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MOLECULAR PREVALENCE AND PHYLOGENY OF SOME TICK-BORNE PARASITES IN RUMINANTS IN SINAI PENINSULA, EGYPT

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ABSTRACT

Background: *Babesia* and *Theileria* are the etiological agents of babesiosis and theileriosis. Among them, some species are highly pathogenic and cause serious economic losses to livestock holders, especially in desert areas of Egypt. **Objective:** The present study aimed to prevalent and phylogeny characterizes three subspecies, *Babesia bovis*, *Babesia bigemina*, and *Theileria annulata*, infecting ruminants in Sinai, Egypt. **Methods:** 230 field blood samples (74 cattle, 48 goats, and 108 sheep) were collected randomly from March 2020 to April 2021. They investigated by three PCR-based assays targeting *B. bovis Rhoptry Associated Protein-4*, *B. bigemina BbigRAP-1a*, and *T. annulata merozoite surface antigen-1* genes. **Results:** The overall prevalence was 16.09% for *B. bovis* and 33.04% for *T. annulata*, whereas *B. bigemina* was absent (0%) in the three host species. The infection rates were 24.32%, 16.67%, and 10.19% (for *B. bovis*), and 40.54%, 50.0%, and 20.37% (for *T. annulata*) in cattle, goats, and sheep, respectively. Mixed infections were prevalent in 11 (4.78%) cattle, 4 (8.33%) goats, and 7(6.48%) sheep. DNAs of six generated fragments were sequenced and recorded in the GenBank database under accession numbers MZ197893, MZ197894, and MZ197895 (for *B. bovis*), and MZ197896, MZ197897, and MZ197898 (for *T. annulata*). The sequence identity values with preserved strains in GenBank ranged from 94.1 to 100% for *B. bovis* and 89.9 to 100% for *T. annulata*, dispersing themselves across several clades in two phylogenetic trees containing sequences from other countries. **Conclusions:** *Babesia* and *Theileria* strains were prevalent with diverse and low rates in ruminants in Sinai.

KEYWORDS: *Babesia bovis*, *Theileria annulata*, Phylogenetic analysis, Sequencing, Egypt.

INTRODUCTION

Babesia and *Theileria* protozoan parasites are the etiological agents of theileriosis and babesiosis, which are characterized by significant morbidity and mortality worldwide in cattle and small ruminants in the world.[1, 2] Some hosts can carry, concurrently, the species of *Babesia* and *Theileria*.[3] Over 100 recognized species of *Babesia* are described from different parts of the world, of which *B. bovis* and *B. bigemina* are the most economically important species worldwide in ruminants.[4] Only six sequenced *Theileria* sp. were identified: *Theileria annulata*, *T. electrophori*, *T. equi*, *T. microti*, *T. orientalis*, and *T. parva*.[5]

The diversity of *Babesia spp*. is dependent on the host-parasite and vector-parasite specificities, well reflected in the geographically restricted distribution of some species.[6] Although *B. bovis* and *B. bigemina* are phylogenetically related and transmitted by the same *Ixodid* ticks vectors, they cause remarkably different diseases mainly in cattle and small ruminants.[7] *B. bovis* is often a severe and highly pathogenic parasite than *B. bigemina* leads to higher mortality among susceptible animals. Both are transmitted biologically by *Boophilus* ticks, in which nymphs and adults transmit *B. bigemina* but only tick larvae transmit *B. bovis*.[8] *T. annulata* infects ruminants and causes a cancer-like illness, known as tropical theileriosis, characterized by enlarged lymph nodes, fever, swollen eyelids, profuse lachrymation, anemia, jaundice, and sometimes death.[9] The long-term survival of *T. annulata* in host animals is facilitated by the protozoan's genetic diversity, which helps the parasite escape the host's immune response.[10]

Microscopic identification of Giemsa stained blood smears could not detect the infection of *Babesia* and *Theileria* accurately in carrier animals or differentiate closely related organisms[11], in particular, when the parasitemia is very low.[12] Besides, the trophozoites of different species of *Babesia* or *Theileria spp*. appeared very similar, and their differentiation was difficult in mixed infections. Thus, the use of molecular tools remains crucial for distinguishing and identification of *Babesia* and *Theileria* spp.[13] In Egypt, several published studies of *Babesia* and *Theileria* have described ticks and animals in different localities[9,14,15], but rarely with sequencing and phylogenetic analysis. The present study investigated *B. bovis*, *B. bigemina*, and *T. annulata* infections in ruminants in Sinai (regardless of age and sex) by using three PCR-based assays and characterized their isolates by sequencing and phylogenetic relationships.

MATERIALS AND METHODS

Ethical approval: The present study followed the International Guiding Principles for Biomedical Research Involving Animals, 2012. The present study has complied with all the relevant national regulations and institutional policies for the care and use of animals under the Ministry of Agriculture and Land Reclamation, Egypt.

Study area: The Sinai Peninsula is a triangular region lies in northeast Egypt that occupies an area of 61,000 km^2 between the Mediterranean Sea to the north and the Red Sea to the south (Figure 1). It contains North and South Sinai Governorates, where many new farms included settled sheep and goats besides newly imported cattle were modern established. Two big Cites, (Ras-Sudr and El Tur) were selected for samples collection because of the increased number of new farms and animals; where small ruminants sourced from Alarish (north Sinai), Alhasna and Nukhul (middle Sinai), and ElTur, Ras Sudr, and Sharm El-Shaikh (south Sinai). Cattle are being introduced from neighbored Nile-Delta Governorates, and thousands are annually imported from Brazil mainly for slaughter and some of them for breeding. There is irregular use of Ivermectin as antiprotozoal drug against ticks and blood parasites.

Figure 1: Map of Sinai Peninsula shows the two main sites were selected for collecting field blood samples (Ras Sudr and El-Tur).

The other places in red color refer to the source of animals before settled in south Sinai: Alarish (north Sinai), Alhasna and Nukhul (middle Sinai), besides Sharm Elshaikh and suburban of Ras Sudr and El Tur.

Animals: Overall of 230 different animals (74 cattle, 48 goats, and 108 sheep) were selected randomly from private farms and animal traders in the study area from March 2020 to April 2021. They belong to 29 mixed flocks, each flock contained between 70 to 300 animals aged between 2 months to 6 years. All animals were apparently healthy except 51 animals (23 cattle, 10 sheep, and 18 goats) showed symptoms such as abortion, diarrhea, fever, bloody urine, enlargement of lymph nodes, and corneal opacity.

Sampling and field data: From each animal, five mL of blood was obtained from the jugular vein in a tube containing EDTA as an anticoagulant and immediately preserved at –20 °C until use. Also, a questionnaire was applied to include all information of animals (Age, sex, and clinical state) and the newly introduced animals into the farm and their sources, besides asking about the regular use of antiprotozoal medicaments.

