MOLECULAR STUDY AND PHYLOGENY OF *Babesia* spp. IN NATIVE SHEEP FROM SULAIMANI GOVENORATE/ NORTHERN IRAQ

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ABSTRACT

This study was conducted to investigate *Babesia* parasites infecting sheep in eight districts of Sulaimani governorate/north Iraq from April to October 2017. Forty flocks of small ruminants were selected to collect blood samples randomly from 450 sheep. The samples were examined for babesiosis by microscopic examination and PCR. Primers based on the 18S rRNA were used for *Babesia* diagnosis, followed by sequencing of the amplicons for confirmation of the PCR product identities. Seventy-four samples (16.44%) showed the presence of *Babesia* piroplasms microscopically, while 116 (25.78%) samples were positive using PCR. Results showed that *B. ovis* was reported in 15.78% (n = 71), and *B. motasi* in 10.0% (n = 45) of the samples. Also, BLAST analysis of the obtained partial sequences of the 18S rRNA gene from current study isolates revealed the existence of both *B. ovis* and *B. motasi*, with a high homology degree of nucleotide identity with other nucleotide sequences of *Babesia* spp. in GenBank database. Distribution of babesiosis, according to the sampling time, revealed that high-frequency rates occur during July and August. Based on the result data, babesiosis was mainly caused by *B. ovis* and *B. motasi.*

Keywords: *Piroplasma,* PCR, microscopic examination, partial sequences

دراسة جزيئية لتطور سلالات . *Babesia spp* للأغنام المحلية المخمجة طبيعياً لمحافظة السليمانية / شمال العراق

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المستخلص

أجريت هذه الدراسة للتحري عن طفيليات البابيزيا التي تخمج الأغنام في ثمانية مناطق من محافظة السليمانية. حيث تم اختيار 40 قطيع لتربية المجترات لأخذ عينات الدم من 450 راس غنم وبشكل عشوائي للفترة من نيسان إلى تشرين الاؤل من 2017. و تم فحص العينات بحثاً عن وجودالطفيلي باستخدام الفحص المجهري و تفاعل البلمرة المتسلسل (PCR) على التوالي. حيث تم استخدام البادئة المستندة إلى 18S rRNA لتشخيص ال *Babesia* ، متبوعًا بتسلسل الامبليكونات (amplicons) لتأكيد هويات منتج ال PCR.ومن نتائج الفحص المجهرى أظهرت أربعة وسبعون عينة (16.44%) وجود كمثريات البابيزيا مجهريا ، بينما كانت 116 (25.78%) عينة موجبة باستخدام PCR. كما وأظهرت النتائج بأن *B. ovis* سجلت في 71 (15.78%) و *B. motasi* في 45 (10.0%) من العينات التي تم فحصها. وكشف تحليل بلاست للتسلسلات الجزئية التي تم الحصول عليها من جين 18S rRNA من عزلات الدراسة الحالية عن وجود كل من *B. ovis* و *B. motasi* مع درجة تماثل عالية لهوية النيوكليوتيدات مع متواليات نيوكليوتيدات أخرى من *Babesia spp* في قاعدة بيانات GenBank. وأظهر توزيع البابيزيا حسب وقت أخذ العينات أن معدلات التكرارات العالية تحدث خلال شهري تموز و اب بناءً على معطيات نتائج الدراسة وكان السبب الرئيسي لداء الكمثريات هوكل من *B. ovis* و *B. motasi* .

