



Full Length Research Article

Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Open Access



Date Received:
23/09/2024;
Date Revised:
18/02/2025;
Date Published Online:
31/10/2025;

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How to Cite:

Salah AH, Hussain MH (2025). Molecular report of resistant isolates of *Theileria annulata* by targeting the cytochrome b gene in cattle and ticks in Babylon, Al-Qasim city of Iraq. Adv. Life. Sci. 12(3): 528-533.

Keywords:

Theileria annulata, Cytochrome b gene, Tick, Buparvaquone, Sequencing

Editorial Note:

The editorial office provided significant language and presentation support to the authors of this article to improve its clarity.

Molecular report of resistant isolates of *Theileria annulata* by targeting the *cytochrome b* gene in cattle and ticks in Babylon, Al-Qasim city of Iraq

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Abstract

Background: Ticks are the carriers of the deadly animal illness theileriosis. *Theileria annulata* in cattle is an obligatory intracellular protozoan parasite carried by ticks (*Hyalomma spp.*) that infects hosts with mild, severe, and fatal illnesses. *Theileria annulata*, also known as tropical theileriosis, is a lymphoproliferative illness that causes severe mortality and morbidity in calves. Currently available antiparasitic medications work well in animals, but owing to developing resistance, animals may die or continue to be carriers. Treatment works best while the disease is still in its early stages.

Methods: In the current study, 2.5 mL of blood was drawn from the jugular vein and placed in an EDTA anticoagulant tube. Tick samples from each animal suspected of having *Theileria annulata* infection were placed in plastic tubes and sent straight to the laboratory for microscopic diagnosis using a blood smear and, later, PCR testing, which yielded a positive result.

Results: PCR targeting the 18S rRNA gene showed amplification in all tested blood samples (100%), confirming infection with *T. annulata*. In ticks, 81.25% (13/16) was positive for *T. annulata*. Furthermore, the cytochrome b gene (resistant gene) was detected in 100% of the calves' samples and 81.25% of the tick samples tested using PCR.

Conclusion: The cytochrome b gene was detected in all (100%) calf samples and 81.25% of tick samples, which may be a contributing factor to mortality in infected calves. Sequencing analysis is a necessary method for the identification of mutations in the ticks and calves' blood samples.



Introduction

Theileria annulata is widely dispersed in the Middle East, Russia, China, and Africa [1]. Various *Theileria* species from the phylum Apicomplexa produce the major tick-borne disease known as bovine theileriosis, which affects domesticated cattle in tropical and subtropical regions [2]. Certain species' effects on cattle mortality and morbidity cause major economic losses in several states of the United States [3].

The infected animals had pyrexia (40.5 - 41.5 °C), nasal and ocular discharges, swelling of the superficial lymph nodes, salivation, anemia, respiratory distress, and eye lesions [4]. Animals that recover from acute illness typically have, in order to maintain the parasite population, *Theileria* piroplasms that act as reservoirs [5].

The development of the superficial lymph nodes was linked to these symptoms. Icterus, anemia, and sporadic hemoglobinuria are symptoms of theileriosis because the parasites are killing the erythrocytes [6]. *Theileria annulata* is thought to be the most virulent *Theileria* species, with a global distribution, and is the cause of tropical bovine theileriosis [7]. Anorexia, a lingering fever, enlarged lymph nodes, diarrhea, and anemia are the signs of clinical tropical theileriosis [8].

Thin blood smears stained with Giemsa and lymph node biopsy smears are mostly analyzed microscopically to determine the diagnosis of acute theileriosis. Although microscopy is the 'gold standard' for the diagnosis of acute infections, it is not sensitive enough to demonstrate the low parasitemia that is usually observed in carrier animals [9]. However, the technique is only reliable for acute case diagnosis. Furthermore, due to a low parasitemia and the tiny number of piroplasms that may be detected in the peripheral blood in preclinical instances, it might be difficult to discriminate between various pathogenic and non-pathogenic piroplasms. Therefore, substantial expertise is required [10]. PCR assays for the diagnosis of *T. annulata* are more precise and dependable than other conventional procedures, and because of this, it has become the method of choice for diagnosing bovine and ovine theileriosis in epidemiological investigations [11-12]. RFLP (Restriction Fragment Length Polymorphism) assay was employed as a diagnostic tool to enable direct, concurrent, highly specific, and sensitive identification of *Theileria spp.* [13].