DNA extraction and primers: Extraction of DNA from blood samples using the QIAamp DNA Mini kit (Qiagen, GmbH, Germany), following the manufacturer's recommendations. The three oligonucleotide-specific primers purchased from Metabion (Germany) and their sequences are in Table (1).

Table 1: Target genes, nucleotide sequences and amplicon sizes of the selected primers.

Target gene	Primers sequences	Size (bp)	Reference
B. bovis BbSBP-4	AGTTGTTGGAGGAGGCTAAT TCCTTCTCGGCGTCCTTTTC	907	Terkawi *et al*., 2011a
B. bigemina BbigRAP-1a	GAGTCTGCCAAATCCTTAC TCCTCTACAGCTGCTTCG	879	
T. annulata tams1	GTAACCTTTAAAAACGT GTTACGAACATGGGTTT	721	Nourollahi-Fard *et al*., 2015

PCR amplification: The infections with *B. bovis*, *B. bigemina*, and *T. annulata* were screened according to.[11, 16] Every volume of 25µl reaction contained 12.5µl of Emerald Amp Max PCR Master Mix (Code No. RR310Akit, Takara, Japan), 1µl of each specific primer, 4.5µl water, and 6µl extracted DNA. The thermocycling conditions for PCR amplifications were as follows: one cycle of peri-denaturation in 5min at 94^0C followed by 35 cycles ((30 s of denaturation at 94^0C, 40 s of annealing at 55^0C, and extension at 72^0C (1 min., 50 s, and 45 s)) for *B. bovis*, *B. bigemina*, and *T. annulata*, respectively. After that, a final extension at 72^0C lasted 10 min for the three parasites. The PCR amplifications were carried out in an Applied BioSystem 2720 thermal cycler. Electrophoresis separated the products of PCR on 1.5% agarose gel (AppliChem, GmbH, Germany) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20µl of each PCR product loaded in each gel slot, and a Gene-ruler 100 bp ladder (Fermentas, Germany), was used to determine the fragment sizes. Then, gels were photographed and analyzed by a gel documentation system (Alpha Innotech, Biometra).

Molecular analysis: Six specific and sharply positive products, three for *B. bovis* and three for *T. annulata* were selected as one fragment from each host at amplicon sizes of 907 bp and 721bp, respectively. DNA sequencing targeted *B. bovis BbSBP-4* and *T. annulata tams1* genes and phylogenetic analysis. QI-quick PCR product extraction kit (Qiagen Inc. Valencia, CA); was used to purify the PCR product directly which, was sequenced in forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). For sequencing, a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA) (Cat. No.4336817); was used. The BLAST® analysis (Basic Local Alignment Search Tool); was used to establish the sequence identity to GenBank accessions at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).[17] The comparative analysis of sequences using the CLUSTALW multiple sequence alignment programs, version 1.83 of the MegAlign module of Lasergene DNAStar software Pairwise, was performed. The neighbor-joining method (NJ) was used in the MEGA 6 program for phylogenetic reconstruction.[18]

RESULTS

The results of the three PCR assays of DNA blood samples showed that out of 230 examined animals, the prevalence was 16.09% for *B. bovis*, 0.0% for *B. bigemina*, and 33.04% for *T. annulata* infection in ruminants in Sinai. *B. bovis* was detected in 18/74 (24.32%) cattle, 8/48(16.67%) goats, and 11/108 (10.19%) sheep. *T. annulata* was detected in 30/74(40.54%) cattle, 24/ 48 (50.00%) goats, and 22/108 (20.37%) sheep. *B. bigemina* did not amplify (0%) in the examined blood samples from the three host species. Goats have the maximum rate of infection with *T. annulata* in contrast to *B. bovis* that prevalent in cattle than others. The mixed infection with the two pathogens was lower and detected only in 9.57% (22/230) of examined animals with nearly results.

The results of the molecular characterization confirmed the presence of each *B. bovis* and *T. annulata* in blood samples of ruminants for the first time in Sinai (Figs 2, 3). The amplified fragments at amplicon sizes of 907 bp and 721 bp were purified and then ligated into a pGEM-T vector for subsequent sequencing. The GenBank database of sequence analysis and the accession numbers for *B. bovis* and *T. annulata* are in Table (3).

Table 2: Molecular detection of the three infections in different host-species.

Parasite	Cattle (n=74)	Goats (n=48)	Sheep (n=108)	Total (230)
Babesia bovis	18(24.32%)	8(16.67%)	11(10.19%)	37(16.09%)
Babesia bigemina	(0.00%)	(0.00%)	0(0.00%)	0(0.00%)
Theileria annulata	30(40.54%)	24(50.00%)	22(20.37%)	76(33.04%)
Mixed infections	11(4.78%)	4(8.33%)	7(6.48%)	22(9.57%)

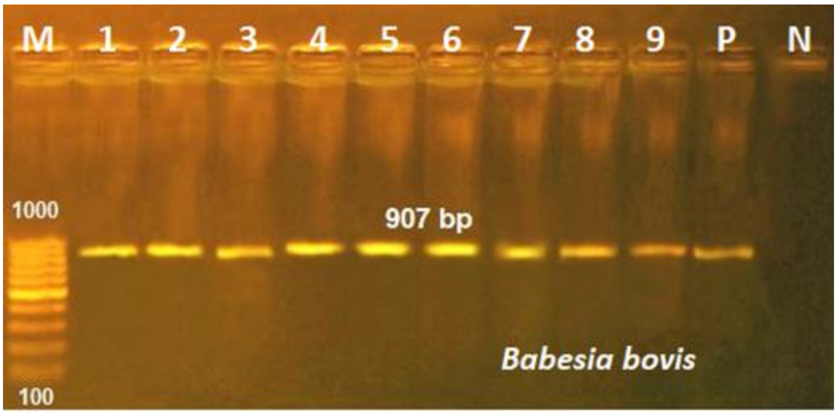


Figure 2: 1.5% Agarose-gel electrophoresis of amplification products obtained from genomic DNA of *B. bovis* of ruminants using *B. bovis BbSBP-4* specific primer (gave 907 bp PCR products).

Lanes: M: molecular weight standards (100bp), Lanes N and P: negative and positive controls; Lane 1-4, 5-7, and 8, 9: B. bovis correspond to cattle, goats, and sheep, respectively.

