MOLECULAR DIAGNOSIS AND PHYLOGENETIC ANALYSIS OF BABESIA SPECIES ISOLATED FROM TICKS OF INFESTED CATTLE IN WASIT GOVERNORATE, IRAQ

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ABSTRACT

The aim of current study is to detect *Babesia bovis*, *B. bigemina*, and *B. divergens* in ticks using molecular polymerase chain reaction (PCR) assay. In a totally 180 cattle examined to collect of tick samples during December 2018 to August 2019, the findings were revealed on 63 (35%) cattle infested with ticks that classified morphologically to belong to the genus of *Hyalomma* and genus of *Rhipicephalus*. From 50 tick samples tested by PCR assay, 41 (82%) were infested by *Babesia* genus including 30 (68.18%) infested with *B. bovis* and 11 (31.82%) infested with *B. bigemina*; whereas, no tick samples were found to be infested with *B. divergens*. To document the local isolated strains, five PCR products of each *B. bovis* and *B. bigemina* positive strains were selected, sequenced and reported in the NCBI under the accession numbers of (MN727083.1, MN727084.1, MN727085.1, MN727086.1, and MN727087.1) and (MN741113.1, MN741114.1, MN741115.1,MN741116.1, and MN741117.1) respectively.

Keywords: *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*, PCR, sequence.

الكشف الجزيئي والتحليل الوراثي لأنواع البابيزيا المعزولة من القراد المتطفل على الابقار في محافظة واسط، العراق

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المستخلص

هدفت الدراسة الحالية الى تحديد اصابة القراد ب *Babesia bovis* ، *B. bigemina* ، و *B. divergens* باستعمال تقنية تفاعل البلمرة المتسلسل الجزيئية. تم اجماليا فحص 180 بقرة لجمع عينات القراد خلال كانون الاول 2018 الى آب 2019، واظهرت النتائج ان 63 (35%) من الابقار كانت مصابة بالقراد الذي تم تصنيفه مظهريا لجنس *Hyalomma* وجنس *Rhipicephalus*. من خلال فحص 50 عينة قراد بتقنية تفاعل البلمرة المتسلسل، بلغت نسبة الاصابة بجنس البابيزيا 41 (82%)، تضمنت 30 (68.18%) مصابة بال *B. bovis* و 11 (31.82%) مصابة بال *B. bigemina* : في حين، لم يتم العثور على عينات قراد مصابة بال *B. divergens* . من اجل توثيق العتر المحلية المعزولة، تم اختيار خمس نواتج تفاعل بلمرة متسلسل موجبة لكلا من *B. bovis* و *B. bigemina*، وتحليل تتابعها وتسجيلها في المركز الوطني لتقانة المعلومات تحت الارقام التسلسلية (MN727083.1 ، MN727084.1 ، MN727085.1 ، MN727086.1 ، و MN727087.1) و (MN741113.1 ، MN741114.1 ، MN741115.1 ، MN741116.1 ، و MN741117.1) على التوالي .

الكلمات المفتاحية: *Babesia bovis* ، *Babesia bigemina* ، *Babesia divergens* ، تفاعل البلمرة المتسلسل ،تحليل وراثي .

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INTRODUCTION

The genus of *Babesia* is one of the more pathogenic tick-borne parasites which infect many domestic and wildlife animals as well as humans resulting in an intraerythrocytic infection, babesiosis (**30**). The most important species that affect cattle are *B. bovis* and *B. bigemina* which present in Africa, America, Asia, and Australia; in addition to *B. divergen*s that distributed in Europe (**4, 19**). *Babesia* species undergo a complex unique life cycle to perpetuate their parasitic existence by propagation and to guarantee host-to-host transmission through specialized infective stages (**38**). This mediated by the combination of two asexual reproduction cycles and one sexual reproduction cycle, which alternate between the vertebrate host and the tick vector (**25, 36**). In cattle, babesiosis can detect acutely based on the clinical symptoms and confirmed by microscopic examination of blood smears and or molecular assays whereas in chronic infection, serological techniques as enzyme-linked immunosorbent assays in addition to molecular techniques as polymerase chain reaction which demonstrates with a great value (**15**). Xenodiagnosis that is, detection of *Babesia* spp. from ticks, either by microscopy, culture in artificial media and animal inoculation, or molecular techniques has been employed for supportive epidemiological evidence in the diagnosis of babesiosis (**8**). However, microscopic and cultural methods are time-consuming, laborious, give highly variable results, and need for extensive expertise to be performed (**27, 37**). Based on 18S rRNA, molecular methods have developed as the method of choice for detection of the parasite genomic DNA, and for confirm of infection in different samples due to their high sensitivity and specificity (**33**). In Iraq, a number of studies have identified the role of ticks in transmission of protozoa such as *Theileria* and *Babesia* parasite to field animals; however, there limited data on *Babesia* isolates found across ticks infested cattle herds (**1, 7**). Therefore, the present study carried out for molecular characterization of ITS1 region of 18S rRNA of *B. bovis*, *B. bigemina*, and *B. divergens* alongside its phylogenetic relationship with the other local and global isolates/strains.

