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Research article

Microscopical and phylogenetic analysis of *Theileria annulata* in Iraqi local breed cattle

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Abstract

The development of cattle industry in Iraq and its neighboring countries may be hampered by *Theileria annulata* (T. annulata) infection. In the present work, the cytochrome b gene (cyt-b) sequences were used to infer the phylogenetic relationships of *T. annulata*. Fifty blood samples collected randomly from 10 farms of Iraqi local breed cattle in Al-Diwaniyah province were analyzed for piroplasm using microscopic and molecular techniques. Microscopic examination revealed that 37 (74%) of the 50 blood samples examined were infected. In addition, the PCR test showed that 23 (62.2%) of the 37 blood samples (which tested positive microscopically) were positive. The analysis of the cyt-b 1092 bp gene of *T. annulata* reported the presence of six genetically related clones. These six clones have been deposited in GenBank as [ON706262, ON706263, ON706264, ON706265, ON706266, and ON706267], and when compared to the *T. annulata* cyt-b gene (XM949625) from the GenBank database, a unique polymorphism at sixteen sites was discovered. Furthermore, the alignment of our amino acid sequences with the reference sequence reveals thirteen non-synonymous mutations in codons 11, 22, 33, 63, 103, 128, 130, 129, 172, 178, and 190, and three silent mutations in codons 124, 128, and 275. Moreover, phylogenetically, our study clones were related to Iraqi, Indian, and Turkish clones. In conclusion, depending on the phylogenetic analysis the current study identified six *T. annulata* parasite isolates in Iraqi local breed cattle that were genetically related to the Indian, Iraqi, and Turkish clones.

Keywords: 18S rRNA gene, Cattle, Cytochrome b gene, Iraq, Phylogenetic tree, Theileria spp.

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INTRODUCTION

The main goal of the livestock industry is to make milk and meat of high quality for people to eat (Wu et al., 2014). Al-Shawi et al., (2016) reported that the Iraqi cattle breed is either exotic, like the taurine (*Bos taurus taurus*) and the native zebu breed (*Bos indicus*), or local, like the Rustaqi and Jenoubi, or crossbred. Researchers and experts haven't paid enough attention to the Iraqi crossbreeds, which are separate breeds. It is well known that theileriosis and other tick-borne diseases greatly reduce the amount of milk that cattle can produce. This causes large economic losses both locally and globally, as well as other problems.

Bovine theileriosis is a tick-transmitted disease, and *T. annulata* and *T. parva* are the two most economically significant species worldwide (Jongejan and Uilenberg, 2004; Elsify et al., 2015). Ticks of the genus *Hyalomma* (Jongejan and Uilenberg, 2004) are responsible for transmitting *T. annulata*, which causes a high fever (up to 40–41.5ºC), fatigue, depression, tears, nasal discharge, lymph node swelling, and anaemias (Brites-Neto et al., 2015). Furthermore, it considers one of the major barriers to the growth of animal wealth that the high illness vulnerability of rare breeds and hybrid cattle results in substantial financial losses (Gharbi et al., 2011). The rate of abnormality in exotic breeds can approach 40–50% if they are not treated against theileriosis (Brown, 1990).

Three main strategies are now being employed to control the tropical theileriosis outbreak. Three main strategies are now The first is the divisive and expensive problem of eliminating vector ticks (Gharbi and Darghouth, 2015). Cattle vaccination is another option (Zhang, 1997). Though the long-term effects of vaccines in endemic areas are controversial, their short-term impact on a wide range of field parasite populations is not (Gharbi and Darghouth, 2015). An antiprotozoal hydroxynaphthoquinone medicine known as buparvaquone is the third and most widely used method for treating newly infected animals.

Hydroxynaphthoquinone's theilericidal activity was first identified in the 1970s (Boehm et al., 1981). After that, a less toxic compound called buparvaquone (trans-2-[4-(4-butyl cyclohexyl)] cyclohexanone) was developed (McHardy and Morgan, 1985) and is commonly used to treat *Theileria* species infections caused in endemic areas (McHardy et al., 1985).

Studies have shown that the fact that *T. annulata* is resistant to buparvaquone is one of the most difficult things about using these kinds of treatments. Buparvaquone may also function by binding to cyt-b, so mutations in the connecting site of ubiquinone are likely the same thing that is responsible for *T. annulate's* resistance to the drug (Roy et al., 2000; Mhadhbi et al.; 2010). So far, cytochrome b (cyt-b) is one of the most useful genes for studying evolution, and its protein product's structure and function have gotten the most attention of any mitochondrial gene (Fivelman et al., 2002). In the cyt-b gene and codon locations, there are both highly conserved domains or regions and highly variable domains or regions that change at different rates. So, this gene has been used in many different kinds of systematic studies, from "deep" phylogenies (Meyer and Wilson, 1990; Degli Esposti et al., 1993) to levels of population and recent divergence (Kumazawa and Nishida, 2000). But cyt-b has problems like base compositional biases, rate variation between lineages,

saturation at third codon positions, and limited variation in first and second codon positions. This means that there is either not enough phylogenetic information for "deep" questions about evolution or not enough information at the population level for the third codon position (Degli Esposti et al., 1993. This has made people question the overly simple idea that this gene can be used as a phylogenetic marker that works everywhere (Ali et al., 2022).