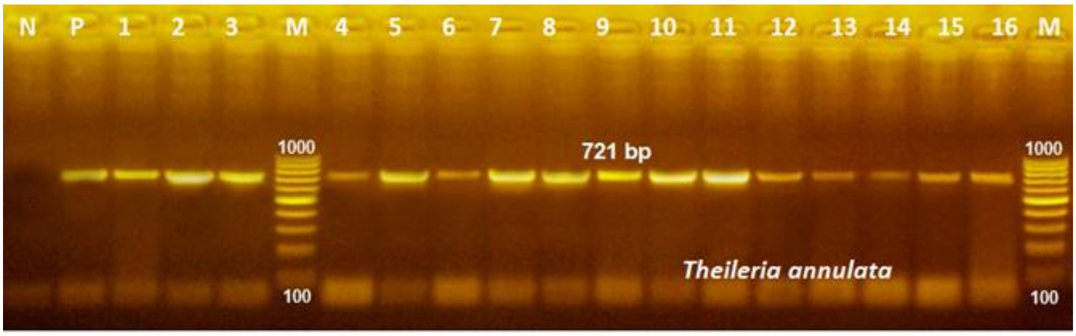


Figure 3:PCR amplification products of 721 bp generated from *T. annulata* DNA in ruminants using *T. annulata tams 1* a specific primer.

Lanes: M: molecular weight standards (100 bp), Lanes N and P: negative and positive controls; Lanes 1-7, 8-10, 11-16: T. annulata amplified from sheep, goats, and cattle, respectively.

Table 3: GenBank database included identity and accession numbers of DNA isolates from different hosts in Sinai.

	Parasite	Host	Place	Origin	Date	Accession no.
1	*B. bovis*	Cattle	El-Tur	South-Sinai	11-2020	MZ197893 *B. bovis Bb1-DRC-Eg*
2	*B. bovis*	Goat	El-Tur	Alarish, North-Sinai	11-2020	MZ197894 *B. bovis Bb2-DRC-Eg*
3	*B. bovis*	Sheep	Ras Sudr	Nukhul, Middle-Sinai	3-2020	MZ197895 *B. bovis Bb3-DRC-Eg*
4	*T. annulata*	Cattle	El-Tur	South-Sinai	11-2020	MZ197896 *T. annulata3-DRC-Eg*
5	*T. annulata*	Goat	El-Tur	Sharm El-Sheikh	11-2020	MZ197897 *T. annulata4-DRC-Eg*
6	*T. annulata*	Sheep	Ras Sudr	Ras Sudr- Sinai	3-2020	MZ198898 *T. annulata7-DRC-Eg*

The present study produced six new nucleotide sequences data of the six isolates are available in the GenBank™ databases under the accession numbers: [MZ197893], [MZ197894], [MZ197895] (for *B. bovis*), and [MZ197896], [MZ197897], [MZ197898] (for *T. annulata*). The sequenced strains of *B. bovis* have distributed in the same cluster but not the same clade (Fig. 4). The two accession numbers (MZ197893 and MZ197894) isolated from cattle and goat shared identity of 100% in one clade, whereas the third strain isolated from sheep (MZ197895), clustered in a different sub-clade with an accession number of AB571871.

In the same manner, the two sequenced *T. annulata* isolated from cattle and sheep in the present study recorded under accession numbers (MZ197896 and MZ197898) reflected to be diverse in the same cluster of *T. annulata* and the same clade (Fig. 5). But the constructed phylogenetic tree revealed that one sequence (MZ197897) isolated from goats clustered in a different subclade. These results showed that MZ197895 *B. bovis* and MZ197897 *T. annulata* are denoting to vary genetically from other isolates collected from Sinai. It has pointed to the possible presence of novel strains of *B. bovis* and *T. annulata* in Sinai.

The present results also showed high similarity among the three Egyptian *B. bovis* strains (99.2%-100%) and relatively identity with other *B. bovis* (93.4%-100%) distributed worldwide. The three sequences of *T. annulata* based on *the T. annulata tams1* gene showed homologies in the range of 94–100% among each other and from 89.9% to 100% with various isolates abroad. The divergence table of *B. bovis BbSBP-4* gene (Fig. 6) showed a divergence from 0 to 0.9 between submitted isolates and 0 to 4.4 with other *B. bovis* preserved in the GenBank database. Also, a close association from 0 to 6.2 between submitted Egyptian isolates of *T. annulata* and from 0 to 9 with other reference samples preserved in GenBank (Fig. 7).

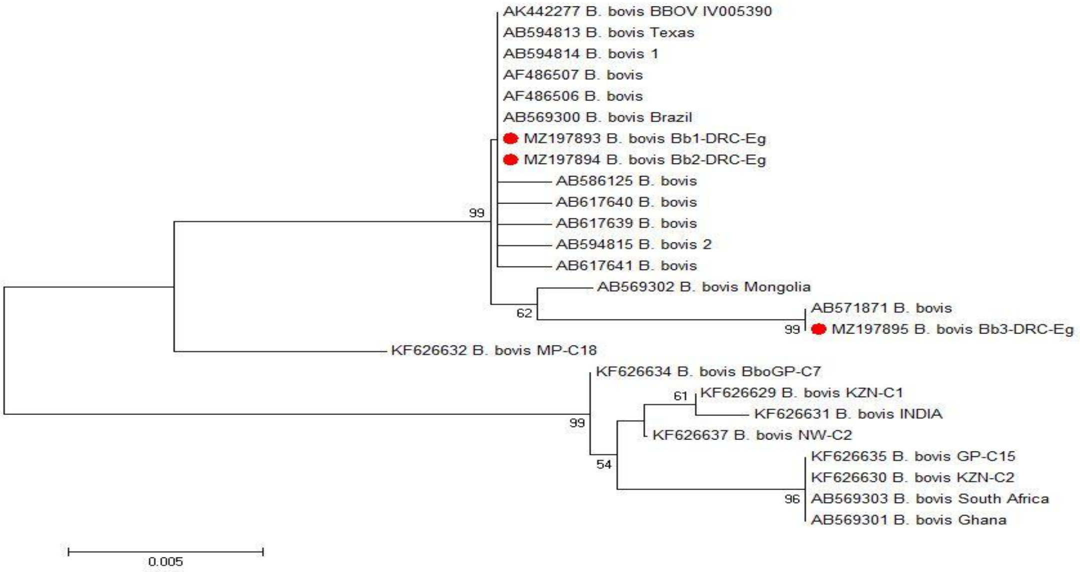


Figure 4: A phylogenetic tree was constructed in MEGA 6.0 using the neighbor-joining method for analysis of the *B. bovis Rhoptry Associated Protein-4* gene. *The sequence obtained in this study is indicated with a red circle.*