Buparvaquone, an antitheilerial medication, has been used successfully in the field to treat tropical theileriosis [14]. Currently, the main medication used to treat tropical theileriosis is buparvaquone. The hydroxy naphthoquinone compounds parvaquone and buparvaquone, which operate primarily on *cytochrome b* (Cyto b) of *Theileria* mitochondria, were first

presented as active medications in the 1970s. Since that time, buparvaquone has been the theileriosis therapy of choice on a global scale [15].

Phylogenetic models could suggest that novel resistance mutations can start from a single source, become immobile through collection, and then spread through parasites. This situation would probably result from the transfer of hosts that had medication-resistant alleles in their genes. Contrarily, as a result of host migration, resistance mutations may continually develop from many sources, become fixed by selection, and transfer amongst parasites [16].

Methods

Clinical examination

Most of the information was taken from the owner before the samples were collected from the suspected infected calves. The information, including the breed of calves, is recorded; the calves are selected from two to ten months of age, breed, and sex, and the signs, like the swollen lymph nodes, anemia, state of mucous membranes, and the presence of ticks on the body, are considered. Research was conducted between April and August 2022, when tick activity was at its peak, with all samples selected from Babylon, Al-Qasim city.

Blood and tick sample collection

The samples were collected between April and August 2022, when tick activity was at its peak. Sixteen blood samples from calves and sixteen tick samples were randomly selected from various pastoral areas in Babylon, Al-Qasim city. The sample size was limited due to restricted outbreak cases during the study period. Approximately 2.5 ml of blood was drawn from the jugular vein into an EDTA anticoagulant tube from each animal suspected of *Theileria annulata* infection and immediately transported to the laboratory. The sample size was limited due to restricted outbreak cases during the study period. Blood samples were kept at -20 °C until they were used in this study. Later, ticks were also collected from the same infected calves in a beaker with the help of tongs through the direct use of ethanol on the ticks and were transported to the lab. To make DNA extraction for PCR, DNA was stored at -20 °C until it was used in the study.

Microscopic detection of *Theileria annulata*

Peripheral blood smears were prepared on glass slides, which were then dried and fixed with methanol for roughly five minutes, then stained with 10% Giemsa stain for about 30 minutes, and studied under 100x oil immersion magnification [17].

DNA extraction and PCR conditions

Using a DNA isolation kit, total DNA was isolated from tick samples and EDTA blood (Genomic DNA mini kit,

blood/cultured, Korea) to prepare the sample for PCR using two primers, 18srRNA for the detection of *T. annulata* and the *cytochrome b* gene for the detection of gene resistance. Amplification of the partial sequence of the *cytochrome b* gene of *Theileria annulata* was performed using two primers (Tcytb F, R). Thiel-cyto F (5' CAGGGCTTTAACCTACAAATTAAC3') and Thiel-cytoR (5' CCCTCCACTAAGCGTCTTTCGACAC3') produce 1092 bp according to Mhadhbi *et al.*, [18].

Molecular detection

The *cytochrome b* gene for the detection of resistant genes in both *Theileria annulata* and tick samples was used for the detection of the parasite's resistance to antiparasitic drugs (buparvaquone). The total volume of reaction for the PCR was 25 µL and is composed of a DNA template of 2 µL, 1 µL of each primer, 12.5 µL of master mix, and 8.5 µL of distilled water.

Results

Examination of Blood Films by Microscopy

The microscopic examination was used to determine whether piroplasms were present. All sixteen blood samples taken from the calves gave a positive result due to the presence of piroplasms in the RBCs. Figure 1 shows the *T. annulata* under 100X oil immersion.

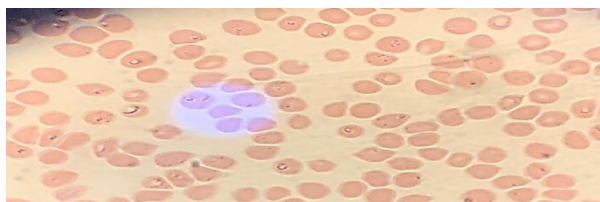


Figure 1: Under an oil immersion 100X microscope, the calf blood sample collected from the jugular vein displays piroplasms of *Theileria* spp. within red blood cells in this figure, following the application of Giemsa stain for the study of *Theileria* spp.

PCR test result

Detection of the *cytochrome b* gene in *T. annulata* blood sample

A 1092 bp fragment of the *cytochrome b* gene was used to detect the resistant gene of *Theileria annulata* in blood samples of calves, using the PCR test; C was the control (-ve), in which water (H₂O) was added instead of DNA, as shown in Figure 2.