الكلمات المفتاحية: البيروبلازما، تفاعل البلمرة المتسلسل، الفحص المجهري، التسلسلات الجزئية،

INTRODUCTION

Protozoan parasites of the genus *Babesia* are known to cause babesiosis (13). The genus includes a large number of classified and unclassified species that infect different wild and domestic animals, humans, and birds. *Babesia* parasites are different in size, shape, and capability to produce disease (14). *Babesia* species are widespread in tropical and subtropical areas of the world. In sheep, babesiosis is a crucial hemoparasitic disease caused by *Babesia ovis, B. motasi*, *B. crassa*, and *Babesia* sp. BQ1 (Lintan) (20). Other species, including *B. foliate* and *B. taylori,* were described microscopically, while there was no molecular data in GenBank supporting the availability of these two *Babesia* species (21). The clinical signs of babesiosis vary, ranging from subclinical infection to life-threatening diseases that may result in severe economic losses among livestock, particularly cattle (7). The clinical signs are not very prominent in small ruminants, although typical signs of pyrexia, hemoglobinuria, and anemia occur in the severe form of the disease (15). The main tick vectors responsible for the transmission of *B. motasi* and *B. ovis* are *Haemaphysalis* sp. and *Rhipicephalus bursa*, respectively (14), although *B. ovis* infection has been confirmed in *Hyalomma anatolicum anatolicum* and *Hyalomma marginatum* too (9). Also, the transmission of *B. motasi* by *Dermacentor silvarum* has been reported (1). Blood smears and clinical symptoms are useful in the diagnosis of acute piroplasmosis, but they are insufficient in subclinical cases. On the other hand, accuracy in diagnosis of specific-species existence in certain regions or countries required sensitive diagnostic method such as sequencing and phylogenetic analysis (3), so molecular methods could allow direct, specific, and sensitive detection of parasites. PCR is the most commonly used molecular assay, and the 18S rRNA genes have been successfully applied to identify and classify several unknown *Babesia* parasites (29). Moreover, detection of *Babesia* infection in carrier animals by DNA amplification is considered a powerful tool for epidemiological investigations, since it is more sensitive and specific than Giemsa-stained blood smears (2). Babesiosis has been documented in Sulaimani province/north Iraq using microscopic examinations. However, a comprehensive molecular study has not been previously conducted to determine the prevalent *Babesia* species in the area, especially in clinically healthy animals. This study was designed to investigate the occurrence of *Babesia* in naturally infected sheep from Sulaimani governorate in the north region of Iraq and to conduct molecular characterization and phylogenetic analysis of the isolates with reference isolates of *Babesia* spp., deposited in the GenBank database.

MATERIAIS AND METHODS

Blood sampling and study area

This study was carried through in eight districts of Sulaimani, north of Iraq, from April to October 2017. Forty small ruminants' flocks were selected in seven districts of the province, namely Arbat, Bakrajo, Bazian, Mawat, Nal Parez, Sayid Sadiq, Sharazoor, and Sitak. A total of 450 sheep blood samples were collected randomly. About 5 mL of blood was withdrawn from the jugular vein and collected in EDTA-coated vacutainer tubes. Samples were collected indiscriminately from male and female one-to-six-year-old sheep. Thin blood smears were fixed on glass slides, and the remainder of each blood sample was stored at -80°C till DNA extraction. The blood smears were fixated in methanol and stained with Giemsa stain using standard procedures. The slides were examined microscopically under 1000× magnification for the presence of *Babesia* organisms.

DNA extraction

DNA was extracted from aliquots of 200 μL of whole blood samples using a DNA extraction kit for blood (GeNet Bio, South Korea) according to the manufacturer's instructions. The DNAs were eluted into 200 μL buffer, at room temperature, and extracted DNA yields stored at -80°C and used as templates when PCRs performed.

Molecular detection

All DNA samples were screened using PCR with primers designed based on the 18S rRNA. The reactions were achieved in a total volume of 20 μL using 2× Prime Tag Premix (GeNet Bio), in a programmable thermal cycler (Prime, UK) in two steps. The first step included amplifying about 1700 bp of