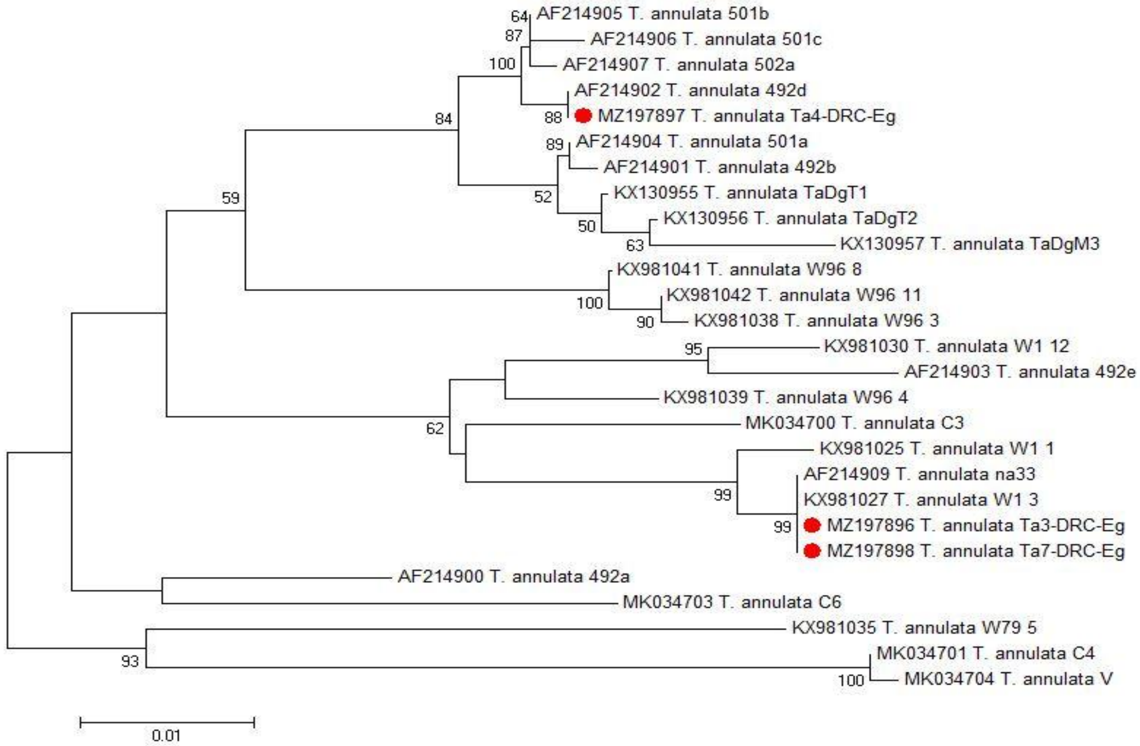
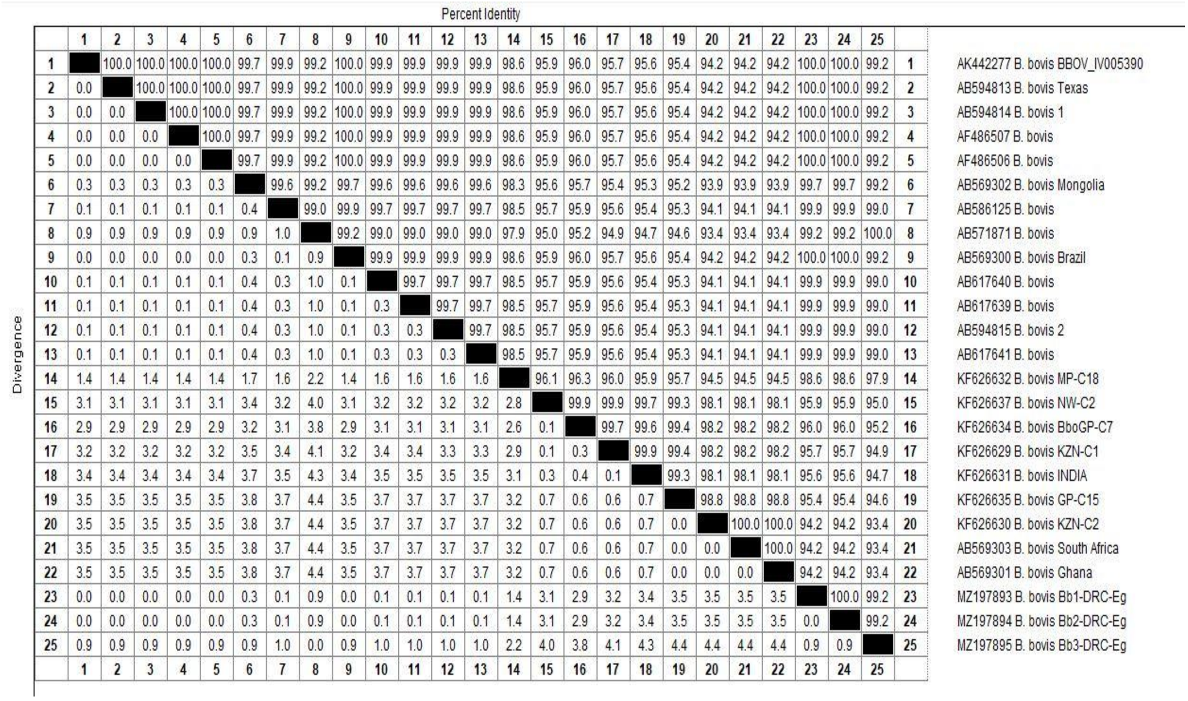
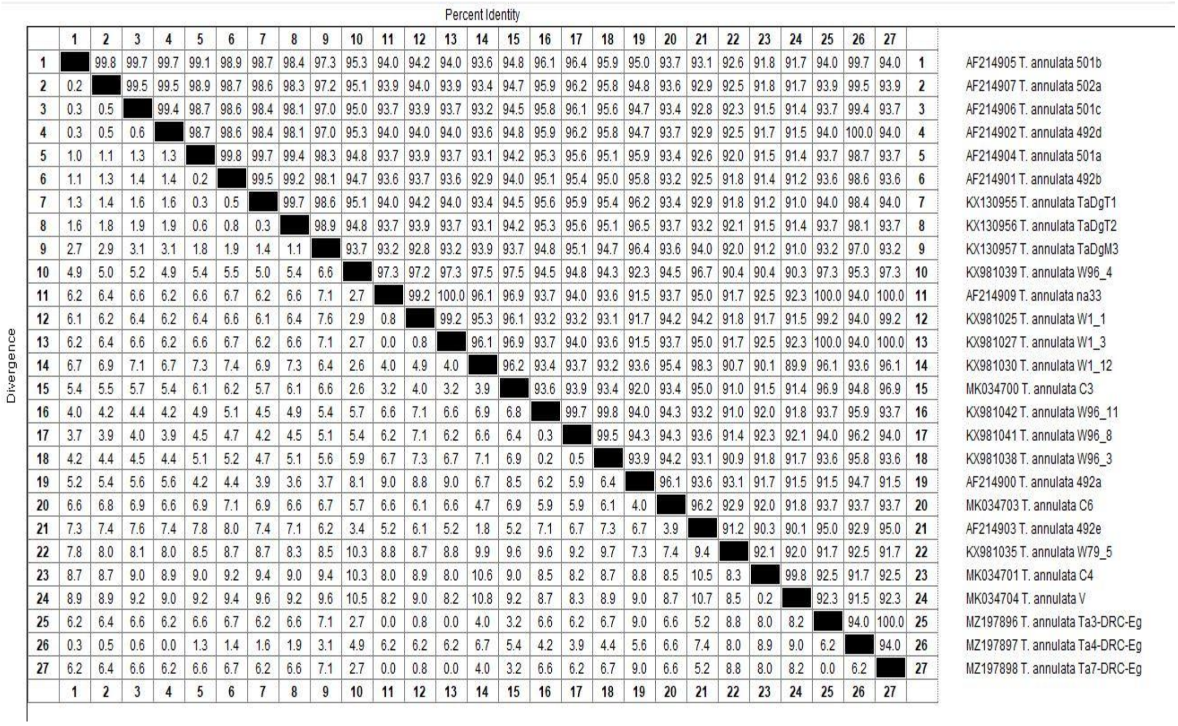