Unfortunately, the number of countries, such as Tunisia (Honeycutt et al., 1995; Mhadhbi et al., 2015), Iran (Sharifiyazdi et al., 2012), Sudan (Chatanga et al., 2019; Salim et al., 2019), and Egypt (Yousef et al., 2012), where buparvaquone hasn't been able to control the disease has grown. The mechanism of buparvaquone resistance and its treatment are not well known. Like the related medications atovaquone (Fry and Pudney, 1992) and the 1,4-naphthoquinone family of hydroxynaphthoquinones (Boehm et al., 1981), buparvaquone was theorized to disrupt the mitochondrial electron transport chain at the cytochrome bc1 complex (Fry and Pudney, 1992). In light of the similarities between buparvaquone and atovaquone, it was hypothesized that a mutation in cytochrome b of *T.annulata*, more precisely at the binding site of ubiquinone, would be linked to resistance to buparvaquone (Honeycutt et al., 1995; Sharifiyazdi et al., 2012; Mhadhbi et al., 2015).

This study aimed to evaluate the phylogenetic tree of *T. annulata* that infects Iraqi local breed cattle based on the DNA sequence of the cyt-b gene, search for mutations in that sequence, and create a link between the *T. annulata* clones used in this study and those used in prior studies.

MATERIALS AND METHODS

Samples collection

50 blood samples were collected randomly from 10 farms of local breed cattle in Al-Diwaniyah province in the south of Iraq from May 2022 to December 2022. A total of 2.5 ml of blood were taken from the jugular vein and placed in EDTA blood collecting tubes, which were then put in a cooling transport box (APTACA Spa, Italy) and transferred immediately to the lab for doing the further assessment regarding the standard blood smear and molecular analysis.

Staining of blood samples with Giemsa

Giemsa stain was used to prepare and stain thin blood smears. The parasites were recognized based on Soulsby's descriptions of their characteristics (Soulsby, 1982). Thin blood spots stained with Giemsa stain were looked at through an Olympus light microscope (version CX21FS1 made in the Philippines) with a 100X oil objective. The scale of the slide and the illustrations were set using the ImageJ program (USA). The results of this microscopic inspection were used to categorize samples as either positive or negative before proceeding with the investigation of hemato-biochemical parameters.

Assessment of Hemato-biochemical Parameters

Blood was drawn into sterile tubes coated with the anticoagulant disodium salt of ethylenediaminetetraacetic acid (EDTA). Hemoglobin (Hb) g/dL, Hematocrit % (HCT), total erythrocyte count (TEC) (RBC× 10^6/µL), and total leukocyte count (TLC x 10^3/µL), MCV (fL), and MCHC (g/dL) of the healthy and theileriosis-affected cattle were measured in the blood samples. These characteristics were examined using standard techniques (Jain, 1986).
To perform the necessary biochemical analysis, the serum was separated from the blood samples by centrifugation at 2500 rpm for 30 minutes. After collecting the serum, it was pipetted into Eppendorf® Flex-Tubes® and placed in the freezer to be analyzed at 20°C. The Reflotron® Plus chemistry analyzer (Gmbh Mannheim, Germany) was used to measure several biochemical parameters, including total protein g/dL, albumin (Alb) g/dL, globulin (Glb) g/dL, aspartate aminotransferase (AST) U/L, alanine aminotransferase (ALT) U/L, urea mg/dL, total serum bilirubin mg/dL.

DNA extraction and PCR conditions

The genomic DNA was extracted from 50 blood samples using a DNA extraction kit with Proteinase-K from Bioneer, Korea. The NanoDrop spectrophotometer 2000 (Thermo Scientific) was used to detect the 260/280 nm absorbance of the extracted DNA to ascertain its concentration and purity (García-Alegría et al., 2020).

The cyt-b (1092 bp) gene primers (Bioneer, Korea) including "F: 5'-CAGGGCTTTAACCTACAAATTAAC-'3 and R: 5'-CCCCTCCACTAAG CGTCTTT CGACAC-'3" were utilized for the detection of the cyt-b (1092 bp) gene (Mhadhbi et al., 2015). Furthermore, a set of primers (Bioneer, Korea) were designed to target the 18S rRNA gene (620 bp) (Alfatlawi et al., 2021) of *T. annulata*" F:5'-ATTGCTTGTGTCCCTCTGGG and R: TCCACCAACTAAGAACGGCC-'3".

