# RESEARCH ARTICLE



# Prevalence and Phylogenetic Analysis of *Babesia ovis* Isolated from Sheep and Goats in Erbil Province, Kurdistan Region- Iraq

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# INTRODUCTION

Babesia parasites are tick-borne diseases and obligate intraerythrocytic protozoa belonging to the phylum Apicomplexa (Lee et al., 2018), capable of invading a wide range of vertebrate hosts, including animals and human, subsequently leading to serious economic problems for the livestock industry throughout tropical and subtropical regions of the world (Munkhjargal et al., 2016). Babesia ovis, Babesia motasi, and Babesia crassa are recognized as the species causing ovine Babesiosis. The first one is highly pathogenic to sheep and goats with mortality rates of 30-50%, while the other two species are non-pathogenic or less pathogenic (Zangana and Naqid, 2011 and M'ghirbi et al., 2013). The infected animal recovers after a period of 5-8 days or dies (Razmi et al., 2003 and Ijaz et al., 2013). The first clinical signs are acute pneumonia (cough and mucus production), the susceptibility depends on the breed, but they often have concurrent infections (Rahbari et al., 2008 and Hassan, 2011). The body temperature rises before the onset of overt parasitemia (Hassan, 2011). Severe anemia, hemoglobinuria may occur for 5 days, followed by death. At the same time, severe leucopenia develops (Rahbari et al., 2008; Hassan, 2011 and Zangana and Naqid, 2011).

Giemsa-stained blood smears as a laboratory diagnosis is used in small ruminant piroplasmosis. However,

# ABSTRACT

A total of 143 blood smears were obtained from sheep and goats (n = 98) and (n = 45), respectively, by jugular venipuncture between February 2019 and December 2019. Samples were examined for parasite occurrences. Results reveal that the prevalence of infection by the parasite was 12.24% and 11.11%, respectively (microscopically), while 20.41% sheep and 17.78% goats (molecular-based identification using 18S SSU rRNA sequence). Both methods revealed the specificity and sensitivity of the diagnostic assays. Phylogenetic analysis of *Babesia ovis* (MN309736-MN309745) displays that the pathogens identified in this study are clustered together, which indicating similar molecular characteristics. Overall, these results provide a better understanding that Babesiosis is highly prevalent in the study area and continuous monitoring of tick-borne pathogen in sheep and goats are essentially required in Erbil Province.

Keywords: 18S SSU rRNA; Babesiosis; Erbil province; Phylogeny; Tick-borne

species identification by microscopy is difficult because different parasites share a similar morphology, and if mixed infections occur. Furthermore, identification can be difficult in carrier animals where the presence of parasites is low and even in acute cases at the onset of the disease (M'ghirbi et al., 2013 and Hasheminasab et al., 2018). The advanced biological techniques such as polymerase chain reaction (PCR) could be used for identification of *Babesia* spp. with a high degree of sensitivity and useful for the identification and classification of several hemoparasites (Habibi et al., 2004; Ahmed et al., 2006; Chen et al., 2014; Naderi et al., 2017 and Niu et al., 2017).

The aim of the study was to detect and identify *Babesia* spp. infecting sheep and goats in Erbil Province, using microscopic and PCR. In blood, the parasite cannot easily be diagnosed by examination of stained blood film and negative microscopic examination does not exclude the possibility of infection and to determine their phylogenetic relationships to related species based on 18S rRNA gene sequence analysis.

# **MATERIALS AND METHODS**

#### **Study Area**

This study was carried out in the Veterinary Department, Shaqlawa Technical Institute, and Paitaxt Technical Institute, Erbil-Kurdistan. The sheep and goats maintained under a traditional extensive husbandry system (kept on pasture and housed at night). Acaricide treatment is usually conducted two in the year (spring and autumn).

#### **Collection of Blood Samples**

One hundred forty-three whole blood samples were collected from sheep (n = 98) and goats (n = 45) by jugular venipuncture between February 2019 and December 2019. At the time of blood sample collection, the animals were manually examined for the presence of ticks.

#### **Microscopic Examination**

The thin blood smears were prepared immediately after the collection of blood samples and fixed in absolute methanol for 5 min and stained by Giemsa stain diluted at 10% with a buffer solution for 30 min. Slides were screened under oil immersion (×100 magnification) for the presence of intraerythrocytic piroplasms (Rahbari et al., 2008 and Shayan et al., 2008). The morphological and biometrical parameters such as the shape and location of parasite in any infected erythrocyte have been considered for differential diagnosis (Haghi et al., 2013).

#### **DNA Analysis and PCR Amplification**

DNA was extracted using Qiagen DNA extraction kits, according to the manufacturer's instructions. For amplification of Babesia spp., the usual primers forward (5-TGGGCAGGACCTTGGTTCTTCT-3) and reverse (5- CCGCGTAGCGCCGGCTAAATA -3) were used to amplify 549bp sequence of the 18S SSU rRNA gene (Ringo et al., 2018). The PCR was performed using AB Applied Biosystems Thermocycler (Veriti 96 Well Thermal Cycler - Singapore). The amplification reaction mixture (25  $\mu$ l) consisted of 12.5 µl of (2X) Go-Tag master mix, 2.5 µl of each primer (forward and reverse), 2.5 µl of template DNA, and 5 µl nuclease-free water. The reaction mixtures were subjected to an initial denaturation step of 15 min at 95°C, followed by 38 cycles of denaturation at 95°C for the 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 1 min. Amplification was completed by a further 5 min step at 72°C. Negative and positive controls were included in each amplification assay. The Conventional PCR products were visualized through ultraviolet transillumination in 1.5% Agarose gel, following electrophoresis and staining with ethidium bromide. The amplified products were commercially sequenced in both directions (BMRg, Padua, Italy) and the generated sequences were examined using Accelrys Gene 2.5

program (Accelrys, Cambridge, UK) and compared against the NCBI database through the use of BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

#### **Phylogenetic Analysis**

Multiple sequence alignments and sequence similarities were calculated using the Clustal W (Thompson et al., 1994) and the identity matrix options of BioEdit (Hall, 1999), respectively. Phylogenetic analyses were inferred by the neighbor-joining method using MEGA version 6 software.