Figure 5: A phylogenetic tree was constructed in MEGA 6.0 using the neighbor-joining method for analysis of the *T. annulata merozoite surface antigen-1* gene. *The sequence obtained in this study is indicated with a red circle.*



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		
1	■	100.0	100.0	100.0	100.0	99.7	99.9	99.2	100.0	99.9	99.9	99.9	99.9	98.6	95.9	96.0	95.7	95.6	95.4	94.2	94.2	94.2	100.0	100.0	99.2	1	AK442277 B. bovis BBOV_IV005390
2	0.0	■	100.0	100.0	100.0	99.7	99.9	99.2	100.0	99.9	99.9	99.9	99.9	98.6	95.9	96.0	95.7	95.6	95.4	94.2	94.2	94.2	100.0	100.0	99.2	2	AB594813 B. bovis Texas
3	0.0	0.0	■	100.0	100.0	99.7	99.9	99.2	100.0	99.9	99.9	99.9	99.9	98.6	95.9	96.0	95.7	95.6	95.4	94.2	94.2	94.2	100.0	100.0	99.2	3	AB594814 B. bovis 1
4	0.0	0.0	0.0	■	100.0	99.7	99.9	99.2	100.0	99.9	99.9	99.9	99.9	98.6	95.9	96.0	95.7	95.6	95.4	94.2	94.2	94.2	100.0	100.0	99.2	4	AF486507 B. bovis
5	0.0	0.0	0.0	0.0	■	99.7	99.9	99.2	100.0	99.9	99.9	99.9	99.9	98.6	95.9	96.0	95.7	95.6	95.4	94.2	94.2	94.2	100.0	100.0	99.2	5	AF486506 B. bovis
6	0.3	0.3	0.3	0.3	0.3	■	99.6	99.2	99.7	99.6	99.6	99.6	99.6	98.3	95.6	95.7	95.4	95.3	95.2	93.9	93.9	93.9	99.7	99.7	99.2	6	AB569302 B. bovis Mongolia
7	0.1	0.1	0.1	0.1	0.1	0.4	■	99.0	99.9	99.7	99.7	99.7	99.7	98.5	95.7	95.9	95.6	95.4	95.3	94.1	94.1	94.1	99.9	99.9	99.0	7	AB586125 B. bovis
8	0.9	0.9	0.9	0.9	0.9	0.9	1.0	■	99.2	99.0	99.0	99.0	99.0	97.9	95.0	95.2	94.9	94.7	94.6	93.4	93.4	93.4	99.2	99.2	100.0	8	AB571871 B. bovis
9	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.9	■	99.9	99.9	99.9	99.9	98.6	95.9	96.0	95.7	95.6	95.4	94.2	94.2	94.2	100.0	100.0	99.2	9	AB569300 B. bovis Brazil
10	0.1	0.1	0.1	0.1	0.1	0.4	0.3	1.0	0.1	■	99.7	99.7	99.7	98.5	95.7	95.9	95.6	95.4	95.3	94.1	94.1	94.1	99.9	99.9	99.0	10	AB617640 B. bovis
11	0.1	0.1	0.1	0.1	0.1	0.4	0.3	1.0	0.1	0.3	■	99.7	99.7	98.5	95.7	95.9	95.6	95.4	95.3	94.1	94.1	94.1	99.9	99.9	99.0	11	AB617639 B. bovis
12	0.1	0.1	0.1	0.1	0.1	0.4	0.3	1.0	0.1	0.3	0.3	■	99.7	98.5	95.7	95.9	95.6	95.4	95.3	94.1	94.1	94.1	99.9	99.9	99.0	12	AB594815 B. bovis 2
13	0.1	0.1	0.1	0.1	0.1	0.4	0.3	1.0	0.1	0.3	0.3	0.3	■	98.5	95.7	95.9	95.6	95.4	95.3	94.1	94.1	94.1	99.9	99.9	99.0	13	AB617641 B. bovis
14	1.4	1.4	1.4	1.4	1.4	1.7	1.6	2.2	1.4	1.6	1.6	1.6	1.6	■	96.1	96.3	96.0	95.9	95.7	94.5	94.5	94.5	98.6	98.6	97.9	14	KF626632 B. bovis MP-C18
15	3.1	3.1	3.1	3.1	3.1	3.4	3.2	4.0	3.1	3.2	3.2	3.2	3.2	2.8	■	99.9	99.9	99.7	99.3	98.1	98.1	98.1	95.9	95.9	95.0	15	KF626637 B. bovis NW-C2
16	2.9	2.9	2.9	2.9	2.9	3.2	3.1	3.8	2.9	3.1	3.1	3.1	3.1	2.6	0.1	■	99.7	99.6	99.4	98.2	98.2	98.2	96.0	96.0	95.2	16	KF626634 B. bovis BboGP-C7
17	3.2	3.2	3.2	3.2	3.2	3.5	3.4	4.1	3.2	3.4	3.4	3.3	3.3	2.9	0.1	0.3	■	99.9	99.4	98.2	98.2	98.2	95.7	95.7	94.9	17	KF626629 B. bovis KZN-C1
18	3.4	3.4	3.4	3.4	3.4	3.7	3.5	4.3	3.4	3.5	3.5	3.5	3.5	3.1	0.3	0.4	0.1	■	99.3	98.1	98.1	98.1	95.6	95.6	94.7	18	KF626631 B. bovis INDIA
19	3.5	3.5	3.5	3.5	3.5	3.8	3.7	4.4	3.5	3.7	3.7	3.7	3.7	3.2	0.7	0.6	0.6	0.7	■	98.8	98.8	98.8	95.4	95.4	94.6	19	KF626635 B. bovis GP-C15
20	3.5	3.5	3.5	3.5	3.5	3.8	3.7	4.4	3.5	3.7	3.7	3.7	3.7	3.2	0.7	0.6	0.6	0.7	0.0	■	100.0	100.0	94.2	94.2	93.4	20	KF626630 B. bovis KZN-C2
21	3.5	3.5	3.5	3.5	3.5	3.8	3.7	4.4	3.5	3.7	3.7	3.7	3.7	3.2	0.7	0.6	0.6	0.7	0.0	0.0	■	100.0	94.2	94.2	93.4	21	AB569303 B. bovis South Africa
22	3.5	3.5	3.5	3.5	3.5	3.8	3.7	4.4	3.5	3.7	3.7	3.7	3.7	3.2	0.7	0.6	0.6	0.7	0.0	0.0	0.0	■	94.2	94.2	93.4	22	AB569301 B. bovis Ghana
23	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.9	0.0	0.1	0.1	0.1	0.1	1.4	3.1	2.9	3.2	3.4	3.5	3.5	3.5	3.5	■	100.0	99.2	23	MZ197893 B. bovis Bb1-DRC-Eg
24	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.9	0.0	0.1	0.1	0.1	0.1	1.4	3.1	2.9	3.2	3.4	3.5	3.5	3.5	3.5	0.0	■	99.2	24	MZ197894 B. bovis Bb2-DRC-Eg
25	0.9	0.9	0.9	0.9	0.9	0.9	1.0	0.0	0.9	1.0	1.0	1.0	1.0	2.2	4.0	3.8	4.1	4.3	4.4	4.4	4.4	4.4	0.9	0.9	■	25	MZ197895 B. bovis Bb3-DRC-Eg
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		

