



STATUS, MOLECULAR AND PHYLOGENETIC ANALYSIS  
OF TRYPANOSOMIASIS IN BUFFALOES (*BUBALUS BUBALIS*)  
IN NINEVEH PROVINCE, IRAQ

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**Summary**

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*Trypanosoma evansi* is a common blood protozoa that infects a broad range of mammalian species in wide geographical regions all over the world. This survey focused for the first time on the molecular and phylogenetic analysis of trypanosomiasis in buffaloes in Nineveh province, Iraq. Through cross-sectional scanning of multiple regions during the period from November 2024 to March 2025, blood was obtained from 175 local water buffaloes of both sexes aged from seven months to five years. The PCR technique was used to detect the *18S rRNA* gene for *Trypanosoma spp.* in all blood specimens using universal primers. The second PCR reaction was done to detect the *ITS1* gene for *T. evansi*. Results demonstrated that the prevalence of *Trypanosoma spp.* was 24/175 (13.71%) at a band of approximately 540 bp, while the prevalence of *T. evansi* in buffaloes in the Nineveh province was 6/175 (3.42%) at a band of approximately 480 bp. Four local *T. evansi* isolate sequences with accession numbers PV593636.1, PV593637.1, PV593638.1, and PV593639.1 were deposited in NCBI GenBank. These local sequences were highly homologous (99.53–100% identity) with sequences from different countries such as Tunisia, Thailand, China, Sudan, Iran, Egypt, and Malaysia. In conclusion, *Trypanosoma evansi* was reported for the first time in buffaloes in the Nineveh province, Iraq. The application of the PCR technique is required for detection and confirmation of such protozoa, due to the lack of precise related clinical signs of the disease in buffaloes.

**Key words:** Nineveh – Iraq, PCR technique, phylogenetic, status, *Trypanosoma evansi*

INTRODUCTION

Among significant protozoal infections, *Trypanosoma* is a blood parasite affecting a wide domain of susceptible hosts, including humans (Sabir *et al.*, 2017). The pathogenic species of these parasites, transmitted iatrogenically by blood-

sucking insects, are responsible for lowered production and economic consequences of wild and domestic livestock. The parasite is distributed globally, including in Africa, Asia, and Central and South America (Elhaig *et al.*, 2013;

Behour *et al.*, 2019; Dyonisio *et al.*, 2020; Moh *et al.*, 2023).

The different species of unicellular haemoflagellate protozoa, including *T. evansi*, *T. brucei*, and *T. equiperdum*, belong to the genus *Trypanosoma* and family *Trypanosomatidae* (Mekata *et al.*, 2013; Rjeibi *et al.*, 2015). These parasites are also rated as Salivaria in this genus, as they are hosted in the tick salivary glands and transferred to the host mammal over bitten vectors (Telleria *et al.*, 2014). In animals, generally, the trypanosomiasis is grouped into tsetse-transmitted trypanosomiasis or African animal trypanosomiasis (AAT) and non-tsetse-transmitted animal trypanosomiasis that mainly exists apart from the African continent (Yaro *et al.*, 2016), with *Trypanosoma equiperdum*, *Trypanosoma vivax*, and *Trypanosoma evansi* being the major parasites in domestic animals (Birhanu *et al.*, 2015). *Trypanosoma evansi* affects a broad range of hosts, including camels, horses, cattle, and buffalo in India, South America, and Southeast Asia (Desquesnes *et al.*, 2013). *Trypanosoma evansi* is assorted into A and B genotypes, with type A being exceedingly revealed in Asia, Africa, and South America (Carnes *et al.*, 2015; Subekti *et al.*, 2024). *Tabanus* spp. (horseflies) and *Stomoxys* spp. (stable flies) are the senior haematophagous flies that iatrogenically transmit *T. evansi* (Changbunjong *et al.*, 2018; Muita *et al.*, 2025).