All PCR reactions were carried out in a 20 µl reaction mixture that included 12 µl of the AccuPower® PCR PreMix (Bioneer, Korea), 5 µl of the template DNA, and 1.5 µl (10 pmole) of each primer. In the thermocycler, the initial denaturation step was set at 95 °C for 5 minutes. Then 30 cycles of denaturing at 95°C for the 30s, annealing at 58°C for the 30s, and extension at 72°C for 1 minute; finally, one cycle of final extension at 72°C for 5 minutes. Under the UV gel documentary, the PCR product bands were detected using a 1% agarose gel stained by ethidium bromide.

DNA sequencing

The sequence of the purified positive PCR products of the cyt-b gene made with the AB DNA sequencing instrument (Bioneer Company, Korea) was used to build the phylogenetic investigation. The tree was constructed using the neighbour-joining method (MEGA v6) and the NCBI-Blast alignment methods, as shown in the Table 1.

Table 1 The accession numbers used in the current study to achieve phylogenetic study

No.	Accession Numbers	Source
1	ON706262	Current Study
2	ON706263	Current Study
3	ON706264	Current Study
4	ON706265	Current Study
5	ON706266	Current Study
6	ON706267	Current Study
7	MK390359	GeneBank
8	MK390361	GeneBank
9	MK390360	GeneBank
10	MK390364	GeneBank
11	MK693123	GeneBank
12	MK693124	GeneBank
13	MK693125	GeneBank
14	MK693126	GeneBank
15	MK693127	GeneBank
16	MK693128	GeneBank
17	MK693130	GeneBank
18	MK693131	GeneBank
19	MK693132	GeneBank
20	MK693133	GeneBank
21	MK693134	GeneBank
22	MK693135	GeneBank
23	MK693139	GeneBank
24	MG787979	GeneBank
25	MG787980	GeneBank
26	MG787981	GeneBank
27	MG787982	GeneBank
28	MG787983	GeneBank
29	MG787984	GeneBank
30	MG787985	GeneBank
31	MG787986	GeneBank
32	MT812969	GeneBank
33	OM937770	GeneBank
34	XM949625	Reference Clone-GeneBank

RESULTS

Clinical Finding

Diseased cattle have a high body temperature of 40.3–41.5ºC, inappetence, asymmetrical enlargement of superficial lymph nodes, particularly the pre-scapular ones, and may have diarrhoea, a pale and/or icteric mucous membrane of the eyes, bulging eyes, lacrimation, ecchymosis haemorrhages on the sclera, and incoordination (Table 2).

Table 2 Number and percentage of *T. annulata*-infected cattle based on clinical finding

No.	Clinical Finding	Number of animal with clinical sings/total number	Percentage (%)
1	Fever, anorexia, swollen superficial lymph nodes, nervous signs, and the presence of ticks over the animals.	31/50	62
2	Fever, diarrhea, bloating, and tick presence	6/50	12
3	Fever, pale mucus membrane, lacrimation, and tick presence	9/50	18
4	Fever, incoordination, nervous symptoms, and salivation	4/50	8
Total		50	100

Giemsa Stain and Microscopic Examination

After microscopic examination, 37 (74%) of the 50 blood samples were found to be infected (Figure 1).

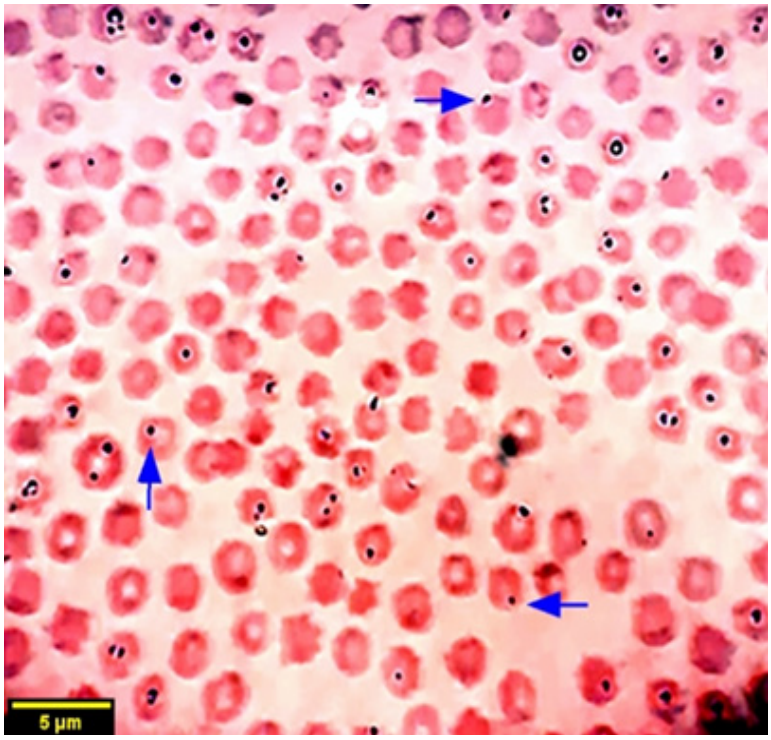

Figure 1 A peripheral blood smear revealed the presence of Theileria spp. (blue arrows) inside RBC (Giemsa stain, X100).