Figure 6: The percent of identity for the three studied Egyptian isolates of *B. bovis* compared to other isolates worldwide based on *B. bovis BbSBP-4* gene. *The accession number of submitted isolates is followed by the respective place of origin (DRC-Eg).*



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27		
1	■	99.8	99.7	99.7	99.1	98.9	98.7	98.4	97.3	95.3	94.0	94.2	94.0	93.6	94.8	96.1	96.4	95.9	95.0	93.7	93.1	92.6	91.8	91.7	94.0	99.7	94.0	1	AF214905 T. annulata 501b
2	0.2	■	99.5	99.5	98.9	98.7	98.6	98.3	97.2	95.1	93.9	94.0	93.9	93.4	94.7	95.9	96.2	95.8	94.8	93.6	92.9	92.5	91.8	91.7	93.9	99.5	93.9	2	AF214907 T. annulata 502a
3	0.3	0.5	■	99.4	98.7	98.6	98.4	98.1	97.0	95.0	93.7	93.9	93.7	93.2	94.5	95.8	96.1	95.6	94.7	93.4	92.8	92.3	91.5	91.4	93.7	99.4	93.7	3	AF214906 T. annulata 501c
4	0.3	0.5	0.6	■	98.7	98.6	98.4	98.1	97.0	95.3	94.0	94.0	94.0	93.6	94.8	95.9	96.2	95.8	94.7	93.7	92.9	92.5	91.7	91.5	94.0	100.0	94.0	4	AF214902 T. annulata 492d
5	1.0	1.1	1.3	1.3	■	99.8	99.7	99.4	98.3	94.8	93.7	93.9	93.7	93.1	94.2	95.3	95.6	95.1	95.9	93.4	92.6	92.0	91.5	91.4	93.7	98.7	93.7	5	AF214904 T. annulata 501a
6	1.1	1.3	1.4	1.4	0.2	■	99.5	99.2	98.1	94.7	93.6	93.7	93.6	92.9	94.0	95.1	95.4	95.0	95.8	93.2	92.5	91.8	91.4	91.2	93.6	98.6	93.6	6	AF214901 T. annulata 492b
7	1.3	1.4	1.6	1.6	0.3	0.5	■	99.7	98.6	95.1	94.0	94.2	94.0	93.4	94.5	95.6	95.9	95.4	96.2	93.4	92.9	91.8	91.2	91.0	94.0	98.4	94.0	7	KX130955 T. annulata TaDgT1
8	1.6	1.8	1.9	1.9	0.6	0.8	0.3	■	98.9	94.8	93.7	93.9	93.7	93.1	94.2	95.3	95.6	95.1	96.5	93.7	93.2	92.1	91.5	91.4	93.7	98.1	93.7	8	KX130956 T. annulata TaDgT2
9	2.7	2.9	3.1	3.1	1.8	1.9	1.4	1.1	■	93.7	93.2	92.8	93.2	93.9	93.7	94.8	95.1	94.7	96.4	93.6	94.0	92.0	91.2	91.0	93.2	97.0	93.2	9	KX130957 T. annulata TaDgM3
10	4.9	5.0	5.2	4.9	5.4	5.5	5.0	5.4	6.6	■	97.3	97.2	97.3	97.5	97.5	94.5	94.8	94.3	92.3	94.5	96.7	90.4	90.4	90.3	97.3	95.3	97.3	10	KX981039 T. annulata W96_4
11	6.2	6.4	6.6	6.2	6.6	6.7	6.2	6.6	7.1	2.7	■	99.2	100.0	96.1	96.9	93.7	94.0	93.6	91.5	93.7	95.0	91.7	92.5	92.3	100.0	94.0	100.0	11	AF214909 T. annulata na33
12	6.1	6.2	6.4	6.2	6.4	6.6	6.1	6.4	7.6	2.9	0.8	■	99.2	95.3	96.1	93.2	93.2	93.1	91.7	94.2	94.2	91.8	91.7	91.5	99.2	94.0	99.2	12	KX981025 T. annulata W1_1
13	6.2	6.4	6.6	6.2	6.6	6.7	6.2	6.6	7.1	2.7	0.0	0.8	■	96.1	96.9	93.7	94.0	93.6	91.5	93.7	95.0	91.7	92.5	92.3	100.0	94.0	100.0	13	KX981027 T. annulata W1_3
14	6.7	6.9	7.1	6.7	7.3	7.4	6.9	7.3	6.4	2.6	4.0	4.9	4.0	■	96.2	93.4	93.7	93.2	93.6	95.4	98.3	90.7	90.1	89.9	96.1	93.6	96.1	14	KX981030 T. annulata W1_12
15	5.4	5.5	5.7	5.4	6.1	6.2	5.7	6.1	6.6	2.6	3.2	4.0	3.2	3.9	■	93.6	93.9	93.4	92.0	93.4	95.0	91.0	91.5	91.4	96.9	94.8	96.9	15	MK034700 T. annulata C3
16	4.0	4.2	4.4	4.2	4.9	5.1	4.5	4.9	5.4	5.7	6.6	7.1	6.6	6.9	6.8	■	99.7	99.8	94.0	94.3	93.2	91.0	92.0	91.8	93.7	95.9	93.7	16	KX981042 T. annulata W96_11
17	3.7	3.9	4.0	3.9	4.5	4.7	4.2	4.5	5.1	5.4	6.2	7.1	6.2	6.6	6.4	0.3	■	99.5	94.3	94.3	93.6	91.4	92.3	92.1	94.0	96.2	94.0	17	KX981041 T. annulata W96_8
18	4.2	4.4	4.5	4.4	5.1	5.2	4.7	5.1	5.6	5.9	6.7	7.3	6.7	7.1	6.9	0.2	0.5	■	93.9	94.2	93.1	90.9	91.8	91.7	93.6	95.8	93.6	18	KX981038 T. annulata W96_3
19	5.2	5.4	5.6	5.6	4.2	4.4	3.9	3.6	3.7	8.1	9.0	8.8	9.0	6.7	8.5	6.2	5.9	6.4	■	96.1	93.6	93.1	91.7	91.5	91.5	94.7	91.5	19	AF214900 T. annulata 492a
20	6.6	6.8	6.9	6.6	6.9	7.1	6.9	6.6	6.7	5.7	6.6	6.1	6.6	4.7	6.9	5.9	5.9	6.1	4.0	■	96.2	92.9	92.0	91.8	93.7	93.7	93.7	20	MK034703 T. annulata C6
21	7.3	7.4	7.6	7.4	7.8	8.0	7.4	7.1	6.2	3.4	5.2	6.1	5.2	1.8	5.2	7.1	6.7	7.3	6.7	3.9	■	91.2	90.3	90.1	95.0	92.9	95.0	21	AF214903 T. annulata 492e
22	7.8	8.0	8.1	8.0	8.5	8.7	8.7	8.3	8.5	10.3	8.8	8.7	8.8	9.9	9.6	9.6	9.2	9.7	7.3	7.4	9.4	■	92.1	92.0	91.7	92.5	91.7	22	KX981035 T. annulata W79_5
23	8.7	8.7	9.0	8.9	9.0	9.2	9.4	9.0	9.4	10.3	8.0	8.9	8.0	10.6	9.0	8.5	8.2	8.7	8.8	8.5	10.5	8.3	■	99.8	92.5	91.7	92.5	23	MK034701 T. annulata C4
24	8.9	8.9	9.2	9.0	9.2	9.4	9.6	9.2	9.6	10.5	8.2	9.0	8.2	10.8	9.2	8.7	8.3	8.9	9.0	8.7	10.7	8.5	0.2	■	92.3	91.5	92.3	24	MK034704 T. annulata V
25	6.2	6.4	6.6	6.2	6.6	6.7	6.2	6.6	7.1	2.7	0.0	0.8	0.0	4.0	3.2	6.6	6.2	6.7	9.0	6.6	5.2	8.8	8.0	8.2	■	94.0	100.0	25	MZ197896 T. annulata Ta3-DRC-Eg
26	0.3	0.5	0.6	0.0	1.3	1.4	1.6	1.9	3.1	4.9	6.2	6.2	6.2	6.7	5.4	4.2	3.9	4.4	5.6	6.6	7.4	8.0	8.9	9.0	6.2	■	94.0	26	MZ197897 T. annulata Ta4-DRC-Eg
27	6.2	6.4	6.6	6.2	6.6	6.7	6.2	6.6	7.1	2.7	0.0	0.8	0.0	4.0	3.2	6.6	6.2	6.7	9.0	6.6	5.2	8.8	8.0	8.2	0.0	6.2	■	27	MZ197898 T. annulata Ta7-DRC-Eg
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27		