MATERIALS AND METHODS

Ethical approval: This study was performed under the regulation of Department of Parasitology in the College of Veterinary Medicine / University of Baghdad, Baghdad, Iraq. The collection of blood samples was approved by the Scientific and Ethical Committee of Veterinary Medicine / Baghdad University, Iraq.

Sample collection:A total of 180 cattle that selected randomly from different areas in Wasit province / Iraq, were subjected for examination to collect of tick samples during December 2018 to August 2019. Ticks of each infested animal were removed carefully by using 70% Ethanol alcohol to avoid of tick's mouthparts damage, and kept into a plastic container labeled with the a serial number particular to each study animal. Ticks of each study animal were considered as one sample (**2, 13**). All sample containers were transported to the laboratory using an ice-box. Data of animal age, sex, and period were recorded. Bodily sites of tick collection were reported also including the ear, neck, leg, tail, anus, udder, scrotum, and abdomen.

Morphological classification: All samples of collected ticks were examined microscopically by Assist Prof. Dr. Haider Mohammed Ali (Department of Parasitology, College of Veterinary Medicine, University of Baghdad), in assistance of the Natural History Museum and Research Center, University of Baghdad, Iraq; to detect the bodily characteristic structures.

Molecular examination: Following the manufacturer's instruction (Geneaid, Taiwan) of the Genomic DNA Mini Kit (Tissue), the samples of ticks were subjected for extraction of purified DNA. Concentration (ng/µl) and purification at an absorbance of A260/A280, of extracted DNA were tested using the Nanodrop system (Thermo-scientific, UK). For DNA amplification, four sets of primers that designed based on internal transcribed spacer 1 (ITS1) region of 18S rRNA gene of the National Centers for Biotechnology Information (GenBank-NCBI) and provided by the Company of Macrogen (South Korea) were used in this study to detect of *Babesia* spp., *B. bovis*, *B. bigemina* and *B. divergens* (Table 1).

Table 1. Primers applied for detection of *Babesia* in ticks samples of infested cattle

Gene		Primer Sequence	Amplicon size	Reference
***Babesia* spp.**	**B.ITS1-F**	**5´ GGCCGTTCTTAGTTGGTGGA 3´**	**357bp**	**(24)**
	B.ITS1-R	**5´ TGTGTACAAAGGGCAGGGAC 3´**		
B. bovis	**BboITS-F**	**5´ TGCGCACTTCATAGAGGGAC 3´**	**513bp**	**(40)**
	BboITS-R	**5´ GAAGTGCTCGCGAGTACGTA 3´**		
B. bigemina	**RLB-F**	**5´ CCGGCGCGTTTTGTTAAAAT 3´**	**371bp**	**(10)**
	RLB-R	**5´ TGAGCCAAGACATCCATCGC 3´**		
B. divergens	**Bob2A-F**	**5´ TTTCCGACTCCTTCAGCACC 3´**	**469bp**	**(23)**
	Bob2A-R	**5´ GATTACCCAGCCCTTTCGGG 3´**		

PCR-Mastermix was prepared by PCR-Premix kit (Bioneer, South Korea) at a final volume of 20µl. PCR steps of ThermoCycler (Bio-Rad, USA) conditions included: initial denaturation (95°C, 5 min) for 1 cycle; then denaturation (95°C/30 sec), annealing (58°C / 30 sec), and extension (72°C/1 min) for 30 cycles; final extension (72°C / 5 min) for 1 cycle; and hold (4°C). PCR products were analyzed in 1% agarose gel stained with ethidium bromide at 80 Volt for 1 hour, and visualized using UV trans-illuminator (ATTA, South Korea). For phylogenetic analysis, PCR products of five positive samples to each species positive *B. bovis* and *B. bigemina* with forward primer were sent to Macrogen Company (South Korea). Sequencing data received by private Email were analyzed by MEGA-6 software using of Clustral W alignment tool to detect of multiple sequence alignment, and Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) to obtain of phylogenetic tree. Homology sequence identity was performed to compare of local strains with the global NCBI-Blast isolates/strains.