Hemato-biochemical Assessment

In this study, infected cattle had a highly significant ($p<0.05$) decrease in mean ± SE levels of TLC, TEC, Hb, HCT, and MCHC levels when compared to healthy cattle, while MCV values were significantly higher ($P<0.05$) (Table 3). Regarding the biochemical analysis, total protein, albumin, globulin, were all significantly lower ($p< 0.05$) in bovines infected with theileriosis compared to healthy bovines, whereas AST, ALT, urea, total serum bilirubin levels were significantly higher ($P<0.05$) (Table 3).

Table 3 Hemato-biochemical parameters (Mean± SEM) of infected and uninfected cattle with *T. annulata*.

NO.	Parameters	Infected Cattle	Non-infected Cattle
1	TLC (× 10^3/µL	4.145 ± 0.322 $^{b\downarrow}$	5.832±0.178 ^{a}
2	RBCs(X10/L)	4.812± 0.101$^{b\downarrow}$	6.975± 0.219 ^{a}
3	Hb (g/dL)	5.648±0.120 $^{b\downarrow}$	9.500±0.193 ^{a}
4	HCT (%)	20.997±0.456 $^{b\downarrow}$	37.240±1.611 ^{a}
5	MCV (fL)	63.519 ± 0.7413$^{b\uparrow}$	44.819±0.875 ^{a}
6	MCHC (%)	24.341±0.227 $^{b\downarrow}$	30.701±0.258 ^{a}
7	Total protein g/dL	5.104 ± 0.061 $^{b\downarrow}$	8.112 ± 0.054 ^{a}
8	Albumin g/dL	2.533 ± 0.103 $^{b\downarrow}$	5.122 ± 0.091 ^{a}
9	Globulin g/dL	2.571 ± 0.114 $^{b\downarrow}$	3.360 ±0.073 ^{a}
10	AST U/L	151.876± 2.631 $^{b\uparrow}$	60.122±1.351 ^{a}
11	ALT U/L	30.804 ± 0.734 $^{b\uparrow}$	18.194±0.457 ^{a}
12	Urea mg/dL	67.186 ± 0.375 $^{b\uparrow}$	37.795±1.004 ^{a}
7	Total serum bilirubin mg/dL	2.28 ± 0.025 $^{b\uparrow}$	0.632 ± 0.029 ^{a}

The small letter (a and b) represent the significant difference between the infected and un-infected cattle. The ($^{\uparrow}$) represents increased values, while ($^{\downarrow}$) represents decreased values.

Polymerase chain reaction (PCR) technique analysis

Among a total of 37 (microscopically positive) samples, 23 (62.2%) passed the PCR test with positive results for both targeted genes (cyt-b and 18S rRNA). As a follow-up to the positive results for the cyt-b gene, an 18S rRNA primer was used to find out if cattle were infected with *T. annulata* (Figure 2). All of the cytochrome b gene amplicons, on the other hand, were successfully sequenced and found to be *T. annulata*.

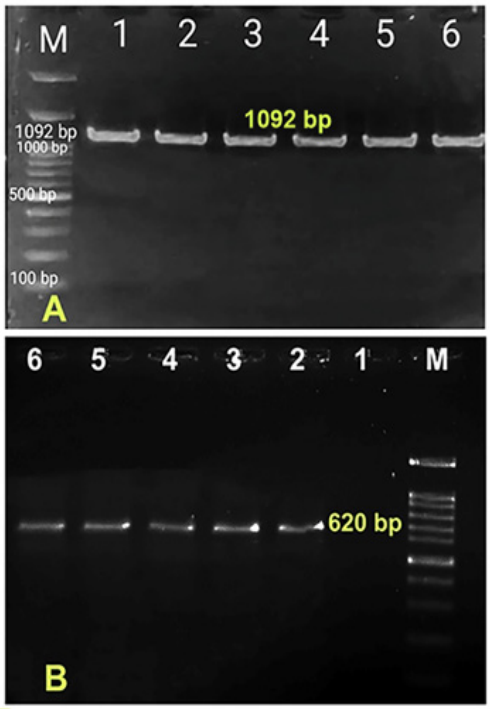

Figure 2 (A) The PCR product of the 1092bp T. annulata cytb gene separated into bands on a 1% agarose gel. Positive samples of the 1092-bp T. annulata cytb gene were found in lanes (1-6), while the ladder was found in lane (M, 1500-100 bp). (B) On a 1% agarose gel, the PCR product showed bands of 620 bp T. annulata 18S rRNA gene. Lane (M) is the ladder (1500-100bp), Lane (1) is negative control; and Lanes (2–6) are positive T. annulata 18S rRNA gene samples.