Figure 7: The percent of identity for the three studied Egyptian isolates of *T. annulata* in comparison with other isolates worldwide based on the *T. annulata tams 1* gene. *The accession numbers of our submitted isolates are followed by their origin (DRC-Eg).*

DISCUSSION

Livestock, particularly cattle, buffaloes, small ruminants, and camels, and their production from milk and meat are economic resources income through a broad belt of tropical and subtropical areas.[19] Most nomads in desert provinces in Egypt are negatively affected by hemoprotozoan parasitic diseases.[13, 14] In Sinai, scarcely studies were conducted only on the geographical distribution of ticks infesting sheep, goats, cattle, and camels in the north and middle Sinai for *B. ovis* and *T. ovis*.[20-23] and a recent study was conducted on 11 animals in south Sinai for *T. annulata*.[28] They have reported 12 tick-species of which *Hyalomma dromedarii* and *H. anatolicum* were the most prevalent hard ticks, and the adult of *Boophilus annulatus* was the highest density among species on cattle also parasitizes sheep and goats.[24]

In the present study, cattle were newly introduced and largely kept without close association with other carrier animals, such as sheep and goats. *Ixodid* ticks transmit *Babesia* and *Theileria* and their distribution is very similar. For this, the transmission of blood parasites could occur by hard ticks in large numbers, especially in spring, and should infect over one ruminant species as reported by.[25] Also, molecular detection based on the present PCR techniques provides reliable results with high sensitivity and specificity in agreement with.[13] It was used successfully in the differential diagnosis between the *B. bovis* and the *B. bigemina* infections shown by the appearance of specific diagnostic bands.[26]

Regarding *Babesia* genomic DNA isolated from the blood of examined ruminants by utilizing two PCRs targeting sizes of 907bp for *B. bovis* and 879bp for *B. bigemina*. Of examined animals, 16.09% were positive for *B. bovis* with a high rate of infection in cattle (24.32%) compared to goats (16.67%) and sheep (10.19%), whereas *B. bigemina* was absent. The findings were nearly similar to those reported in Egypt by different techniques:[27] found *Babesia sp*. in 25.33 % of cattle and an infection rate of 11.16% with *B. bovis*. In contrast, *B. bigemina* was the most prevalent parasite (7.97%) reported.[1] In northern west Egypt, the infection with *Babesia sp.* in camels was 18.43%, out of them (59.1%) were *B. bovis* and (40.9%) were *B. bigemina.*[13] Also, it was present in 2.81% of camels in southern east Egypt.[28] In El-Qalubia governorate, 22.47% of buffaloes and 51.28% of cattle have *Babesia* infection. In the middle Delta of Egypt, a nearly similar result of *Babesia* (12.6%)[19], contrary to a lower prevalence (5.30% and 3.97%), documented in the west Delta and Upper Egypt.[29][30] recorded a 10% infection rate for *Babesia* using PCR, and[31] revealed an infection rate of 11.11% for bovine babesiosis. In other tropical and subtropical areas, nPCR showed a prevalence of 26.7% in Brazi[32], 78.5% in Portugal[33], 29% in Pakistan[34], and 18.8% in the Philippines.[35]