Statistical analysis

Data of this study were collected, tabled, figured, and analyzed using the computerized, Microsoft Office Excel (version ^{2007}) and IBM/SPSS (version ^{16}) software. Chi-square (x^2) test was applied to estimate significant differences at a probability of $P \leq 0.05$ (16).

RESULTS AND DISCUSSION

Among 180 cattle, 63 (35%) animals were revealed to be infested with ticks that distributed on different bodily parts of study animals (Figure 1). Based on morphological characteristics, two types of *Ixodes* hard ticks were detected on infested cattle include *Hyalomma* spp and *Rhipicephalus* spp. In Iraq, tick-borne protozoa such as *Babesia* spp and *Theileria* spp represent an economic crisis for different animals (1, 7). For genus of *Babesia* or it species associated with ticks in cattle, there are only a few studies (11, 26). This calls for further investigations on the distribution and diversity of *Babesia* species and their relevance to public and animal health in the region. In the present study, ticks were screened using molecular technique for the presence of *Babesia* spp. to investigate the vector-cattle association. Our findings showed that there a high relative percentage of study animals were infested with tick distributed in different bodily parts, significantly hind-limbs, perineal region, and udder. These data indicated that Iraq is endemic with tick particularly *Ixodes* hard ticks that attached to the animal through the hind-limbs and udder as these parts near to the ground. Although some ticks are opportunistic feeders on several different hosts, many will target a specific host (7). A tick can detect a potential host with sensory organs that pick up traces of carbon dioxide given off by the animal, as well as heat, moisture and vibrations. However, our findings were in agreement with that detected previously in different areas especially in Asia (12, 31), and Europe (32, 35) as the major tick species infesting crossbreed cattle are *Rhipicephalus* and *Hyalomma*.

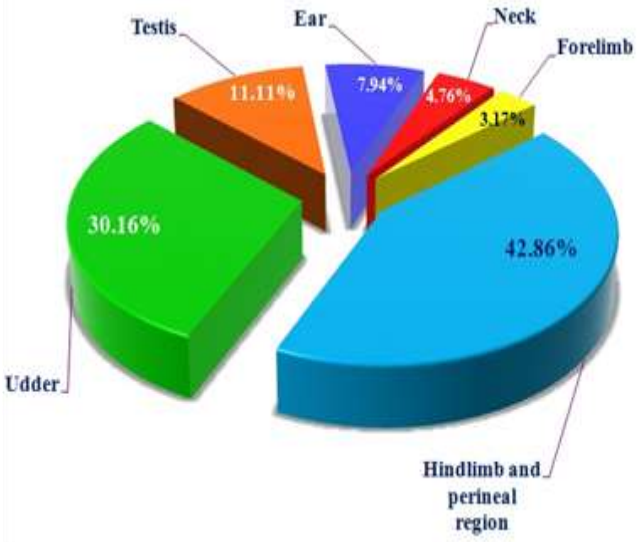

Figure 1.Tick distribution among body regions of infested cattle

The effect of certain risk factors on resistance or sensitivity of cattle to *Babesia* was aimed in this study. Our findings detected that there was a significantly higher (P≤0.05) infested cattle at an age of 3-<5 (45.45%) and 1-<3 (45.07%) years; during August (95%) and July (65%) months, and among females (37.96%) more than males (25.58%), (Table 2)

Table 2.Association between cattle infestation with ticks and risk factors

Factor	Category	Total No.	Infested	Non-infested
Age	< 1	29	3 (10.34%) *	26 (89.66%)
	1- < 3	71	32 (45.07%) **	39 (54.93%)
	3- < 5	55	25 (45.45%) **	30 (54.55%)
	≥ 5	25	3 (12%) *	22 (88%)
Period	December - 2018	20	3 (15%)	17 (85%)
	January - 2019	20	0 (0%)	20 (100%)
	February	20	0 (0%)	20 (100%)
	March	20	1 (5%)	19 (95%)
	April	20	4 (20%)	16 (80%)
	May	20	8 (40%)	12 (60%)
	Jun	20	15 (60%)	5 (40%)
	July	20	13 (65%) *	7 (35%)
	August	20	19 (95%) **	1 (5%)
Sex	Males	43	11 (25.58%) *	32 (74.42%)
	Females	137	52 (37.96%) **	85 (62.04%)