Cytochrome b sequence analyses

The 1092 bp nucleotide sequences that make up the whole cyt-b gene were amplified and sequenced. The results of the gene sequence analysis of cyt-b (1092 bp) revealed six *T. annulata* clones, which were then compared to other cyt-b gene sequences in the GenBank database. All of the information was put into the GeneBank database with the accession numbers ON706262, ON706263, ON706264, ON706265, ON706266, and ON706267 (Table 1). When the current study's six clones were compared to the *T. annulata* cyt b gene (XM949625) and other closely related cyt b gene sequences found in the GenBank database, a unique polymorphism at sixteen locations was discovered. Furthermore, the alignment of our amino acid sequences with the reference sequence reveals thirteen non-synonymous mutations in codons 11, 22, 33, 63, 103, 128, 130, 129, 172, 178, and 190, which are mostly concentrated in the ON706265 isolate. While a three silent mutations were found in codons 124, 128, and 275 (Table 4).

Table 4 Mutations registered in the cytochrome b gene of the study isolates [ON706262-67] compared to the reference *T. annulata* cyt-b gene isolate (XM949625)

The reference *T. annulata* cyt-b gene isolate (XM949625)																
Sequence	34	63	67	103	190	304	373	385	387	392	517	526	534	554	570	826
Codon	11*	22*	23*	33*	63*	103*	124	128	129*	130*	172*	175*	178*	184*	190*	275
	TAC	ATA	CCG	AAG	CGT	CTG	TAT	TCG	GGA	GCT	ATA	GAT	AAT	TAT	TTT	TAT
ON706262	.C.	.C.	.A.	.G.	.A.	…	.A.	.G.	.A.	…	…	.G.	.C.	…	.C.	.G.
ON706263	.C.	.C.	.A.	.G.	.A.	…	.A.	.T.	…	…	…	…	…	…	…	.G.
ON706264	.C.	.C.	.A.	.G.	…	.C.	.A.	.T.	.A.	…	…	…	…	…	…	.G.
ON706265	…	…	.A.	…	…	.C.	.A.	.T.	…	…	…	…	…	…	…	.G.
ON706266	…	…	.A.	…	.A.	…	.A.	.T.	.A.	.A.	.A.	…	…	.C.	…	.G.
ON706267	.C.	.C.	…	.G.	.A.	…	.A.	.T.	…	…	…	…	…	…	…	.G.

* Nonsynonymous mutation

Phylogenetic analysis

An edited alignment of 34 cyt-b protein sequences spanning 350 amino acids was used to build the phylogenetic tree using the neighbour-joining method (Table 1). The phylogenetic analysis compared the reference clone from the Gene Bank (XM949625) to six Iraqi isolates (accession numbers ON706262-ON706267) (Table 1). Isolate 4 (ON706265) was found to be similar to the clone from Iraq, while isolate 5 (ON706266) was found to be similar to the clone from India (MG787985) (OM937770). Similarities between the Ankara clone (reference) XM949625 and other Turkish clones, such as MK693127, MK693128, MK693126, MK390359, MK390361, MK390360, and MK390364, are shown in Figure 3 for Isolates 1, 2, 3, and 6.