Concerning *T. annulata*, the PCR produces positive results with lengths of 721bp in animals harboring infection using the specific primer in agreement with[16] in Iran and[36] in Tunisia, and[37] in Egypt, who used the same primer. In the present study, 33.04% of examined animals harbored theileriosis using PCR; 40.54% in cattle, 50.00% in goats, and 20.37% in sheep. This result was higher than that cited in Egypt; 30.98%[38], and 9.56%[39] recorded an infection rate in cattle in Menoufia, Behera, Giza, and Sohage governorates, Egypt. Also, it was higher than those previously recorded in different provinces in Egypt (16.05%)[19] In El-Qalubia governorate, *Theileria* infected 14.61% of cattle and 7.69% of buffaloes.[40] Also, it detected in 10.25% of examined cattle in different governorates using PCR[1] and 27.13% in El-Wadi El-Gadid province, Egypt.[41] The present results are lower than that recorded in several governorates in Egypt: 87.5% of sheep in El-Gharbia governorate[42], and 86.1% of goats showing clinical symptoms of theileriosis in Giza governorate[43], 43.1% of apparently healthy Port Said governorate[44], and 65.6% of bovine theileriosis in Upper Egypt.[45] *Theileria* sp. was identified with percentages of 23.4% in Tanzania[46], 37.8% in eastern Turkey[47], and 17.3% in Tunisia[31] using PCR. The fluctuation in the prevalence rates maybe because of the differences in localities, susceptibility of the animal species, changes in detection method, and environmental humidity and temperature that influence the migration of vectors into new areas of reclamation in Sinai.

The population structures of many protozoan parasites are predominantly clonal.[48-50] Surveys of some vector-borne parasite species such as *T. annulata* and *B. bovis* have shown high levels of genetic diversity.[51] *Babesia* sp. and *Theileria* sp. are widespread in Southern Europe, North Africa, and Southern Asia, thus presenting a significant threat to livestock productivity.[52]

In the present study, the molecular characterization studies primarily involve selective targets of *B. bovis BbSBP-4* and *T. annulata tams1* genes that are highly specific for *B. bovis* and *T. annulata*, with no cross-reactivity with other spp. Among positive amplification, it conducted six sharp positive fragments on sequencing and phylogenetic analysis. Sequence comparisons of two amplified fragments, their GenBank accession no. MZ197893 Bb1-DRC-Eg for cattle and MZ197894- Bb2-DRC-Eg for goat revealed high conservation of Egyptian isolates identical with six DNA fragments encoding *BbSBP-4* from north and south-America and east and south of Asia. Their GenBank accession numbers are; AB594813 and AK442277 *B. bovis* from Texas, AF486506 *B. bovis* from north-America, AB569300 *B. bovis*-Brazil, and AB594814 *B. bovis*, AF486507 *B. bovis* from Thailand. The third sample with GenBank accession no. MZ197895 Bb3-DRC-Eg for sheep was identical with GenBank accession no, AB571871 *B. bovis* from Thailand.[11, 53-55] The divergence between local and reference samples ranged from 0 to 4.4. In comparison, in a very recent study[28] sequencing and phylogenetic analyses revealed one strain of *B. bovis* under accession number MF737083 in GenBank isolated from a camel in Egypt was closer to some strains isolated from Brazil. It referred to a possible transmission to local ruminants through thousands of Brazilian cattle imported annually to Egypt.

The *polypeptide Tams1* is an immunodominant major merozoite piroplasm surface antigen of *T. annulata.* Its uses are for epidemiological studies and phylogenetic analyses.[2] Several studies found that the *Tams1* gene is highly polymorphic; raising questions concerning the suitability of *Tams1* gene-targeted primers to detect all *T. annulata* isolates.[56] The authors advised to use two different probes, not one, for more discrimination of infection.[2, 57,58] On the contrary, a PCR assay using one probe targeting the *tams-1* gene could be reliable in the present study. However, results of the phylogenetic analysis showed that the *Tams1* sequences distributed into four clusters contain many sub-clades where isolates distributed in several groups and may exhibit geographic specificity. *Tams1* gene sequences got from three continents revealed the effect of geographic specificity when comparing *Tams1* gene sequences with particular sequences types that may belong to definitive regions.[2]

With regard to sequence data in the present study, we found that local *T. annulata* strains of cattle, goats, and sheep were closely identical to some Tunisian *T. annulata* strains. However, two accession numbers of MZ197896 *Th. ann* Ta3-DRC-Eg/ cattle and MZ197898 Ta7 DRC-Eg/ sheep were analogous with the two accession numbers AF214909 na33 (Tunisia) and KX981027 W1-3 (Uk).[4, 59] The third sequence of MZ197897 Ta4-DRC-Eg/ goat is identical to AF214902 Ta 492d (Tunisia)[59] with a sequence identity of 100%. In addition, the divergence between local and reference samples ranged from 0 to 0.9. Sequence comparisons of the three amplified *T. annulata tams1* with 24 accession numbers in GenBank shared to 89.9 to 100% sequence identity. Comparable recent studies recorded that phylogenetic analysis grouped the Egyptian (MN251047) *T. annulata* _cattle_ 2019 and (MN251046)_*T. annulata* _buffalo_ 2019 in a separate clade and shared from 93% to 100% genetic similarity with other *T. annulata* strains by[37] and from 90.68 to 97.75% by.[41] Nucleotide differences recorded in the present study were; 11 for MZ197897 Ta4-DRC-Eg, and 28 for each of MZ197896 Ta3-DRC-Eg and MZ197898 Ta7 DRC-Eg.

CONCLUSION

The present study has lighted on some tick-borne parasites (*B. bovis*, *B. bigemina*, and *T. annulata*) in ruminants in Sinai, Egypt using three specific PCR assays in blood–DNA samples sourced from reared cattle, goats, and sheep. It showed that *T. annulata* was more prevalent than *B. bovis*, whereas none of the infections with *B. bigemina*. Cattle were the most infected animals with *B. bovis*, whereas goats have the maximum infection rate with *T. annulata*. The mixed infections with the two parasites were less in examined animals. These findings are consistent with a low prevalence rate despite animals sourced from different regions in Sinai. The management and animal species are likely to be risk factors for variation in prevalence rates. Sequence analysis of selected fragments from each host species and parasites was BLASTn based on the data published in the GenBank on *Babesia spp.* and *Theileria spp*. *B. bovis RAP-4* and *T. annulata tams1* genes were diverse among the samples and across several clades in the phylogenetic tree containing sequences from other countries. It represented 100% identity between *B. bovis* isolates of cattle and goat than sheep, whereas the two *T. annulata* isolates from cattle and sheep were identical than goats isolate. Also, it referred to a possible transmission of a *B. bovis* Brazilian strain to local ruminants.

AUTHOR CONTRIBUTIONS

The author has the idea, planned, designed the work, analyzed the molecular studies, and has contributed to writing and approved the manuscript.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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