Significance * (P≤0.05)

Control measures have always focused on the tick vectors, and thorough understanding of how the ticks interact with their host is vital to continued efficacy of control measures. Significant correlation of both age and sex with tick burden is key information that might be eventually used to improve herding strategies. Several studies have performed to detect the significant effect of the age of cattle on tick burden, and found a lower tick burden in calves compared to older cattle, and a higher infestation rate in older animals of different ruminant livestock species (3, 17, 22, 28). Most studies on tick burden in cattle have not evaluated the effect of live weight. Rocha *et al.* (29) showed that in addition to age, live weight also had a significant effect on tick burden as the heavier animal have higher tick burden, and hypothesized that factors contributing to the higher tick burden in heavier animals could be either a compromised immune system in heavier older animals or that larger animals have a wider skin surface with a denser vasculature. Association between sex and tick burden on cattle suggested the role of stress due to milk production and gestation, and the implication of grazing and management strategies. Information on the prevalence of tick-borne pathogens in potential vector ticks of the period is essential for the epidemiology of tick-borne diseases. Attempts have been made to develop models to understand spatial dynamics of habitual suitability for ticks, emphasizing ecological preferences and sensitivity to abiotic conditions. Previous research efforts investigated population dynamics, parasite-host interactions, seasonal fluctuations, and physiological response to climate factors (6, 39). These studies added growing body of work that has elucidated many important variables in this complex ecological system. Global climate changes were certainly altering the spatial arrangement of suitable habitat for these important vectors (9, 18, 34). Of total tick samples, only 50 were selected randomly and tested molecularly using PCR assay. Our findings were revealed on 41 (82%) tick samples infested with *Babesia*; 30 (68.18%) for *B. bovis* and 14 (31.82%) to *B. bigemina*. However, no positive tick samples were positives to *B. divergens* (Table 3, Figures 2, 3, and 4).

Table 3.Total PCR results for testing hard tick samples of 50 infested cattle

Targeted pathogen	Total No.	Positives	Negatives
***Babesia* spp.**	**50**	**41 (82%)**	**9 (18%)**
B. bovis		**30 (60%) ** **	
B. bigemina		**14 (28%) ***	
B. divergens		**0 (0%)**	

Significance *(P≤0.05)

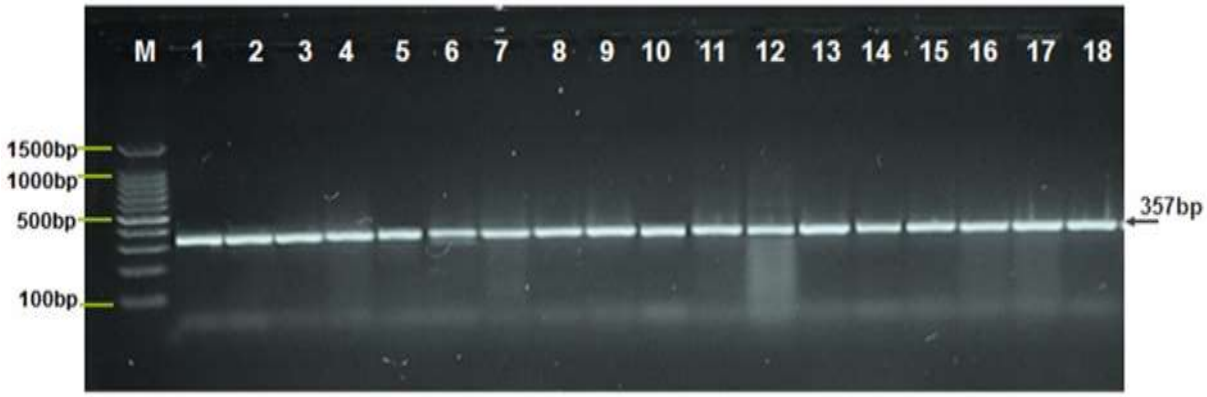

Figure 2.Agarose gel electrophoresis (1.5%) of PCR products to detect of *Babesia* spp.
M: Marker ladder (100-1500bp), Lanes (1-18): Positive samples at a product size of ~ 357bp