*Trypanosoma evansi* is a common pathogenic trypanosome that affects small ruminants, horses, camels, cats, dogs, pigs, and Asian elephants all over the world (Desquesnes *et al.*, 2013). Typically, the bovine species is regarded as an active reservoir, and infections of *T. evansi* are clinically characterised by chronic to lethal acute forms, weakness, weight loss, periodic fever, and enlarged

lymph nodes (Rjeibi *et al.*, 2015; Kamyngkird *et al.*, 2020). Cattle are not commonly sensitive, although the cerebral manifestation has been recorded in Southeast Asia. On the other hand, water buffalo (*Bubalus bubalis*) in this zone repeatedly exhibited acute attacks with a marked mortality rate (Reid, 2002). Additionally, the buffaloes are reservoir hosts for *T. evansi* without obvious infection (Alsaad *et al.*, 2021; Suwan *et al.*, 2023). However, significant impact and financial losses to the agricultural industry from *T. evansi* are also a concern in buffaloes (Villareal *et al.*, 2013). Economic sequels associated with *T. evansi* infections are ascribed to its capability to spread through apparently healthy carriers silently in most instances (Desquesnes *et al.*, 2013), to decrease productivity and reproduction performance, and minimise carcass traits (Patel *et al.*, 2022). Moreover, remarkable losses to the owners in terms of abortion, decreased fertility, dropping milk yield, anaemia, muscle weakness, and loss of appetite with death of about 50–70% acutely affected animals are reported (Desquesnes *et al.*, 2013).

To date, there is no clear planning for diminishing the transmission of trypanosomiasis, and the dominance is relying on precise diagnosis, treatment, and coping with the vector transporter (Büscher *et al.*, 2017; Dially *et al.*, 2017). Furthermore, it has been clarified that the frequent mass application of antitrypanosomal therapy such as isometamidium chloride and diminazene aceturate could generate resistance among *T. evansi* isolates, as documented in different countries (Subekti, 2014; Tihon *et al.*, 2017; Nuryady *et al.*, 2019). Conventionally, the detection of *T. evansi* parasite infestation is done through its recognition of in the blood or fluid of affected animals. The parasitological

techniques are however not constantly able to detect the infection in some cases that display low parasitaemia and in the chronic stage of the infection (El-Ashram *et al.*, 2019; Wibowo *et al.*, 2024). As a result, the quality and sensitivity of these diagnostic methods are unacceptable, especially when confirmation of the presence of a parasite during new animal admission or after application of control and eradication strategy are required (Viljoen & Luckins, 2012). It was affirmed that the identification of affected animals is of priority due to the existence of carrier animals and availability of biting flies. Multiple laboratory diagnostic techniques have been developed for distinguishing *T. evansi* infection; the DNA polymerase chain reaction and sequencing have been widely used to allocate the phylogenetic links and verify genetic differences of the parasite (Amer *et al.*, 2011; Desquesnes *et al.*, 2022).

In Nineveh province, the populations of water buffalo are reared in small or semi-intensive farms mostly near the rivers. Due to its significant function in the agricultural economy in many regions, including the study area, through milk and meat supply and due to the absence of information on the former status and phylogenetic report of trypanosomes in these bovines, the objective of this study was to report the prevalence and phylogenetic analysis of trypanosome infections in local water buffaloes (*Bubalus bubalis*) in Nineveh province of Iraq using the PCR technique.

## MATERIALS AND METHODS

### *Ethical approval*

The Institutional Animal Care and Use Committee agrees with this study, with Ref: UM.VET. 2024.094.

### *Animals, study zone, and sample collection*

For the purpose of this study, through cross-sectional scanning of multiple regions in Nineveh province from the period starting in Nov. 2024 till the end of Mar. 2025, precisely five milliliters of blood were obtained from 175 local water buffaloes (*Bubalus bubalis*) of both sexes and age ranging from 7 months to 5 years, representing 10 private small herds, by sterile medical syringes and in sterile blood vacutainer tubes containing EDTA anticoagulant. The herd history, prophylaxis scheme, and observed signs were documented throughout the visits.