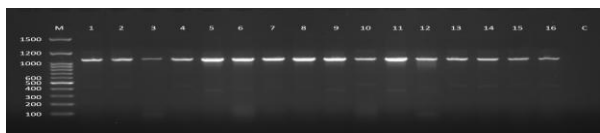


Figure 2: Gel Electrophoresis image (1.7% agarose gel) shows positive (+ve) samples (lanes 1-16) of *Theileria annulata* (PCR amplicon size, 1092 bp), targeting the *cytochrome b* gene in calves as hosts. C was control negative (-ve) where DNA was replaced with water (H₂O). Molecular marker M. (Favor prep, KOREA).

Detection of *T. annulata* by *Cytochrome b* in tick samples

The *cytochrome b* gene was used on tick samples, a 1092 bp fragment, to detect the resistant gene of *Theileria annulata* in tick samples using PCR tests. C was control negative (-ve), in which water (H₂O) was added instead of DNA. Figure 3 shows the positive result (1-16), except (5, 9, 15), which give a negative (-ve) result.

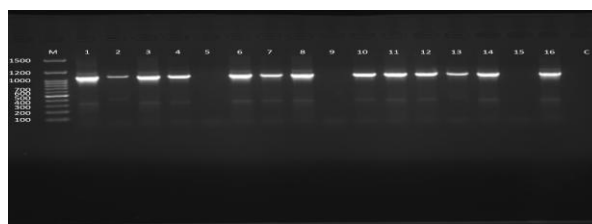


Figure 3: Gel Electrophoresis image (1.7 %) shows positive samples (lane 1-16 except lane 5, 9, 15, which are negative) of *Theileria annulata* (PCR amplicon size = 1092 bp) targeting the *cytochrome b* gene in the tick vector. C was control negative (-ve), and DNA was replaced with water (H₂O). Molecular marker M. (Favor prep, KOREA).

Cytochrome b Gene Amplification, Analysis, and Sequencing

A subset of 20 positive PCR products was selected for DNA sequencing and molecular evolutionary analysis. The screened sequences were cleaned of background noise and submitted to the GenBank database. Based on acquired accession numbers, a comparative investigation was further conducted with other global strains: DNA sequencing analysis, e.g., building of a phylogenetic tree, was carried out with multiple alignment of sequences using Clustal W as well as Molecular Evolutionary Genetics Analysis version 11 (MEGA X). Sequences thus obtained were submitted to alignment with susceptible as well as resistant global strains to identify similarities as well as discrepancies. In building a phylogenetic tree, reference sequences thus obtained using NCBI-BLAST were utilized. Analysis was carried out using the software quoted accordingly [19].

Amino acid sequence analysis

Most isolates in Figure 4 showed T mutations that evolved into A in sequences with accession numbers (OQ627715, OQ627723) in cattle and (OQ627726, OQ627727, OQ627730) in ticks. As shown in Figure 4, the substitutions observed in our dataset occur at codons 175, 177 (F→C in tick isolates), 200 (V→M in cattle), and 224 (W→G in cattle); no change is evident at codon 119 in the alignment. "A" was present in every sequence; however, the accession number (OQ627717) in the cattle sequences at position 175 was different. A mutation in the accession number (OQ627728, OQ627731) at position 177 caused the amino acid (C) to

emerge in the tick isolate rather than (F). Cattle with the accession numbers OQ627716 and OQ627720 had a mutation where the amino acid M was observed rather than V. Cattle with accession numbers OQ627715 and OQ627721 have a mutation where G was observed rather than W.



Figure 4: Multiple sequence alignment of the identified *Theileria annulata* (cytochrome b gene) translated amino acid sequence compared with homologous global sequence (resistant and sensitive strains). Highlighted with four colors to indicate the similarity.

Phylogenetic analysis

Tree(s) for heuristic search were generated automatically by execution of the Neighbor-Joining and

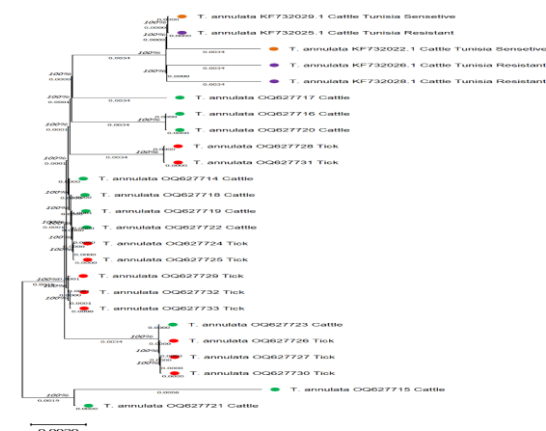


Figure 5: Neighbor-Joining and BioNJ algorithms-based evolutionary study, as deduced by its application. The displayed tree is the one with the highest log probability (-974.43).