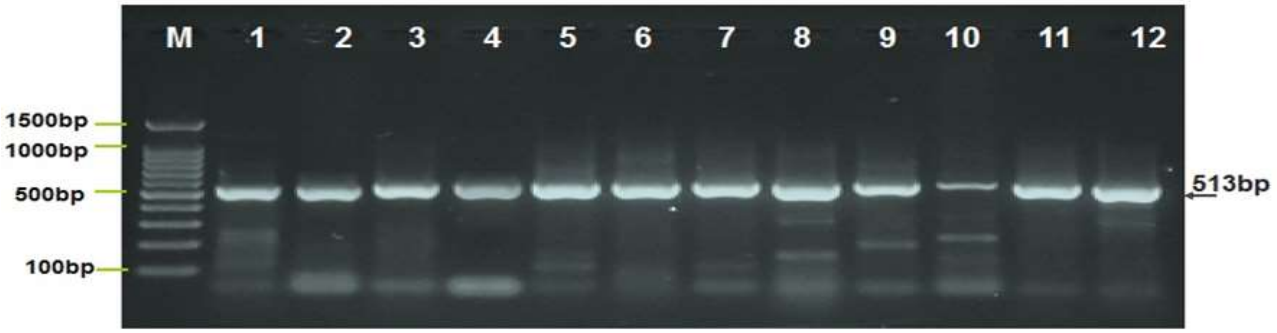

Figure 3.Agarose gel electrophoresis (1.5%) of PCR products to detect of *B. bovis*
M: Marker ladder (100-1500bp), Lanes (1-12): Positive samples at a product size of ~513bp

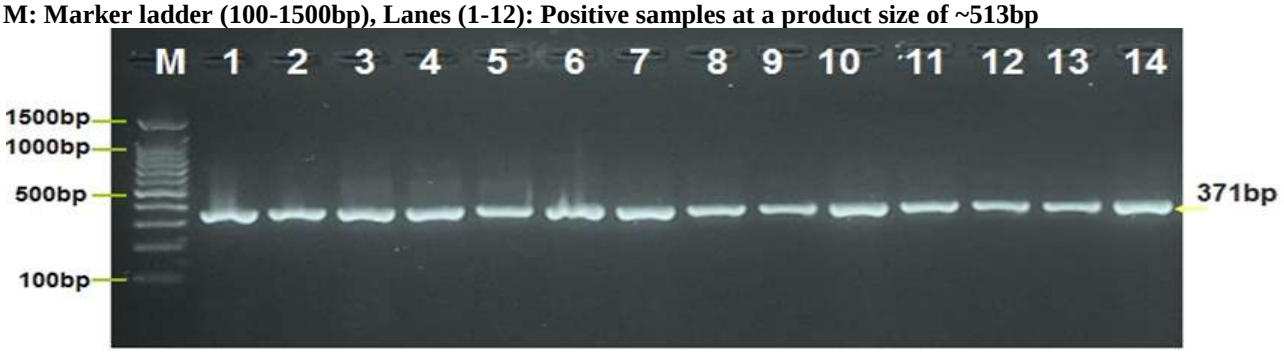

Figure 4.Agarose gel electrophoresis (1.5%) of PCR products to detect of *B. bigemina*
M: Marker ladder (100-1500bp), Lanes (1-14): Positive samples at a product size of ~371bp

Findings of PCR assay indicated that both *B. bovis* and *B. bigemina* were circulating significantly in tick vectors. In Iraq, *B. bovis* was the only *Babesia* species isolated from tick samples of infested cattle (11). Therefore, this study was the first report of *B. bigemina* in ticks infested cattle. For epidemiological surveying, application of specific and sensitive molecular assay has become necessary. ITS1 region of 18S rRNA gene used in present study was successfully detected *Babesia* and its species in ticks. Because ITS1 have great variability in nucleotide and length, ITS1 sequences were used for discriminating different geographic isolates of piroplasmids, identifying new species, and differentiating between piroplasm species and subspecies (20). This has made the 18S rRNA gene, which is one of the genes having ITS1 region, to be widely used for establishment of phylogenetic analysis, evaluation of the evolutionary process, and for the determination of taxonomic identities (21). The absence of positive samples of *B. divergens* might be attributed to that this parasite is not existed in Iraq, no tick vectors are found, and mistakes in sequenced primer. Based on sequence data, local *B. bovis* strains were named and reported in NCBI as IQKB No.1 (MN727083.1), IQKB No.2 (MN727084.1), IQKB No.3 (MN727085.1); IQKB No.4 (MN727086.1), and IQKB No.5 (MN727087.1) strains. Multiple sequence alignment analysis of nucleotides reported similarity and mutations in ITS1 region of 18S rRNA gene (Figure 5).