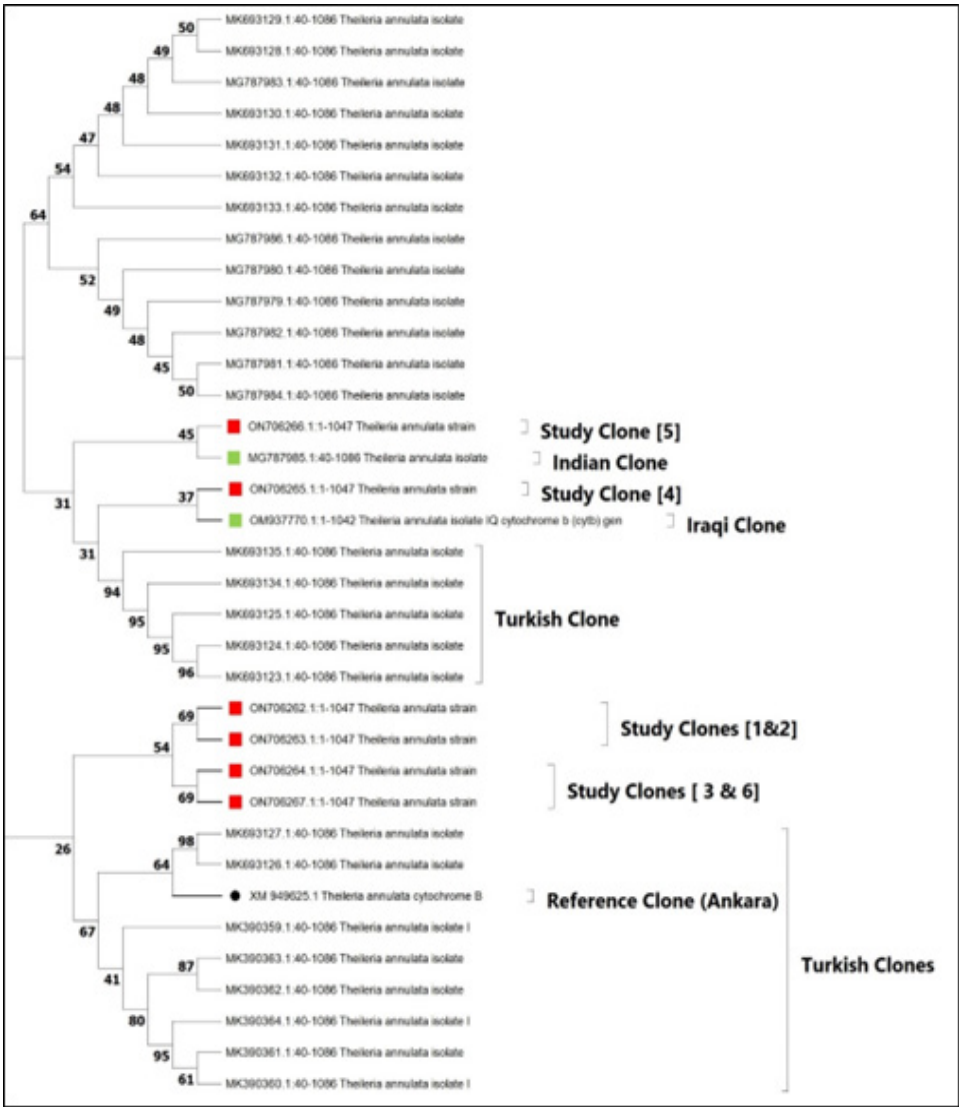

Figure 3 *T. annulata* phylogenetic tree based on Cytochrome b nucleotide sequences from GeneBank and 7 Iraqi isolates(*) using the Neighbor-joining (N-J) method and 1000 bootstrap replicates. (*) The Iraqi isolates were marked with a red box for the isolates of the current study and a green box for the Iraqi isolate from the GenBank.

Correlation between study *cyt-b* gene mutations and clinical signs

In this study, the clinical symptoms were linked to the clades and point mutations found by phylogenetic analysis, as shown in Table 5 and figure 4. Table 5 shows that the mutations that happened in the *T. annulata* cyt-b gene was led to changes in the amino acids. These changes resulted in proline to threonine substitutions at codon 11 and a glycine to arginine alteration at codon 33 of *T. annulata (cyt-b)*. Furthermore, in codons 63, 124, and 275, valine replaced isoleucine. Furthermore, at codon 103, cystine was replaced by arginine, and in codon 387, glycine was replaced by tryptophan, both within a highly conserved site. Moreover, asparagine is converted to lysine in codon 534, histidine to lysine in codon 552, and tryptophane to phenylalanine in codon 570.

Table 5 The correlation between the amino acid substitutions that indicated in the *cyt-b* gene of *T.annulata* of the current study clones and the recorded clinical sings in the Iraqi local breed cattle.

Clones	Recorded Clinical Signs	Mutations/Codon												
		Sequence	33	99	189	309	372	387	390	516	534	552	570	825
		Codon No.	11	33	63	103	124	129	130	172	178	184	190	275
ON706262	Fever, Enlarged lymph nodes, nervous signs		*pro*	*gly*	*iso*	*cys*	*val*	*gly*	*ser*	*	*asn*	*his*	*tyr*	*val*
ON706263			*pro*	*gly*	*iso*	*cys*	*val*	*try*	*ser*	*	*lys*	*his*	*tyr*	*val*
ON706264			*pro*	*gly*	*val*	*arg*	*val*	*	*ser*	*	*lys*	*his*	*tyr*	*val*
ON706267			*pro*	*gly*	*val*	*arg*	*val*	*try*	*asn*	*	*lys*	*his*	*tyr*	*val*
ON706266	Fever, Enlarged lymph nodes, salivation		*thr*	*arg*	*iso*	*cys*	*val*	*	*asn*	*lys*	*lys*	*lys*	*phe*	*val*
ON706265			*thr*	*arg*	*iso*	*cys*	*val*	*try*	*asn*	*	*lys*	*his*	*tyr*	*val*

Pro: proline; gly: glycine; Iso: isoleucine; val: valine; ser: serine; his: histidine; try: tryptophane; thr: threonine; lys: lysine; phe: phenylalanine; arg: arginine; cys: cystine' asn: asparagine

