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Molecular detection and phylogenetic analysis of Theileria annulata in water buffaloes based on the cytochrome b gene

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Abstract Tropical theileriosis is a tick-transmitted disease that leads to considerable economic losses in livestock production in endemic areas. Although it is known that cattle are more resistant, information on whether Iraqi buffaloes also carry T. annulata is limited based on molecular studies. This study was conducted to detect T. annulata in naturally infected water buffaloes in Iraq by using the cytochrome b gene as a genetic marker and to examine its differences from other reported sequences.

Ninety-four buffalo blood samples were collected from September through December 2025 after the buffaloes had been clinically suspected of theileriosis. After Genomic DNA extracted, the cytochrome b gene of T. annulata was amplified using polymerase chain reaction (PCR). The resulting amplicons were sequenced and aligned with reference sequences stored in GenBank. A maximum-likelihood phylogenetic approach was then used to perform phylogenetic inference. PCR analysis confirmed T. annulata infection in 16 of 94 buffalo samples (17.02%). Sequence analysis showed high similarity (97.58–100%) between Iraqi isolates and previously reported global strains. Phylogenetic analysis indicated that Iraqi isolates clustered within the T. annulata clade and had close genetic relationships with isolates from several countries. The study confirms the presence of T. annulata in Iraqi buffaloes and emphasizes the usefulness of the cytochrome b gene as accurate molecular detection and phylogenetic analysis. These findings enhance understanding of the molecular epidemiology of tropical theileriosis in Iraq.

Keywords: Theileria annulata, cytochrome b, water buffalo.

Introduction Amassive problem of livestock production worldwide is the presence of tick borne diseases in tropical and subtropical regions where the ixodid ticks thrive (1). These infections damage the health and production of animals, leading to massive losses in the economy due to reduced milk and meat production, reproductive issues, increased veterinary care, and fatalities (2). The distribution of tick-borne diseases in endemic foci is conditional on the multidimensional interactions of the efficient tick vectors, vulnerable hosts, and the pathogenic protozoa (3). Theileriosis in cattle and buffaloes in Asia, the Middle East, North Africa and some areas of southern Europe is caused by *T. annulata*

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theileriosis (4). The parasite is primarily transmitted by Hyalomma ticks and may lead to serious morbidity and death in cattle and buffalo (5). the most frequently clinical sings include fever, swollen nodes, anemia, and a weakened immune system in clinical practice, but the severity of it is dependent on the host species, as well as the state of the immune system (6,7). Usually, buffaloes are more resistant to the infection than cattle, and they tend to have fewer or no visible symptoms (8). Molecular diagnostics such as PCR and DNA sequencing are essential to an accurate detection and epidemiological study (9,10). Mitochondrial genes are used as common markers since they are found in multiple copies; they possess clear signs of evolution. The cytochrome b gene has been frequently used in the identification of *T. annulata* as well as in comparing the isolates across the world (11,12). The phylogenetic trees that are constructed using these mitochondrial sequences provide us with useful hints regarding the relationship of the strains of parasites and their transmission between hosts and regions (13). These understandings enhance surveillance and aid in the

development of improved control of the tropical theileriosis (14,15). Although buffaloes are economically significant in Iraq, lack of molecular data on the infection, this study was conducted with an objective of detecting the presence of the cytochrome b gene in the buffaloes in Iraq that were naturally infected with *T. annulata* and comparing the obtained sequences with the isolates in the world.

Material and Methods

Ethical approval

The University of Al-Qadisiyah, College of Veterinary Medicine, approved the use and care of the animals in this study. On 28 December 2025, the study was authorized under No. 5633.

Collection sample

The total number of blood samples collected was 94 water buffaloes (Bubalus bubalis) found in the Al-Qadisiyah Province, Iraq, in the months of September and December, 2025.Whole blood samples were aseptically taken from the jugular vein using sterile disposable needles and placed on EDTA-coated collection vials. All the specimens were appropriately labelled and then kept under refrigeration conditions at the Molecular Parasitology Laboratory, College of Veterinary Medicine, University of Al-Qadisiyah, where they were stored at the temperature of -20 0 C until the following DNA extraction processes.

