154	25	16.23
	Female	67	21	31.34 *
Age / Year	< 1	76	9	11.84
	>2	145	37	25.52 *
Bread	Local	98	13	13.27
	Imported	123	33	26.83 *

* Denotes statistical significance at level (P<0.05).

We identified three species of Trypanosoma by using conventional PCR, including *Trypanosoma vivax, T. congolense,* and *T. brucei*, which accounted respectively for (76.09%), (6.52%) and (4.35%). In contrast, mixed infection with different Trypanosoma species was detected at a rate of 13.04%. *Trypanosoma vivax* was significantly higher than the other species. (Table 4, Figure 2)

Table 4: Prevalence of *Trypanosoma* species in cattle

Trypanosoma species	Positive samples	Prevalence (%)
Trypanosoma vivax	35	76.09 *
T. congolense	3	6.52
T. brucei	2	4.35
Mixed infection	6	13.04
Total	46	100.00

* Denotes statistical significance at level (P<0.0001).

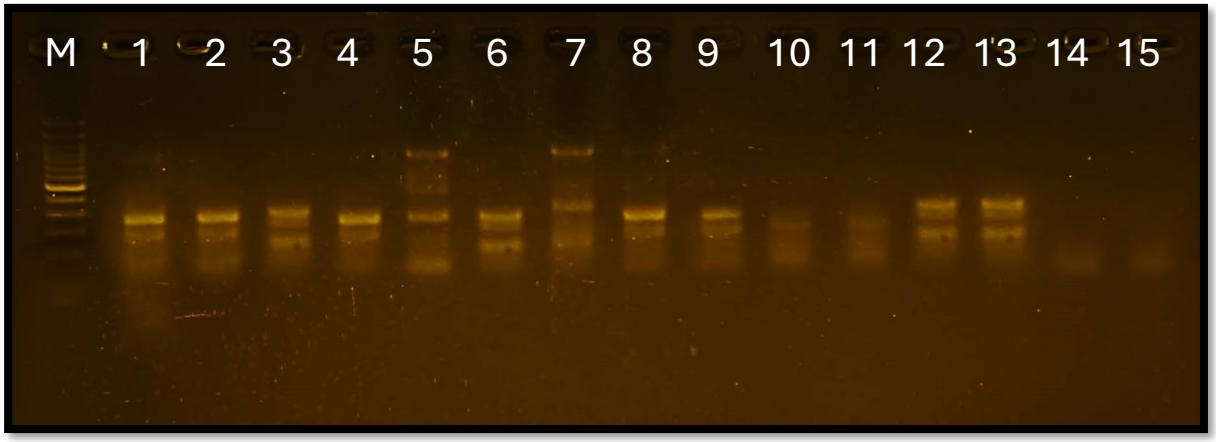

Figure 2: Amplification of ITS1-CF and ITS1-BR primers, M=marker, 1,2,3,4 ,6,8-13 positive to *T. vivax* (250 bp). 5,7 mixed infections with *T. vivax* (250 bp), *T. brucei* (480 bp) and *T. congolense* (700 bp). 14,15 negatives to *Trypanosoma*.

Sequencing DNA and adding it to GenBank:

The Sanger sequencing method was used to sequence the PCR product of the positive samples, which were delivered to Psomagen in the USA. In short, the matching primer was sent with 25 µl of the target gene's PCR product. Sequencing results were saved as text files in the FASTA format. A particular accession number was obtained by registering the chosen strains with GenBank, NCBI. According to the findings of the Trypanosoma 18S rRNA, ITS1, and 5.8S rRNA gene genetic sequencing, there was a genetic sequence present in the cattle samples from Mosul. Only three isolates were identified from the total samples submitted for genetic sequencing; these isolates were listed in GenBank under the serial numbers LC830952.1 for *T. congolense*, LC830948.1 for *T. vivax* and LC830954.1 for *T. brucei*. The multiple sequence alignment of the Trypanosoma gene revealed: Common, variable, and missing regions within the sequences, such as for the other local strain, and constant regions of the nucleotide base sequence of the gene for the local strain.

Table 5: Relationship of GenBank LC830952.1 of *T. congolense* in cattle with the world GenBank

Sample Accession Number	Parasite Identified	Query Cover %	Identic Number %	GenBank Accession Number	Country Identification
LC830952.1	*T. congolense*	98	98.65	MG255203.1	Cameron
		98	89.1	U22315.1	Kenya
		98	89	MG283145.1	Cameron
		94	89.4	JN673389.1	Tanzania
		93	89.2	MZ147876.1	Chad
		93	88.6	MZ147877.1	Chad
		93	88.1	OK483225.1	Rwanda
		94	87.6	JN673388.1	Tanzania

Results of the molecular nucleotide sequence of the 18S rRNA, ITS1, 5.8S: rRNA gene of the *T. congolense* in the database: The results showed the presence of gene sequences that have a percentage of similarity to the nucleotide sequence of the strains that were diagnosed in the study when BLAST was performed in the database compared to the nucleotide sequences of the international strains, which were identified during this study and which were used for the purpose of constructing the genetic phylogenetic tree. The results showed that there was a similarity rate between the local isolates bearing the registration number in GenBank LC830952.1 where the highest similarity rate was reached. 98.65% with the strain registered in the World GenBank under the name MG255203.1 and isolated in Cameron, followed in terms of similarity by the strain under the genetic name U22315.1, reaching 89.1% and also isolated in Kenya. As for the strain under the name MG283145.1 in the GenBank (Table 5).