Figure 5.Multiple sequence alignment for and global NCBI-BLAST*B. bovis* isolate/strain

Genetic relationship analysis was performed based onpartial sequence of18S rRNA gene of the local *B. bovis*strains and global NCBI-BLAST isolate/strain. Findings of phylogenetic tree were closed relative similar at 99% andhave total genetic changes at (0.005-0.015%), (Figure 6).

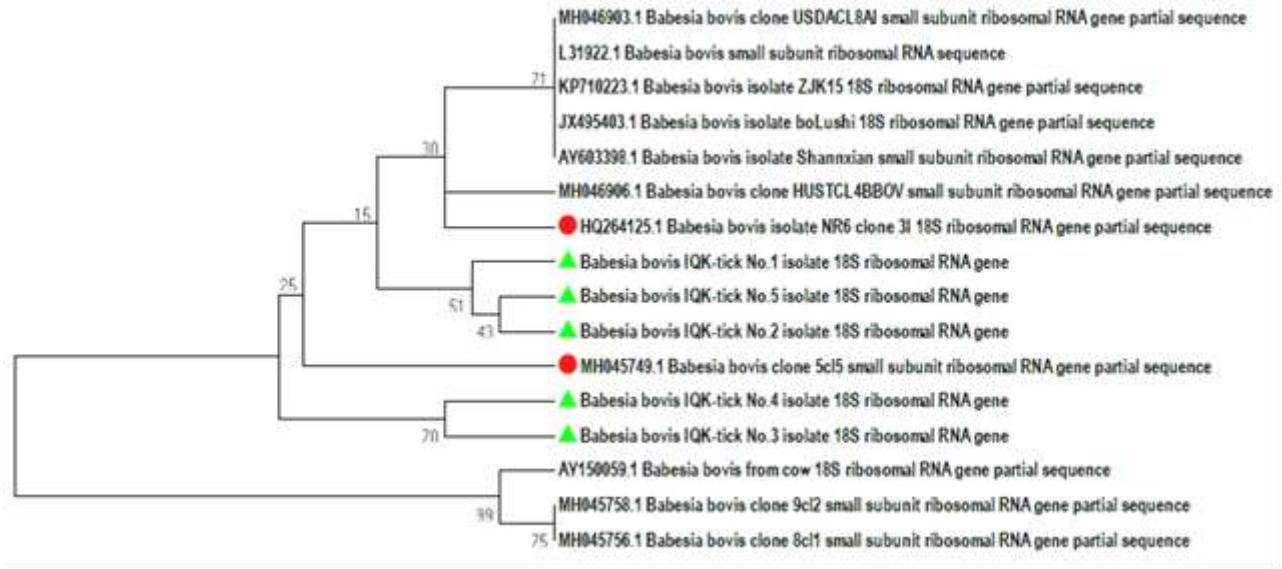

Figure 6.Phylogenetic tree of the local and global NCBI-BLAST*B. bovis* isolate/strain

NCBI-BLAST homology sequence identity showed that the local *B. bovis* IQKB No.1 (MN727083.1), IQKB No.2 (MN727084.1), IQKB No.3 (MN727085.1), IQKB No.4 (MN727086.1), and IQKB No.5 (MN727087.1) strains were respectively identical at 98.52%, 98.51%,98.25%, 98.74%, and 98.75%to the global GenBank (HQ264125.1) USA *B. bovis* NR6 clone "3I" isolate (Table 5).