DNA Extraction from Blood

The (200µL) of peripheral whole blood was used to extract genomic deoxyribonucleic acid (DNA) using the Blood DNA Extraction Kit (AddBio, South Korea) according to the instructions of the manufacturer. The protocol involved enzymatic digestion with proteinase K, column-based purification, and elution of the purified nucleic acid in 50 µL of elution buffer. The obtained DNA solution was kept at -20 C before further molecular analyses.

Polymerase Chain Reaction technique (PCR

Cytochrome b (cyt b) gene of *T. annulata* was amplified using the traditional polymerase chain reaction (PCR) to confirm the existence of parasite DNA in the test samples. The total volume of each reaction was 20 uL, which contained 10 uL 2 x PCR Master Mix (AddBio, South Korea), which is a solution of Taq DNA polymerase, dNTPs, MgCl 2, and reaction buffer. A final concentration of 0.5 pmol of primers including Thiel-cytoF (5 CAGGGCTTTAACCTACAAATTAAC 3) forward and Thiel UN-cytoR (5 CCCCTCCACTAAGCGTCTTTCGACAC 3) reverse primers was added (Table 1). Extraction genomic DNA in the amount of two microliters was used as the template and the total volume was brought to 20 µL using nuclease-free water (12). The programme was started with an initial denaturation phase at 95 C of 3 minutes to ensure complete separation of the strands. This was then repeated 39 times with a denaturation at 95 C of 35 sec, annealing of the primer at 55 C of 35 sec to allow specific binding of the primer to the target sequence and extension at 72 C of 35 sec, through which Taq polymerase

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enhanced the synthesis of the DNA. The final extension of the program was 72 C, 5 min to make sure every fragment amplified is fully extended (Table 2). Agarose gel electrophoresis was performed on the PCR products which were expected to be around 1092 bp long. The samples were placed on 1.5% agarose gel that was prepared in 1 x TBE buffer and was subjected to 100 V and 80 mA electrophoresis. Each gel contained a 100bp DNA ladder (GeneDirex, South Korea) that was used to enable proper sizing of the amplified fragments. The visualisation of the bands in ultraviolet light was done to verify the existence and the approximate size of the PCR products. In order to support the validity of the PCR results and identify any form of contamination, negative control (NC) was included in every run (Figure 1). This control reaction included nuclease-free water instead of DNA template such that any amplification observed was due to the presence of target DNA and not contaminants.

Table 1: Utilizing primers for identification of *T. annulata* (12).

Name	Sequence	Amplicon size	Target gene
Thiel-cytoF	CAGGGCTTTAACCTACAAATTAAC	1092 bp	Cytochrome b gene (mitochondrial)
Thiel-cytoR	CCCCTCCACTAAGCGTCTTTCGACAC		

Table 2: PCR thermocycler conditions.

PCR step	Temperature	Time	Repeat
Initial denaturation	95ºC	3 min	1
Denaturation	95ºC	35 sec	39
Annealing	55 C	35 sec	
Extension	72ºC	35 sec	
Final extension	72ºC	5 min	1

Sequencing and Phylogenetic Analysis

To partially sequence the cytochrome b gene, we selected ten PCR-Positive samples. Once the PCR products had been cleaned up (approximately 1092 bp), we performed Sanger sequencing using the same primers that we used in the amplification process. Chromatograms were visually inspected, edited and trimmed manually to remove ambiguous bases to eliminate any dubious bases. Nucleotide sequences were deposited to GenBank (NCBI), and assigned accession numbers PX961336–PX961345. Sequence similarity was evaluated with BLAST of the NCBI database. To calculate the alignment, we applied ClustalW implemented in MEGA 11, and to estimate phylogenetic relationships, we used the Maximum Likelihood method and the Tamura-Nei substitution model. This final alignment consisted of 20 nucleotide sequences with 372 positions. bootstrap analysis with 1000 replicates was used to test the robustness of the inferred tree topology.