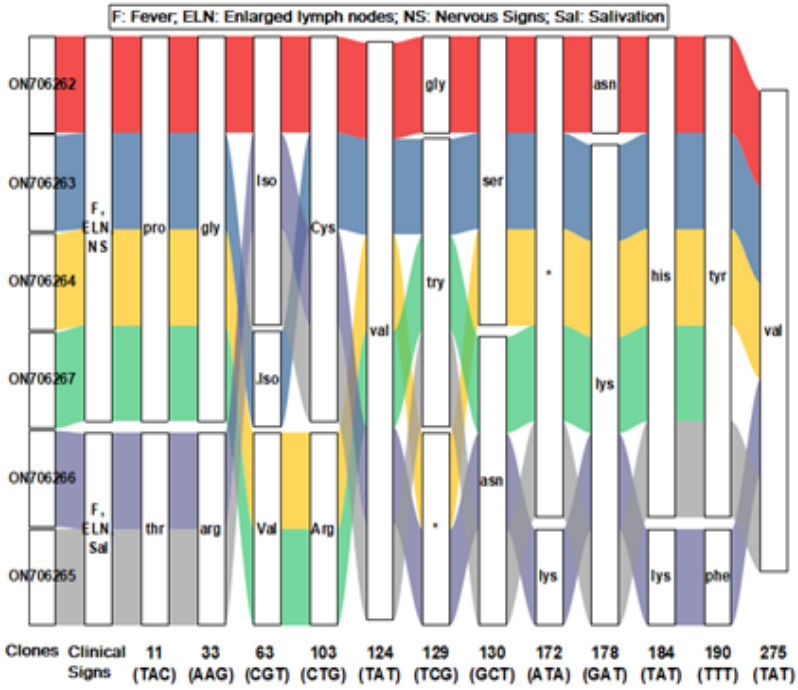

Figure 4 A categorical graph shows the relationship between study clones, clinical signs, and amino acid substitution caused by *T.annulata* cyt-b gene mutations that infect Iraqi local breed cattle

DISCUSSION

Similar results were obtained in the haematological examinations of several other investigations (Hassan, 2012; Mahmoud et al., 2019; Yang et al., 2022). In calves affected by theileriosis, however, both the parasitemia and the erythrocyte count and hematocrit were found to have substantial inverse relationships (Kachhawa et al., 2016). Leukopenia was noted in infected animals, which may have been caused by the systemic damage of piroplasm-infected RBCs by macrophages in lymph nodes, spleen, and other organs of the immune system, coupled with reduced hematopoietic activity (Hussein et al., 2007; Kachhawa et al., 2016), the toxic potential of metabolites of *Theileria* species, and continual haemorrhage due to permanent bloodsucking ticks (Narang et al., 2019).

Other researchers have done their biochemical tests and found the same things we did (Qayyum et al., 2010; Al-Emarah et al., 2012; Hassan, 2012). In animals with the condition, abnormal liver function, metabolic dysfunction, and a lack of appetite are just a few causes of low glucose serum concentration (Agina et al., 2020). *T. annulata* causes liver failure in calves that are naturally infected (Parmar et al., 2019), which may explain why these animals also have low levels of total blood protein and high levels of haemoglobin. Some other causes of hypoproteinemia are malnutrition, diarrhoea, and extra-vascular proteinaceous fluid in body cavities. Increases in the amino acid and glucose-metabolizing enzymes serum alanine aminotransferase (AST) and alanine aminotransferase (ALT) were found. Coagulative necrosis, hepatic cord distortion, and substantial lymphocyte infiltration in the periportal areas are all signs of *T. annulata* infection-related injury to the liver (Abdel-Hamied et al., 2020).

Alfatlawi et al., (2021) wrote about the results of the current study. They said that blood smears from cattle blood samples were positive. Using the partial gene sequencing analysis also made it possible to find four different isolates (Afshari et al., 2021). Gharbi et al., (2006) found a lower percentage in northern Tunisia than what was found in this study. In the same way, PCR tests showed that *Theileria* piroplasms were present in 11.4% of cattle in Sudan's North Kordofan State (Mohammed-Ahmed et al., 2018), which was a lot less than the percentage found in this study. In Sudan, Abaker *et al.,* (2017) found that 7.4% of blood smears and 39% of PCR tests were positive for *Theileria* spp. Piroplasms (Samiurahman Amiri et al., 2021). This disparity in proportions may be caused by a variety of factors, including the number of samples examined, the targeted breed, the proportion of the vector, good management, and the studied population immunity.

The mitochondrial cyt-b gene has been used a lot to study the relationships between species at the family and genus levels (Alfatlawi et al., 2021). Buparvaquone-resistant strains of *T. annulata* are likely to show up and spread, making the current crisis even worse. There's a good chance that a lot of work is going into making molecular techniques and phenotypic assessments to find resistance, figure out how common it is in a population, and model how it will spread. In the current study, the cyt-b gene in the Iraqi clones showed polymorphism at four different positions, as determined by analyzing their DNA sequences. According to the results of our amino acid sequence alignment, three of them are non-synonymous mutations that alter the cyt-b polypeptide chain.