Piroplasma 18S rRNA with the primer sets BaTh F 5'-CCTGGTTGATCCTGCCA GTAGTC-3' and BaTh R5'-CCTTCTGCAGGTTCACCTA CGG -3'. Five microliters of the DNA template were used, and the PCR protocol started with an initial denaturation 95°C/5 min, with 38 cycles of 95°C/1 min, 60°C/30 sec, 72°C/2 min, and a final extension of 72°C/5 min. The second step included PCR amplification for DNA samples being positive in the first PCR step by using the previously described primer sets Bbo-F5'-TGGGCAGGACCTTGG TTCTTCT-3' and Bbo-R 5'-CCGCGTAGCGCCGGCTAAATA-3' to amplify a 549 bp fragment of the 18S rRNA gene of *B. ovis* (6). For *B. motasi*, primer sets described by Shayan et al (28) were applied by semi-nested PCR. In the first reaction, 5.0 μL of DNA with primer sets P1 5'-CACAGGGAGGTAGTGACAAG-3' and P2 5'-AAGAATTTCACCTATGAC AG -3' were used to amplify 389-402 bp of DNA fragment. The second reaction was run with 1.0 μL of the PCR product as a template with primer sets P3 5'-AAGAATTTCACC TATGACAG-3' and P4 5'-CGCGATTCCG TTATTGGAG-3' for amplifying a 205 bp fragment. PCR protocols for *Babesia* species differentiation were executed following previously described procedures (6 and 28) with some modification. Finally, PCR products were separated by loading 10 μl on 1% agarose gels in 1 × Tris/Borate/EDTA buffer for electrophoresis and visualized using ethidium bromide for checking the amplicon size, and by comparing them to a 100 bp DNA ladder.

Sequencing and phylogenetic analysis
PCR amplicons from eight *Babesia*-positive samples five from *B. ovis* and three from *B. motasi* were selected randomly for nucleotide sequencing by Sanger DNA sequencing system in South Korea, using the primer sets for amplifying 1700 bp of *Piroplasma* 18S rRNA. The obtained partial sequences identified in the study were deposited in the GenBank database, and the accession numbers MN545599, MN545600, MN545603, MN551067, MN560046 were obtained for *B. ovis* isolates. Moreover, the *B. motasi* isolates received the accession numbers MN548430, MN551068, and MN548425.The nucleotide sequence identities and similarities were studied by nucleotide sequence homology using BLASTn at the network server of NCBI (National Center for Biotechnology Information). Sequence alignment was performed using CLUSTAL Omega multiple sequence alignment online tool, and phylogenetic analysis was done using MEGA X software (18). The maximum parsimony method was used to construct phylogenetic trees for *Babesia spp.,* and Bootstrap with 1000 replications was used to estimate the confidence of the nodes, and branches of the trees.

RESUITS AND DISCUSSION
Seventy-four (16.44%) out of the total 450 examined sheep included in this study were positive for ovine babesiosis by microscopic examination. Positive smears showed different intraerythrocytic forms of hemoprotozoa, which were morphologically compatible with *Babesia* piroplasms. In different to our finding (5) reported (26.9%) from Al-Najaf province. The incidence of babesiosis was also reported from other countries using microscopy, in Iran (46.0%, 24.67%) were reported by (16 and10), in Turkey 27.3% was reported (25), also in India, the prevalence rate of babesiosis was 26.8% (15). In difference to our finding low prevalence of (9.67%, 2.9%) were reported by (22 and 11) from Pakistan and Tunisia respectively. Following the finding of Shayan et al (28), polymorphic characters of small ruminant *Babesia* were prominent, including double pyriform with an acute or obtuse angle, single pyriform, and ring form. Different sizes of round forms were also evident. Previously, according to morphological data, the large *Babesia* species was routinely diagnosed as *B. motasi* and small-sized as *B. ovis*. Also, the presence of polymorphisms could be the main problem in *Babesia* species differentiation microscopically, which can be dissolved by PCR analysis (10). In this regard (28) described that the large-sized *B. ovis* resembled *B. motasi* in morphological and biometrical parameters. Detection of *Babesia* infection in carrier animals through DNA amplification is a powerful tool for epidemiological investigations, as it is more sensitive and specific than Giemsa-stained blood smears examination (2). The PCR