### *Polymerase chain reaction and sequencing*

The genomic detection launched first with extraction of parasite DNA in all blood specimens utilising procedure steps information of the DNeasy Blood & Tissue Kit (Qiagen, Germany). Thereafter, a set of primers targeting the partial *18S* rRNA gene was submitted to the polymerase chain reaction (PCR) technique Kin1F (GCGTTCAAAGATTGGGCAAT) and Kin2R (CGCCCCGAA AGTTCACC) with the expected site 540 bp layout by McLaughlin *et al.* (1996) for *Trypanosoma* spp. Conventional PCR was performed using the 2× PCR Master Mix Kit, (ABM, Canada) with 2 µL of DNA and 1 µL of each primer in a net volume of 25 µL. The steps of the amplification cycle comprised one cycle of initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 sec., 68 °C for 60 sec., 72 °C for 60 sec., and 72°C for 10 min with one cycle of final extension (Mahmood & Alobaidii, 2022). Moreover, a second PCR reaction was implemented for detecting the *T. evansi* DNA utilising the protocol qualified by (Rjeibi *et al.*, 2015). For this reaction, primers

that magnify the 480 bp portion of the *ITS1* rDNA gene of *T. evansi* were employed: the specific forward primer ITS1F (CCGGAAAGTTCACCGATATTG) and the reverse primer ITS1R (TGCTGCG TTCTTCAACGAA) (Njiru *et al.*, 2005). The PCR technique was performed in 25  $\mu$ L final volume containing 0.2  $\mu$ mol/L of forward and reverse primer, Taq polymerase (Thermo Fisher Scientific, USA) 0.5 U, and 3  $\mu$ L of DNA template. The amplification scheme started with initial denaturation for 5 min at 94 °C, followed by 35 cycles 94 °C for 40 s, 58 °C for 40 s, 72 °C for 90 s, and 72 °C for 5 min for one final extension cycle, similarly to the steps mentioned by Sallemi *et al.* (2017). Verification of the PCR products was achieved on 1.2% agarose gel electrophoresis. Subsequently, the positive samples were sequenced (Macrogen company, South Korea). For the sequence analysis of local *T. evansi* sequences, different online programs were used, such as the Bio-Edit program, NCBI Blastn, and ClustalW tool. Moreover, the submission portal was used for depositing four sequences of the *ITS1* gene of *T. evansi* in NCBI GenBank. The phylogenetic tree of *T. evansi* was created using MEGA 12 software, with bootstrapping 1000 replications (Kumar *et al.*, 2024), and partial DNA sequence of *Trypanosoma lewisi* (KP098536.1) LC244 /China was used as an outgroup (Zhang *et al.*, 2015).

#### Statistical analysis

Data from the current work was descriptively analysed in a Windows 10 Excel sheet.

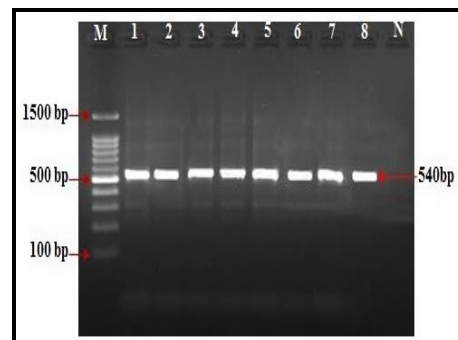
## RESULTS

For the first time, using the polymerase chain reaction technique in the present

study, *Trypanosoma* spp. and *T. evansi* were successfully detected at the molecular level in 175 blood buffalo samples from the Nineveh province, Iraq targeting the *16S* rRNA gene and *ITS1* rDNA gene, respectively. Results showed that the prevalence of *Trypanosoma* spp. was 24/175 (13.71%) (Table 1), at a band approximately 540 bp (Fig. 1) while the prevalence of *T. evansi* was 6/175 (3.42%) (Table 1), at a band approximately 480 bp (Fig. 2).

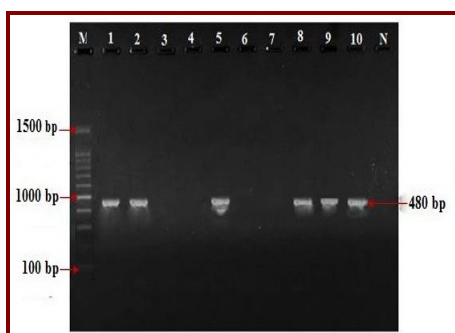
**Table 1.** Prevalence of *Trypanosoma* spp. and *Trypanosoma evansi* using PCR technique in 175 tested samples

Type of protozoa	Positive samples	Prevalence %
<i>Trypanosoma</i> spp.	24	13.71
<i>Trypanosoma evansi</i>	6	3.42



**Fig. 1.** Gel electrophoresis of the 16S rRNA of *Trypanosoma* spp. of the first round. M: Marker, Lanes 1-8: Positive samples for *Trypanosoma* spp. at 540 bp., N: Negative control sample.