Also, at codons 48, 68, and 108, a silent mutation was discovered. It has been postulated that a rise in the prevalence of T. annulata-caused bovine theileriosis could lead to a greater prevalence of mutations in the aforementioned areas or other functional regions of the protein, rendering the medication ineffective. Our strains may have developed resistance to the medicine due to its extensive use in the field; this is especially likely given that Iraq is an endemic region for bovine theileriosis (Hassan, 2012; Mahmoud et al., 2019; Ahmed et al., 2021; Alfatlawi et al., 2021). Chatanga et al., (2019) found numerous point mutations in the cytb gene to suggest the development of buparvaquone resistance in the parasite. Recent studies by Mhadhbi *et al.,* (2015) show that alterations in the drug-binding site, like changing out a few amino acids, might result in a drug's inability to interact properly with the cyt-b components, resulting in a decrease or loss of pharmacological activity (Razmi and Yaghfoori, 2013).

Researchers showed that the buparvaquone-resistant *T. annulata* strains have Q0 cytb gene regions of mutation (Yousef et al., 2012; Salim et al., 2019). Our results are in line with prior studies (Kessl et al., 2005; Sharifiyazdi et al., 2012). *T. annulata* strains recovered from patients whose treatments did not work were found to have single mutations in their cytochrome b gene. Sequence analysis confirmed the presence of mutations in the cyt-b gene's drug-binding Q0 region. Iranian scientists discovered the mutations (Afshari et al., 2021). The Turkish research (Hacilarlioglu et al., 2012) also discovered two further nonsynonymous changes: at position 135, valine was changed to alanine, and at position 253, proline was changed to serine. According to (Sharifiyazdi et al., 2012) mutations in the causative agent's drug-binding regions were the cause of an outbreak of bovine tropical theileriosis in Fars Province, southern Iran.

Transforming *T. annulata* into a drug-resistant strain may be responsible for the severe clinical signs that occur in the infected animals. This transformation requires exchanging the hydrophobic amino acids leucine and proline with the hydrophilic serine. This decrease in hydrophobicity may explain why buparvaquone has a lower affinity for cyt-b. The hydrophobicity of the bond between atovaquone and cyt-b has been demonstrated (Kessl et al., 2005). However, an in silico model confirms that these mutations prevent the medication from binding by altering the protein's shape and shrinking the binding pocket (Walker et al., 1998). The high rate of spontaneous mutations in the mitochondrial genome has been attributed to a variety of factors, including poor proofreading in the nucleus (Weir et al., 2011), multiple copies of the cyt-b gene in mitochondria (Sivakumar et al., 2019), and the production of hydroxyl radicals in the mitochondrial respiration chain (Raha and Robinson, 2000). Perhaps the resistance is a result of selection pressure brought about by the extensive use of buparvaquone over more than three decades.

CONCLUSIONS

The current study was able to record 6 isolates of *T. annulata* parasite in Iraqi local cattle, where their diagnosis was confirmed using cytochrome b and 18srRNA genes and DNA sequencing, and when comparing the six isolates obtained and the strains taken from Gene Bank. There were sixteen genetic mutations discovered, 13 of which were nonsynonymous and three of which were silent. According to the study, one of the isolates was genetically similar to the Indian strain, three to the Iraqi strain, and two to the Turkish strain.

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AUTHOR CONTRIBUTIONS

Hadeel Hadi Albayati, Alaa Mohammed Al Khafaji, and Amal kamel were concerned with conceptualization, Hadeel Hadi Albayati and Alaa Mohammed Al Khafaji were conducted methodology; Hadeel Hadi Albayati and Amal kamel conducted formal analysis; Alaa Mohammed Al Khafaji, Amal kamel and Hassan Al-Karagoly were responsible for investigation, data curation, and study validation; Hadeel Hadi Albayati and Hassan Al-Karagoly were involved in the visualization and original draft preparation; Hassan Al-Karagoly worked on writing review and editing; Hadeel Hadi Albayati and Hassan Al-Karagoly assumed supervisory responsibilities; Hadeel Hadi Albayati and Hassan Al-Karagoly were followed project administration. All authors gave approval to the final version of the manuscript.

CONFLICT OF INTEREST

The authors have no competing interests to declare that are relevant to the content of this article.

DATA AVAILABILITY

Data used to support the findings of this study are available from the corresponding author upon request".

ETHICS APPROVAL

The author confirmed that the current study was conducted in accordance with the relevant guidelines and regulations and with the approval of the College of Veterinary Medicine, University of Al-Qadisiyah. The blood samples were collected from the animal according to the above mentioned ethical guidelines by the first author of this research" Albayati".

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