Table 5.Homology sequence identity

Local isolate	Genbank Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Isolate	Accession number	County	Identity
IQKB No.1	**MN727083.1**	**NR6 clone "3I"**	**HQ264125.1**	**USA**	**98.52%**
IQKB No.2	**MN727084.1**	**NR6 clone "3I"**	**HQ264125.1**	**USA**	**98.51%**
IQKB No.3	**MN727085.1**	**NR6 clone "3I"**	**HQ264125.1**	**USA**	**98.25%**
IQKB No.4	**MN727086.1**	**NR6 clone "3I"**	**HQ264125.1**	**USA**	**98.74%**
IQKB No.5	**MN727087.1**	**NR6 clone "3I"**	**HQ264125.1**	**USA**	**98.75%**

Based on sequence data, local *B. bigemina* strains were named and reported in NCBI as IQKg-tick No.6 (MN741113.1), IQKg-tick No.7 (MN741114.1), IQKg-tick No.8 (MN741115.1), IQKg-tick No.9 (MN741116.1), and IQKg-tickNo.10 (MN741117.1) strains. Multiple sequence alignment analysis for nucleotides reported a similarity and substitutions/mutations in ITS1 region of 18S rRNA gene (Figure 7).

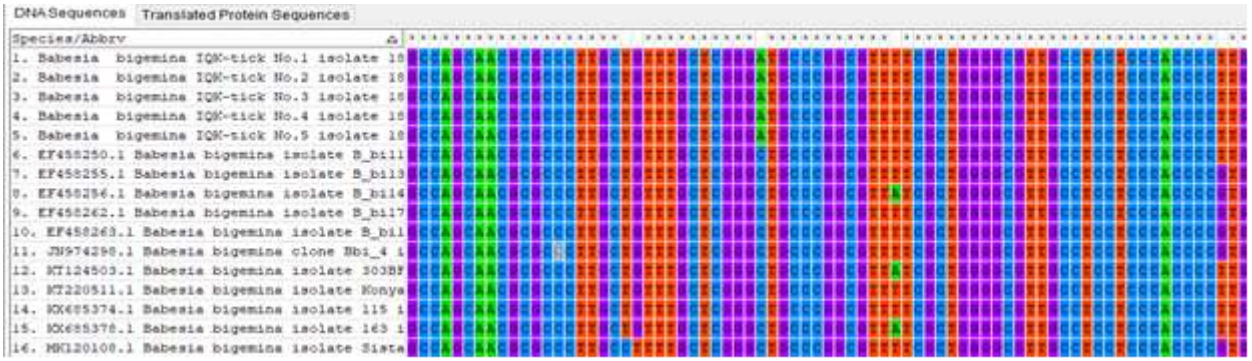

Figure 7.Multiple sequence alignment for and global NCBI-BLAST*B. bigemina* isolate/strain

Genetic relationship analysis was performed based on partial sequence of18S rRNA gene of the local *B. bigemina* strains and global NCBI-BLAST isolate/strain. Findings of phylogenetic tree detected that there a close relative similarity at 99% andtotal genetic changes/mutationsat (0.005-0.015%), (Figure 8).