results of 450 examined sheep revealed that 25.78% (n = 116) were infected with *Babesia* spp. Of these, *B. ovis* was reported in 15.78% (n = 71) of the studied sheep, while 10.0% (n = 45) of examined sheep were positive for *B. motasi*. Based on result data, *B. ovis* and *B. motasi* were reported as the main causative agents of small ruminant babesiosis, with various prevalence rates in different geographical areas. The prevalence rate of 45.0% was reported by Alkhaled and Abdul-Hassan (4) from Iraq, with infection rates of 15.0% and 5.0% for *B. ovis* and *B. motasi* respectively, also in Spain, Nagore et al. (19) reported prevalence rates of 2.5% for *B. ovis* and 2.0% for *B. motasi* by RLB. However, indifferent to the study data, higher infection rate of *B. motasi* than *B. ovis* were reported by Ros-García et al. (24) et al which were 12.3% and 6.3% for both species respectively. Other researchers reported higher incidences of *B. ovis* than the current study in Iran (12) reported an incidence of 18.5%, and (8) found that 17.5% of sheep were infected. In Turkey, a prevalence rate of 48.0% was reported by Sarayli et al. (25). In Pakistan, Iqbal et al. (17) reported prevalence rate of 50.0% by PCR. Also, in Tunisia, an incidence of 17.4% was reported by Rjeibi et al. (23). Different factors

might cause variations in the frequency of babesiosis, such as the animals breed, the immunological status of the host, geographical diversity, and parasite strains (26). This study result constituent with the finding of Faraj and Al-Amery (13) that PCR technique was with sensitivity even in low level of parasitemia in compare to Giemsa stained blood smear for detecting *Babesia* organisms. The existence of subclinical babesiosis in an endemic area might be related to enzootic stability phenomenon, due to the presence of a relatively high number of infected ticks (11). Due to the existence of many infected ticks, host immunity is being maintained at a high level through repeated challenges. So, with the traditional management system, where tick control is loose, the disease represented to be less serious. Month's wise prevalence of babesiosis revealed high-frequency rates of *Babesia* spp. during July and August. In contrast, the lowest frequency rate was found in October (Table 1), which might be related to the favorable conditions for tick vectors activation and disease transmission during this period. Ovine babesiosis as a significant tick-born disease result in high economic losses globally due to high morbidity and mortality (27).

Table 1. Distribution of babesiosis in sheep sampled from April to October 2017 in Sulaimani province, north Iraq using ME and PCR

Month	Number of samples	Diagnostic method			
		ME	%	PCR	%
April	36	5	13.89	9	25.00
May	55	8	14.55	13	23.64
June	52	9	17.31	13	25.00
July	90	18	20.00	26	28.88
August	91	19	20.88	26	28.57
September	72	9	12.50	17	23.61
October	54	6	11.11	12	22.22
Total	450	74	16.44	116	25.78

BLAST analysis of the obtained sequences showed high degree of nucleotide sequence homology with other nucleotide sequences of *Babesia* organisms deposited in the GenBank database. The present study isolates of *B. ovis* with GenBank numbers MN545599, MN545600 shared identities between 99.23% and 99.51% with isolates KP998111 and KP998112 from the middle region of Iraq. Also, the isolates were 98.84% and 98.37% identical with isolates from the northern region (MN309741, MN309736, MN309738,

KC778787). The isolate MN551067 of the current study shared >99.05% identity with previously described isolates from the northern and middle region of Iraq. Moreover, isolate MN545603 from the current study shared identities of 98.98% and 98.37% with isolates from the middle region (KP998111, KP998112) and the northern region (MN309738, MN309736, MN309741, KC778787) of Iraq, respectively. Concerning *B. ovis* new isolates homology with isolates from other countries, they shared the identities