Table 3 : Nucleotide Sequence identity (%) in local T. annulata in buffalo as determined by NCBI BLAS, with corresponding GenBank accession numbers (PX961336, PX961337, PX961338, PX961339, PX961340, PX961341, PX961342, PX961343, PX961344, and PX961345) and compared with other global sequences.

Sequence number	Obtained Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical to	GenBank Accession number	Country	Identity (%)
1	PX961336	*Theileria annulate*	MK390362	Egypt	98.92
2	PX961337	*Theileria annulate*	LC431535	Sudan	100
3	PX961338	*Theileria annulate*	MK693128	Turkey	97.58
4	PX961339	*Theileria annulate*	PQ811607	China	97.58
5	PX961340	*Theileria annulate*	PQ663744	Pakistan	100
6	PX961341	*Theileria annulate*	MH778945	India	99.73
7	PX961342	*Theileria annulate*	MG787979	India	99.46
8	PX961343	*Theileria annulate*	PQ811602	China	99.73
9	PX961344	*Theileria annulate*	OP113848	India	99.46
10	PX961345	*Theileria annulate*	MK693131	Turkey	99.73

Results

The cytochrome b gene was amplified from 94 examined blood samples, 16 of which (approximately 17.02%) of those samples were found to be positive with *T. annulata*. The positive samples had a clear band of 1092bp, whereas the negative control did not show amplification at all (Figure 1). Ten of the samples that yielded positive results in the PCR were sequenced. Once the data had been trimmed and edited, we obtained high-quality partial sequences of the cytochrome b gene. These subsequently were uploaded to GenBank under the accession numbers PX961336-PX961345. A BLAST analysis showed that the sequences were all of *T. annulata*. The studied Iraqi isolates showed 97.58 -100% sequence identity with previously reported isolates in GenBank. A highest similarity score (100 %) identity coincided with Sudan and Pakistan

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isolates and slightly lower identity scores with Chinese and Turkish isolates (Table 3). A phylogenetic analysis of the Maximum Likelihood method with the Tamura-Nei model was carried out. The Iraqi buffalo isolates that formed part of the *T. annulata* clade were closely related to reference sequences in China, Egypt, India, Pakistan, Sudan, and Turkey. Bootstrap values were used to support the major branches of the tree (Figure 2).

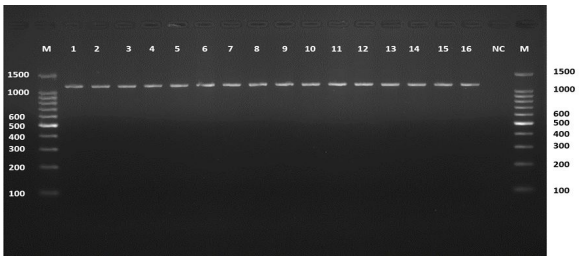

Figure 1: Gel electrophoresis (1.5 %) depicting PCR-amplified T. annulata cytochrome b gene in buffalo blood samples. lanes 1- 16 represent positive samples, NC is negative control contain H2O was added instead of DNA, M is molecular marker (GeneDirex, South Korea) (PCR amplicon size = 1092 bp).

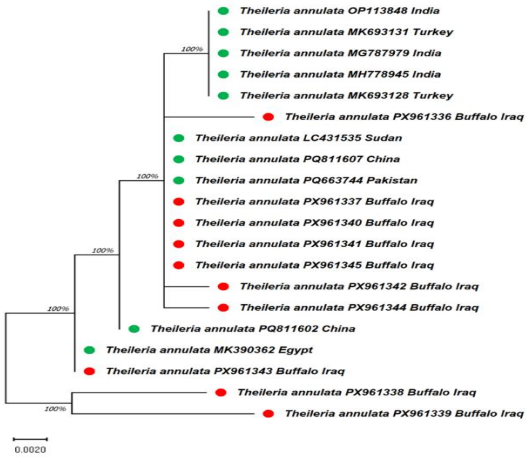

Figure 2: phylogenetic tree of T. annulata isolates from buffalo, constructed using Maximum Likelihood method and Tamura-Nei model. branch lengths are proportional to the number of substitutions per site and bootstrap values indicate the proportion of sites with at least one unambiguous base at each internal node. This analysis involved 20 nucleotide sequences with 372 positions in the final alignment. Phylogenetic inference was performed using MEGA11.