Results of the molecular nucleotide sequence of the 18S rRNA, ITS1, 5.8S: The results showed the presence of gene sequences that have a percentage of similarity to the nucleotide sequence of the strains that were diagnosed in the study when BLAST was performed in the database, compared to the nucleotide sequences of the international strains, which were identified during this study and which were used for the purpose of constructing the genetic phylogenetic tree. The results showed that there was a similarity rate between the local isolates bearing the registration number in GenBank (LC830948.1), where the highest similarity rate was reached 100% with the strain registered in the World GenBank under the names MK880189.1, MK880188.1, U22316.1 isolated in Kenya, and other strains isolated from Africa, Tanzania and Burkina Faso (Table 6).

Table 6: Relationship of GenBank LC830952.1 of *T. vivax* in cattle with the world GenBank

Sample Accession Number	Parasite Identified	Query Cover %	Identic Number %	GenBank Accession Number	Country Identification
LC830948.1	*T. vivax*	100	100	MK880189.1	Kenya
		100	100	MN213748.1	Africa
		100	100	MK880188.1	Kenya
		100	100	U22316.1	Kenya
		100	100	PP210926.1	Tanzania
		100	100	MN213747.1	Africa
		100	99.5	KM391827.1	Ethiopia
		94	100	JX910371.1	Burkina Faso

Results of the molecular nucleotide sequence of the 18S rRNA, ITS1, 5.8S: rRNA gene of the *T. brucei* in the database: The results showed the presence of gene sequences that have a percentage of similarity to the nucleotide sequence of the strains that were diagnosed in the study when BLAST was performed in the database compared to the nucleotide sequences of the international strains, which were identified during this study and which were used for the purpose of constructing the genetic phylogenetic tree. The results showed that there was a similarity rate between the local isolates bearing the registration number in GenBank (LC830954.1) where the highest similarity rate was reached 98.4% with the strain registered in the World GenBank under the names AF306774.1 and AF306775 isolated in Sudan, followed in terms of similarity by the strain under the genetic name AF306776, reaching 98.2% and also isolated in Sudan (Table 7).

Table 7: Relationship of GenBank LC830952.1 of *T. brucei* in cattle with the world GenBank

Sample Accession Number	Parasite Identified	Query Cover %	Identic Number %	GenBank Accession Number	Country Identification
LC830954.1	*T. brucei*	100	98.4	AF306774.1	Sudan
		100	98.4	AF306775	Sudan
		100	98.2	AF306776	Sudan
		100	98.2	AF306777	Sudan
		99	97.9	MZ151468	Uganda

The results of the study showed that the phylogenetic tree that was constructed showed that the 18S rRNA, ITS1, 5.8S rRNA gene for the three genotypes that were isolated during the study possessed phylogenetic and evolutionary characteristics with the recorded genotypes, as the percentage of evolutionary relationship ranged from 99.3-100%, knowing that the strains that have characteristics similar to the local strains declined among Africa, Cameron, Sudan, Uganda, Tanzania, and Ethiopia.

DISCUSSION

African animal trypanosomiasis is a disease that impacts all domestic animals, harms the cattle sector in many nations worldwide, and results in significant financial losses. Therefore, attention has been paid in many countries of the world, as well as countries of the African continent, to diagnosing the disease, given that the disease is considered an emergency disease, and because the symptoms of trypanosomiasis in host mammals can be confused with those of several blood parasites or even other livestock illnesses, making a clinical diagnosis challenging. Therefore, laboratory diagnostic procedures are crucial for confirming suspected cases during epidemiological surveys, enabling the detection of the parasite in hosts and vectors (Okello *et al.,* 2022).

At the PCR level, the total prevalence was substantially higher than the microscopy level ($P<0.05$). This was anticipated, given that molecular techniques are known to have a higher sensitivity, as previously documented by (N'Djetchi *et al.,* 2017; Shaeel *et al.,* 2020).