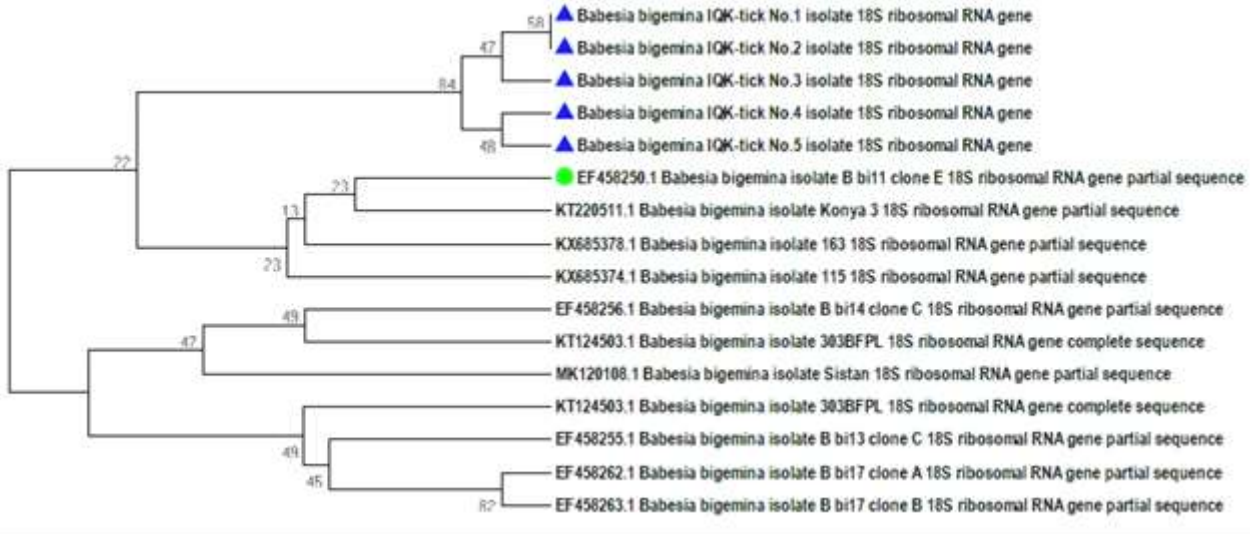

Figure 8.Phylogenetic tree of the local and global NCBI-BLAST*B. bigemina* isolate/strain

NCBI-BLAST homology sequence identity showed that the local *B. bigemina* IQKg-tick No.6 (MN741113.1), IQKg-tick No.7 (MN741114.1), IQKg-tick No.8 (MN741115.1), IQKg-tick No.9 (MN741116.1), and IQKg-tickNo.10 (MN741117.1) strains were respectively identical at 98.42%, 98.42%, 98.10%, 98.73%, and 98.72% to the global GenBank (KT220511.1) Turkey *B. bigemina* Konya 3 clone ”87- Big-ITS” isolate (14), (Table 6).

Table 6.Homology sequence identity

Local isolate	Genbank accession number	NCBI-BLAST Homology Sequence identity (%)			
		Isolate	Accession number	County	Identity
IQKg-tick No.6	**MN741113.1**	**Konya 3 clone ”87-Big-ITS”**	**KT220511.1**	**Turkey**	**98.42%**
IQKg-tick No.7	**MN741114.1**	**Konya 3 clone ”87-Big-ITS”**	**KT220511.1**	**Turkey**	**98.42%**
IQKg-tick No.8	**MN741115.1**	**Konya 3 clone ”87-Big-ITS”**	**KT220511.1**	**Turkey**	**98.10%**
IQKg-tick No.9	**MN741116.1**	**Konya 3 clone ”87-Big-ITS”**	**KT220511.1**	**Turkey**	**98.73%**
IQKg-tick No.10	**MN741117.1**	**Konya 3 clone ”87-Big-ITS”**	**KT220511.1**	**Turkey**	**98.72%**

Regarding to sequence data, we found that local *B. bovis* strains were close relatively identical to American *B. bovis* (HQ264125.1) “NR6” isolate from the blood of white-tailed deer (14). de León *et al.* (5) reported that white-tailed deer and other ungulates have a role in the epidemiology of bovine babesiosis is a critical consideration as the United States

deals with increased incidence of vector tick outbreaks. Despite these molecular data supporting conspecificity of the *B. bovis* found in ticks and whit-tailed deer, questions remain. We suggested that our strains might have descended from the ancestor of the American-isolates, and that there are certain factors might play a role in distribution of this ancestor around the world such as moving of the animals and humans in addition to importation and exportation processes. Surprisingly, the local strains of our study were incompatible with that detected previously in cattle-feeding ticks in Al-Diwaniyah province, Iraq (11), demonstrating that there many strains of *B. bovis* circulate in Iraqi cattle livestock and their infested-ticks. Also, uncontrolled importation of domestic and wildlife animals might participate in existence of new isolates/strains in Iraq. For *B. bigemina*, the local strains were showed a high relative identity with the Turkish (KT220511.1) Konya 3 strains that isolated from cattle based on *B. bigemina* ITS genes. Zhou *et al.* (40) reported that *B. bigemina* isolates are genetically conserved in Turkey, and these isolates might belong to the same genotype. According to these findings, we suggested that ticks have a great part in transmission of infection between cattle herds of neighboring-joined regions/countries. However, the role of wild animals, as reservoir or carrier, in transmission of infection is unknown.

CONCLUSION

Molecular diagnosis of *Babesia* and it species, *B. bovis* and *B. bigemina*, has demonstrated the prevalence of these parasites in prevalent tick species, and the risk of cattle to contact with ticks. Results of the molecular and phylogenetic analysis confirmed *B. bigemina* in hard tick samples of infested study cattle for the first time in Iraq. Phylogenetic analysis of local *B. bovis* and *B. bigemina* strains has pointed out to the possible existence of novel strains in the region. Therefore, further molecular and phylogenetic studies are needed.

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