Discussion

The molecular test showed that of the 94 samples of water buffalo blood under examination, 16/94 (17.02 %) were positive for the gene cytochrome b (cyt b) gene. Regular observation of the predicted 1092bp PCR band was noted in all positive samples and lack of amplification was seen in the negative samples, thus confirming the specificity of the assay. The prevalence rate identified in this paper is comparable to the previous reports in Iraq and the nearby regions where the infection rates have been reported to vary based on the ecological conditions, the host species, and the diagnostic tests (16,17). At a larger geographical level, the distribution of the prevalence of the infection of cows and buffaloes with the *T. annulata*, and in such countries as Sudan, Pakistan, Egypt, and Turkey, previous epidemiological studies have identified the positivity rate over the range of 10-35 % (18,19). Such difference can be presumably explained by several interacting factors, such as the abundance and distribution of tick vectors, livestock management systems, and seasonal sampling and sensitivity of diagnostic methods used.

BLAST analysis revealed that all sequences were identified as *T. annulata* with high rate of nucleotide similarity ranging between 97.58 and 100% to the already known reference sequences in GenBank. It is noteworthy that the Iraqi isolates showed a hundred percent sequence match (100%), with some exhibiting slightly smaller similarity values with the isolates from countries, such as China and Turkey (12,20,21). This means that the Iraqi isolates are genetically conserved and there are close evolutionary relations with the isolates spread in other countries in the neighborhood and the region.

maximum likelihood approach with the Tamura Nei substitution model was also used to investigate the phylogenetic relationships

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between the obtained sequences and strains throughout the world in relation to global references. The resulting phylogenetic tree showed all Iraqi buffalo isolates had a strong clustering point in the clade of *T. annulata*. Additionally, the local isolates were clustered closely with the strains reported in Sudan, Pakistan, India, Turkey, China, and Egypt, indicating shared evolutionary origin and narrow genetic variation between the populations living in different geographic locations (22,23).The phylogenetic tree showed a bifurcating pattern which was backed by high values of bootstrap, which exhibited strong grouping of the analysed sequences, These findings agree with previous studies that have reported comparatively low genetic variability of the cyt b gene among isolates of *T. annulata* in different geographic locations (24,25).Close phylogenetic relations between the Iraqi isolates and the ones documented in a few other countries indicates that there are regional epidemiological ties, which can be attributed to either livestock movement or transboundary animal trade, or the ubiquitous presence of competent tick vectors. In a practical context, molecular detection of the presence of the tick-borne diseases in Iraq on buffalo population is significant with regards to the surveillance and control of tick-borne diseases in Iraq. The cyt b gene has been useful in this regard as a molecular marker not only in identifying the species and in phylogenetic reconstruction but also in tracking the genetic mutations linked to resistance of buparvaquone, which is the primary chemotherapeutic agent against tropical theleriosis (11,12,20). Thus, extended molecular surveillance is highly advisable in support of the successful disease-management efforts, such as in-built tick control programs and the prudent use of antiparasitic therapy.

Conclusion

This study provides molecular evidence of *T. annulata* infection in buffaloes in Iraq, based on analysis of the cytochrome b gene. The results highlight the genetic similarity of locally circulating *T. annulata* strains and confirm the usefulness of mitochondrial markers in the characterization of strains of this parasite by molecular means and a phylogenetic tree. generating molecular data on buffaloes in Iraq helps fill an important knowledge gap and improves understanding of the epidemiology of tropical theileriosis in endemic areas. The finding supports future surveillance programs and improves diagnosis, control, and monitoring of possible drug resistance.

Conflict of interest

No conflict of interest is disclosed by the authors.

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