Microscopic analysis of the blood smear techniques (wet, thin, thick, and buffy coat layer smears) revealed that 12.22% of cattle tested positive for Trypanosoma spp. Our results exceeded those of earlier research conducted on Iraqi cattle, which showed a prevalence rate of 3.33% for *T. congolense* infection (Hasan, 2012) and 3.33% of infection with T. theileri and T. cf. cervi

(Shaeel *et al.,* 2020). It also exceeds the 6.6% of infection with Trypanosoma evansi in Aswan, Egypt (Adel *et al.,* 2019), and 10.3% of infection with Trypanosoma spp. in Somalia (Osman *et al.,* 2023). However, it was lower than the rate reported in the Gamo Zone, southwestern Ethiopia, which was 19.1% for *T. congolense* and *T. vivax* (Tsolo *et al.,* 2024).

According to the PCR assay, 20.81% of the cattle in this study tested positive for Trypanosoma spp., which is consistent with earlier research that found a prevalence of 21.04% in Mosul, Iraq. (Mahmood and Alobaidii, 2022), 22.31% in Côte d'Ivoire (Kouadio *et al.,* 2014) and 23.8% in Somalia (Hassan-Kadle *et al.,* 2020). However, our finding is higher than that reported in Wasit Province/Iraq (9.33%) (Shaeel *et al.,* 2020), but it is lower than that reported in Uganda (29.6%) (Kizza *et al.,* 2021) and Sudan (57.7%) (Osman *et al.,* 2016). The variation in prevalence observed in different countries demonstrates that it is typical for trypanosomiasis incidence rates to vary throughout different biological zones and at different seasons within the same nation. Therefore, climatic differences in the survival of the parasite and the vector can be impacted by various geographic locations and husbandry measures (Kouadio *et al.,* 2014).

In this study, females were significantly more infected than males; this study agrees with earlier research (Shaeel *et al.,* 2020; Kizza *et al.,* 2021; Mahmood and Alobaidii, 2022). Although the exact causes are unknown, the females' frequent pregnancies and milking may have put them under much stress. Moreover, farmers often neglect the management of females, as many focus on the health of males, who are primarily employed as a source of meat or for natural insemination.

When considering age as a risk factor, we found that animals older than two years had a higher prevalence of trypanosomiasis than animals younger than one year. Files (Vectors) are more attracted to adult cows because they produce more odor plumes than calves (Simukoko *et al.,* 2007). It is believed that an animal's ability to fend off infection from the same pathogens is enhanced by the presence of active immunity, which develops with age as a result of recurrent exposure to the pathogen. While maternal immunity is crucial for preventing illnesses during the first six to twelve months of life, this type of passive immunity drastically declines beyond the age of one year (Flynn and Sileghem, 1994; Niewiesk, 2014).

The results indicated that imported breeds were significantly higher than local breeds. The variation in results between the various breeds in the current study might be connected to variations in management and/or genetics. The inherent evolutionary benefit of environmentally adapted breeds must be taken into consideration when evaluating the significance of resistance/ tolerance at the breed level. Due to their evolutionary origins, locally adapted, independent breeds possess a significantly higher degree of genetic resistance and adaptation than imported breeds, which are commonly found in tropical locations where endemic illnesses are prevalent (Jovanović *et al.,* 2009).

In Iraqi cattle, *Trypanosoma brucei* and *T. congolense* (Al-Badrani, 2012), *T. vivax* (Rhaymah & Al-Badrani, 2012) and *T. theileri* and *T. cervi* (Shaeel *et al.,* 2020) were reported in previous studies. In this study, the results indicated that *Trypanosoma vivax* (76.09%), *T. congolense* (6.52%) and the Trypanosomes that were disseminated in the research region were *T. brucei* (4.35%). According to the study, *Trypanosoma vivax* was the most common species in the Nineveh Governorate. This is consistent with research findings that indicate *T. vivax* is more common in many nations worldwide (Thumbi *et al.,* 2008; Fentahun *et al.,* 2012; Aki *et al.,* 2016; Ekra *et al.,* 2023; Santos *et al.,* 2024). The study area (Nineveh

Governorate) and all Iraqi provinces are free from the biological vector (tsetse flies). In contrast, its capacity to adapt and establish itself in the tsetse fly-free zone may be a contributing factor to the high prevalence of *T. vivax* in the studied area (Fentahun *et al.,* 2012). Moreover, it may be transmitted by mechanical vectors (biting flies). It may also be related to the prevalence of mechanical vectors (such as Stomoxys and tabanid flies) in this region and their capacity to disseminate the illness. The study results also indicated a mixed infection with two or three species of Trypanosomes, with a ratio of 13.04%. The presence of mixed infections of parasites focuses attention on epidemiologists, who must concentrate on specific species to identify various illnesses caused by different parasites and genotypes within species that are typical in the field (Cox, 2001).