In the current work, six positive PCR products of the *ITS1* rDNA for *T. evansi* were sequenced, and parallel to the individual sequence analysis using online NCBI BLASTn, the alignment score between local *T. evansi* sequences using the



**Fig. 2.** Gel electrophoresis of the ITS1 rDNA of *Trypanosoma evansi*, of the second round. M: Marker, Lanes 1,2,5,8–10: Positive samples for *T. evansi* at 480 bp., N: Negative control sample.

Multiple Sequence Alignment program was 100. In addition, four sequences were deposited in NCBI GenBank with accession numbers (PV593636.1, PV593637.1,

PV593638.1, and PV593639.1) (Table 2). These local sequences were highly homologous (99.53–100% identity) with those sequences of Tunisia (KJ741365.1), Thailand (AY912277.1; MN121259.1), China (MN446740.1), Sudan (AF306774.1 and AF306775.1), Iran (KX898420.1 and MW272928.1), Egypt (PP825840.1 and PP825842.1), and Malaysia (PP837626.1) (Table 3).

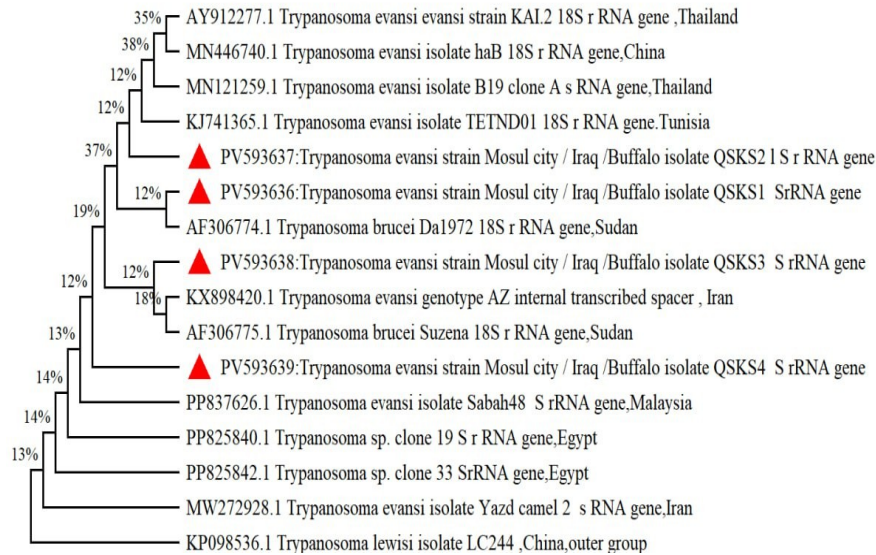
A phylogenetic tree was constructed using the maximum likelihood in the MEGA11 software. It was found that the obtained sequences of *T. evansi* were closely related (99.53–100%) to the sequences available in the GenBank database, as previously mentioned. The tree was rooted with *Trypanosoma lewisi* (KP0985361) LC244/China, used as an outgroup (Fig. 3).

**Table 2.** The *ITS1* rDNA nucleotide sequence for *Trypanosoma evansi* local isolates from Mosul, Iraq, and buffaloes that were entered into the GenBank

Accession No. of <i>ITS1</i> rDNA gene	Pathogen	Isolate
PV593636.1	<i>Trypanosoma evansi</i>	QSKS1
PV593637.1	<i>Trypanosoma evansi</i>	QSKS2
PV593638.1	<i>Trypanosoma evansi</i>	QSKS3
PV593639.1	<i>Trypanosoma evansi</i>	QSKS4

**Table 3.** Homology between the local *Trypanosoma evansi* and other sequences of the same pathogen in NCBI GenBank using NCBI BLASTn