BioNJ algorithms on a matrix of pairwise distances calculated using the JTT model and shown in Figure 5. As a tree topology suitable for further investigation, a tree topology with the maximum value of log likelihood was selected. Below each branch, branch lengths have units of substitutions per site, and the tree is displayed at scale. There were 25 amino acid sequences in this

investigation. The final dataset contained 302 locations in total. MEGA11 software was used to perform evolutionary analyses [19].

Discussion

The most hazardous species for ruminants of all sizes is *Theileria annulata*. *Theileria* parasites can be identified by microscopic analysis of thin blood smears, lesions, clinical signs, and case history, but a similarity in the clinical signs was observed. The physical resemblance of piroplasms, false-negative diagnoses caused by the parasite's rarity (particularly in carrier animals), and difficulties recognizing mixed infections are some of the problems associated with microscopic detection [20]. Ticks present the main factor of transmitting disease, and they seem endemic in Iraq, with widespread distribution due to the suitable climatic circumstances during nine months of the year, because of the appropriate temperature and moisture [21].

Therefore, precise and sensitive diagnostic tests, such as PCR, could be used to diagnose and differentiate between different types of piroplasms [13]. The stained blood smears contained intraerythrocytic stages of *Theileria annulata*. *Theileria* parasite infection can be diagnosed through microscopic examination of thin blood smears, as shown in Figure 1. Several molecular markers, including the cytochrome b genes, have been employed in recent years to ascertain the phylogenetic relationships among the piroplasm population [22]. For assessing evolutionary relationships between species at the levels of families and genera, the mitochondrial cytochrome b gene is frequently used, as reported by Castresana *et al.*, [23].

In location 192, a mutation was noted in accession number (OQ627714, OQ627715, OQ627716, OQ627717, OQ627718, OQ627719, OQ627720, OQ627721, OQ627722, OQ627723) in calves and (OQ627723, OQ627724) in the tick V amino acid, while the sensitive strain showed the similar mutation in the accession number (KF732022.1 and KF732029.1) and resistant strain (KF732025.1, KF732026.1, KF732028.1) in cattle in Tunisia. In position 200, there is a mutation in the accession number (OQ627716, OQ627720) in cattle, where V mutated into M, and all the samples of V amino acid appeared. There is a mutation at position 224 in accession numbers (OQ627715, OQ627721) in which a G amino acid was observed in cattle in the position of W, and all the samples have a G amino acid. There is a mutation in accession number KF732022.1 in a resistant strain of cattle in Tunisia that mutated into S, while other samples take the leucine (L) amino acid.

These results are consistent with findings made by Mhadhbi *et al.*, [24] and Chatanga *et al.*, [25]. They discovered an identical mutation at codon 143. They

also discovered that fifty isolates had mutations at codons 129 (18/50) and 227 (3/50), but some positions in all examined sequences had no alterations. The resistant isolate (JQ308839) had the proline (P) 253 and serine (S) mutation, but the sensitive isolate (JQ308837) had no mutations. All discovered mutations suggest a more error-prone nature of this gene, according to Goodman *et al.*, [26], who underlined that the *cytochrome b* gene is encoded by many copies on the mitochondrial genome, and the mitochondrial polymerase enzyme has low fidelity.

These results support that the *cytochrome b* gene can be utilized as a genetic marker to differentiate resistant *T. annulata* isolates. Small sample sizes were one of our study's limitations due to a shortage of clinical cases that may be used as examples and a lack of finances. Therefore, we suggest that future studies consider using a larger sample size and an open time to involve all seasons. Additionally, we recommend that field researchers check for buparvaquone resistance in various cattle breeds. The phylogenetic tree between various species of *Theileria* parasites using the *cytochrome b* gene sequence was very useful. It should be noted that a bootstrap value is used to determine how reliable a phylogenetic tree is. Values between 70% and 89% are considered to be quite significant, and those above 90% are considered extremely significant. Furthermore, numbers that are less than 70% are thought to be less significant and consistent [27].

Acknowledgement

This research is supported by Professor Dr. Amjed Alsultany of the University of AL-Qadisiyah for molecular work on blood and tick samples.

Author Contributions

All authors developed the concept, designed the methodology, collected, analyzed the data, and prepared the original draft of the manuscript.

Competing Interest

As stated by the authors, they have no conflicting interests.

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