ranging from 98.33% to 99.29% with corresponding Turkish isolates AY260178.1, KY867435.1, MN611761, DQ409332.1, KY283960, MG569902, AY998124, and EF092454. Also, our isolates shared 98.38% to 99.25% similarity with Tunisian isolates KF723611 and KF723612. Similarities ranging from 97.88% to 99.05 with isolates from Spain (AY150058 and AY533146) were observed.
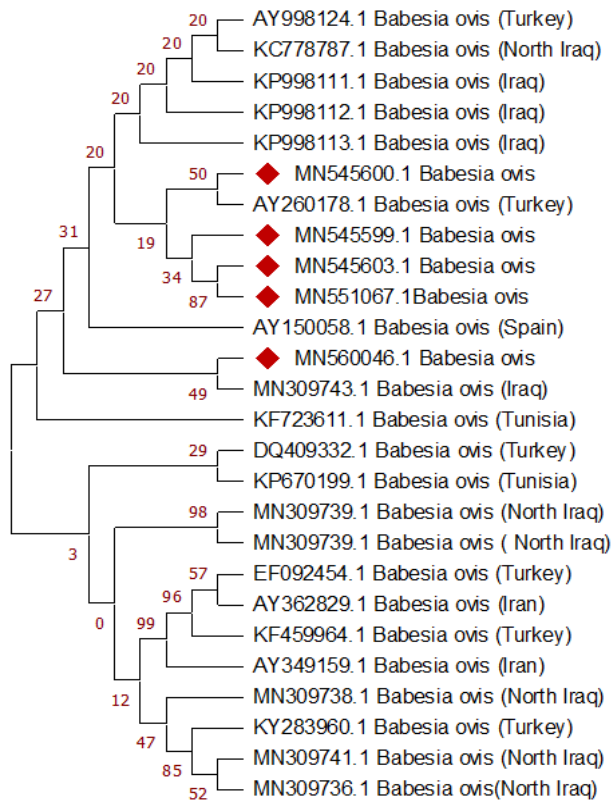

Figure 1. Phylogenic tree based on the 18S rRNA gene partial sequences of the study isolates of *Babesia ovis* with previously registered sequences in the GenBank database. The relationship of the genotypes was determined by maximum parsimony with bootstrap test of (1000 replicates) in MEGA X

The *B. motasi* isolates with accession numbers MN548430 and MN551068 showed 99.17% homology with the Netherlands isolates AY260180 and AY260179. Also, isolate MN548425 showed 99.11%, 99.04%, and 98.90% homology with isolates MF120941, AY533147, and KT725853 from China, Spain, and Hungary, respectively. Moreover, the isolate with GenBank No. MN551068 shared 99.04% homology with Spain isolate AY533147. The phylogenic analysis of new *B.*

ovis sequence isolates represented the presence of diversity. The isolates were clustered in different subgroups of phylogenic branches (Fig. 1), whereas *B. motasi* isolates with accession no. MN548425 and MN548430 were clustered separately, and The MN551068 isolate was clustered in another subgroup of the phylogenic tree (Fig. 2).
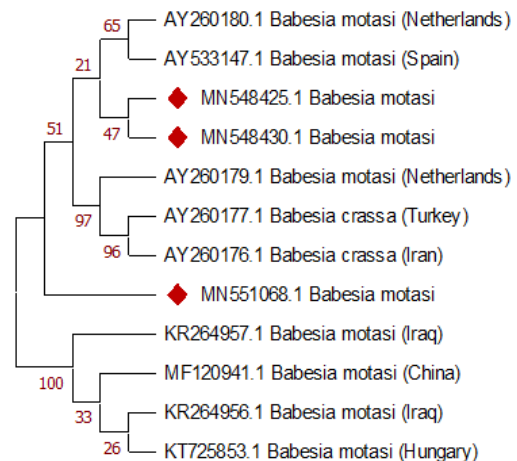

Figure 2. Phylogenic tree based on the 18S rRNA gene partial sequences of *Babesia motasi* isolates with previously registered sequences in the GenBank database. The relationship of the genotypes was determined by maximum parsimony with bootstrap test of (1000 replicates) in MEGA X

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