According to the findings of the Trypanosoma parasite's 18S rRNA, ITS1, and 5.8S rRNA gene genetic sequencing, samples from cattle in Mosul city included a genetic sequence. Only three isolates out of all the samples submitted for genetic sequencing were discovered, and this variance in the similarity rate reflects the parasite's genetic diversity, which is caused by genetic alterations and mutations (Morenikeji *et al.,* 2023). The various sequence alignment results also revealed that the gene included missing sections in addition to constant, common, and variable regions. These findings align with those published by Kidambasi *et al.* (2020), who suggested that variations in the isolation site and environmental factors could be the cause of the differences between the isolated strains and the global strains. (Ibrahim *et al.,* 2021) said that the nucleotide sequence of the virus is very susceptible to producing genetic mutations, which helps the parasite adapt throughout infection to evade the immune response and the formation of new strains. In several nations around the world, there have been outbreaks of novel diseases or emergency infections of varying severity. The study's phylogenetic tree of the 18S rRNA, ITS1, and 5.8S rRNA genes revealed that the three strains belong to the same subgroup and are among particular species listed worldwide (Silbermayr *et al.,* 2013). This association provides unmistakable proof that these animals are highly genetically related. In addition, the study's isolated strains' positions in the phylogenetic tree showed genetic divergence between them (Agbo *et al.,* 2001). Geographical location may play a part in the divergence and convergence of local and global strains, which suggests that they share a shared evolutionary origin from a genetic perspective (Urakawa and Majiwa, 2001). Therefore, through this study, it becomes clear to us that the existence of Trypanosoma and its threat to cattle breeding, which is one of the main reasons for its existence, is the need for adequate control. This was done without taking into account the dangerous genetic strains that are present in the animals' surroundings or the absence of a careful hygiene program that is appropriate for the parasite's spread and the severity of the infections it causes (Kizza *et al.,* 2021). As regards cattle in Iraq, random importation is carried out without strict quarantine procedures (Fikru *et al.,* 2016). Furthermore, our research has demonstrated that the parasite exhibits recombinant genetic strain patterns due to the presence of multiple strains (Ngomtcho *et al.,* 2017). Additionally, one possible cause of the parasite's repeated exacerbations is the failure of veterinary clinics to employ anti-protozoal medications.

CONCLUSIONS

According to the study, cattle in the Nineveh Governorate were infected with various species of *Trypanosoma*, with *T. vivax* being the most common species.

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CONFLICTS OF INTEREST

The authors of this publication declare that neither the writing nor the data analysis had any conflicts of interest.

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الكشف الطفيلي والجزيئي عن أنواع التريبانوزوما في الأبقار في محافظة نينوى، العراق

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البيانات المتوفرة عن داء المثقبيات البقري وأسبابه في محافظة نينوى والعراق محدودة. وهذه هي المرة الأولى التي يتم فيها التعرف على المثقبيات من خلال البحث الجزيئي فيما يتعلق بمصادر الأبقار. تم فحص عينات الدم من ٢٢١ رأس ماشية بالمجهر واختبارات تفاعل البوليميراز المتسلسل التقليدية (PCR) في مناطق مختلفة في محافظة نينوى بالعراق خلال الفترة من أكتوبر ٢٠٢٣ إلى أغسطس ٢٠٢٤. وكانت نتائج الدراسة ١٢,٢٢٪ بالمجهر و٢٠,٨١٪ باستخدام تفاعل البوليميراز المتسلسل التقليدي مع زيادات كبيرة في اختبار تفاعل البوليميراز المتسلسل. وفيما يتعلق بعوامل الخطر الحيوانية (الجنس والعمر والسلالة) من العينات الإيجابية لتحليل تفاعل البوليميراز المتسلسل، تم تسجيل زيادات كبيرة عند مستوى ($P < 0.05$) في مجموعات الإناث ٣١,٣٤٪ والعمر (<٢ سنة) ٢٥,٥٢٪ والسلالة المستوردة ٢٦,٨٣٪. وكان *Trypanosoma vivax* هو النوع السائد في منطقة الدراسة. تم الحصول على نتائج التسلسل الجيني كملفات نصية بصيغة FASTA. تم إرسال السلالات المختارة إلى GenBank وNCBI للتسجيل والحصول على أرقام الوصول المحددة، LC830952.1 لـ *T. congolense*، و LC830948.1 لـ *T. vivax* و LC830954.1 لـ *T. brucei*. تم مقارنة نسبة التشابه في تسلسل النوكليوتيدات للسلالات التي تم تشخيصها في الدراسة عند إجراء BLAST على قاعدة البيانات مع تسلسلات النوكليوتيدات للسلالات الدولية، والتي تم تحديدها أثناء هذه الدراسة والتي تم استخدامها لغرض بناء الشجرة الوراثية التطورية. أظهرت النتائج وجود معدل تشابه في الاختلافات بين العزلات المحلية والسلالات الأخرى والتي تقتصر على دول القارة الأفريقية.