Gene	Isolate number	Identity %	Accession no.	Country name
<i>ITS1</i> rDNA gene	PV593636.1	100.00	KJ741365.1	Tunisia
		99.76	AY912277.1	Thailand
		99.76	MN121259.1	Thailand
	PV593637.1	99.53	MN446740.1	China
		99.53	AF306774.1	Sudan
		99.53	AF306775.1	Sudan
	PV593638.1	99.53	KX898420.1	Iran
		99.53	MW272928.1	Iran
		99.53	PP825840.1	Egypt
	PV593639.1	99.53	PP825842.1	Egypt
		99.53	PP837626.1	Malaysia



**Fig. 3.** Phylogenetic tree of the ITS1 rDNA gene of *Trypanosoma evansi* for the local sequences (▲), from other sequences of the same pathogen in NCBI GenBank, and partial DNA sequences of *Trypanosoma lewisi* (KP098536.1) LC244/China was used as an outgroup.

## DISCUSSION

Diseases caused by *Trypanosoma* spp. in buffaloes and other animals induce remarkable impacts on their output and result in significant financial losses, which in turn affect the agricultural industry worldwide (Villareal *et al.*, 2013). Several reports on *Trypanosoma evansi* in Iraq and other countries recorded different rates of prevalence of these protozoa. In Basrah, Iraq, it was revealed that the rate of *T. evansi* in buffaloes was 87.2% using the sandwich ELISA test (Alsaad *et al.*, 2021). Further, in Brazil, it was 79.31% and 76.72% for ELISA and rapid tests, respectively (Serra *et al.*, 2024). In Pakistan, prevalence was 7.7% identified by the PCR technique (Shahzad *et al.*, 2010). In the Philippines, *T. evansi* was detected in 79 water buffaloes (*Bubalus bubalis*) tested using the PCR (Villareal *et al.*,

2013). Variation in the above-mentioned interpretations between these studies is usually attributed to the influence of sample size, vector ecology, host immunity, management, laboratory investigation methods, and vector control strategy. Our explanation is parallel to the vision of Desquesnes *et al.* (2013), Alsaad *et al.* (2021), Esmael *et al.* (2021), Hasan (2021), Mahmood & Alobaidii (2022) and Hassan *et al.* (2023). It has been shown that abortion, decreased milk production, neurological disorders, morbidity, and mortality are considerable medical and economic losses to the producer farms (Desquesnes *et al.*, 2013).

The molecular investigation in the present work successfully detected *T. evansi* in buffaloes at the molecular level in the Nineveh province through PCR of blood samples targeting the *ITS1* rDNA gene, indicating the sensitivity of this technique for diagnosis and identification of this

parasite, even those with low parasitaemia. This finding is consistent with findings of Sivajothi *et al.* (2016), Patel *et al.* (2020) and Desquesnes (2021) that the predominant diagnosis tool *T. evansi* in ruminants in the field is mostly by thick and thin stained blood smears, but certain circumstances, such as variable and low scales of parasitaemia, especially in sub-acute or chronic stages, could affect the detection of trypanosomes in the samples. Additionally, the absence of precise clinical signs and post-mortem lesions gives rise to the need for a more powerful, sensitive, and specific methods such as the PCR technique that are able to detect and identify *Trypanosoma* even in early-stage, subclinical, asymptomatic, and/or carrier infections in buffaloes and vectors (Baticados *et al.*, 2011; Patel *et al.*, 2020). Moreover, Fernandez *et al.* (2009) and El-Ashram *et al.* (2019) reported mixed infections and novel strains of *T. evansi*.

The phylogenetic tree of the local sequences of *T. evansi* was found to share common phylogenetic characteristics and an extremely close evolutionary relationship (99.53–100%) with the other *T. evansi* sequences recorded in the NCBI GenBank for various countries, including Tunisia (Rjeibi *et al.*, 2015), Thailand (Nguyen, 2019), China (Ekra *et al.*, 2024), Sudan (Agbo *et al.*, 2001), Iran (Bahari *et al.*, 2021), and Malaysia (Mohd Rajdi *et al.*, 2021).

## CONCLUSIONS

This survey is the first molecular record of *T. evansi* in buffaloes in this study region. Furthermore, it could be helpful to later epidemiological studies as well as to identify the link between local isolates and other *T. evansi* through the phylogenetic analysis. Further investigation at the mo-

lecular level is required for detection of trypanosomes and to confirm the clinically suspicious cases in other farm animals.

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