#### **Nucleotide Sequence Accession Numbers**

*Babesia* sequences identified in this study were deposited in the GenBank database of the National Center for Biotechnology Information (https://submit.ncbi.nlm.nih. gov/). The GenBank accession numbers were assigned to the following genes: MN309736-MN309745.

#### RESULTS

Out of the 143 blood smears examined, 12 samples (8.39%) were positive for *Babesia* in sheep and 5 samples (3.5%) were positive in goats, respectively, as showed in Table 1.

All samples positive with microscopic examination of blood smears were also positive with the PCR test. Eight cases in sheep and three cases in goats became positive by the molecular method but were negative by the microscopic method. The performance of both tests in terms of detection of positive cases has been compared in Table 1. Statistically (*t*-test statistics) showed the significant difference between the performance of the PCR technique and microscopy for the diagnosis of hemoparasites.

Twenty-eight of 143 samples showed amplification of a 549-bp fragment with the primer forward (5-TGGGCAGGACCTTGGTTCTTCT-3) and reverse (5- CCGCGTAGCGCCGGCTAAATA -3), specific for *Babesia* spp. Among the 28 samples (20 sheep and 8 goats), respectively, were positive for *B. avis* by PCR.

All the amplicons of *Babesia* spp. from sheep and goats were chosen for genetic analysis. The comparison of the 18S SSU rRNA sequence in sheep shows 100% homology between (Mn309736 and Mn309741) with the recently reported sequence (KY283960) from Turkey

Table 1: Prevalence of Babesiosis among the examined host, using the different diagnostic techniques

Host type	No. of examined sample (clinically affected)	Positive sample microscopically	Prevalence (%)	Positive sample by molecular- based identification	Prevalence (%)
Sheep	98	12	12.24	20	20.41
Goats	45	5	11.11	8	17.78
Total	143	17	11.89	28	19.58

(Bilgic et al., 2017), as shown in Figure 1 and have one nucleotide difference (Mn309738 and Mn309740) in 165 (A to G), additionally have two nucleotide difference with (Mn309737) in 96 and 165 (A to G). The *B. ovis* sequence described in this study from Erbil (Mn309739) had three nucleotide differences. As well as, Figure 2 shows the comparison of *B. ovis* sequences accession number (Mn309742, Mn309743, Mn309744, and Mn309745) in goats with the accession number (KY581552) in Iran. A phylogenetic tree of *Babesia* was constructed from the 18S *rRNA* gene sequences of our amplicons and those available in GenBank [Figures 3 and 4].

#### DISCUSSION

*B. ovis* causing Babesiosis is the most frequent pathogen in sheep and goats with the rate 12.24% and 11.11%, respectively, using microscopical examination, these agreed with Hassan (2011), who showed that the rate of infection in sheep was (11.1%) from a total of 360 sheep in Iraq and Naderi et al. (2017) who was recorded 38 (9.9%) from a total 306 sheep in Iran because this observation could participate with veterinary service (both government and private) in this region to eradicate the vector by dipping of the animals twice in the year. The present results disagreed with that previously mentioned by Esmaeilnejad et al. (2015) in Iran, that Babesia spp. was 52 (18.5%) from a total of 280 samples, and with that of Hasan (2013) who revealed that the infection rate (22.1%) of Babesia spp. in sheep from a total of 384 in Syria. Furthermore, Shahzad et al., 2013, who was showed that 32 (16%) were positive from a total of 200 samples in Pakistan. Such variation in the prevalence may be attributed to several factors, including geographical variation and consequently,

	10	20	30	40	50	60	70	
KY283960, Sheep, Turkey	TGGGCAGGACCTTGG	TTCTTCTTTC	<b>TTGGTTTTG</b>	TACCTGGGTA	ATGGTTAATAG	GAACGGTTG	GGGGCATTCGTA	TTT
MN309736, Sheepq1, Iraq								
MN309737, Sheepq6, Iraq	•••••							
MN309738, Sheepq7, Iraq								
MN309739, Sheep8, Iraq								
MN309740, Sheep10, Irag								
MN309741 Sheep12 Trag								
incostin, succeptin, indq								
	110	120	130	140	150	160	170	1
				1				
KY283960, Sheep, Turkey	TAGATTTGTTAAAGAG	GGACCACTO	CGAAAGCAT	FIGCCAAGGA	GTTTCCATTA	ATCAAGAAC	AAAGTTAGGGG	ATC
MN309736, Sheepq1, Iraq								
MN309737, Sheepq6, Iraq	•••••			• • • • • • • • • • •			3	
MN309738, Sheepq7, Iraq							3	
MN309739, Sheep8, Iraq							3	
MN309740, Sheep10, Iraq							3	
MN309741, Sheep12, Iraq								
	210	220	230	240	250	260	270	2
EV283060 Sheen Tunker	TACTCCTAACCTTAA	CCATCOCC	CTACCATT	accorcorco	TTCCCACTCC	CTCACCACC	TGAGAGAAATC	20.05
kizosydo, sheep, furkey	TAGICCIAACCIIAA	ACCATOCCO	CTAGGGAT I	3000010010	ATTCCGACTCC	CICAGCACC	IIGAGAGAAAIC	ммм
MN309736, Sneepq1, Iraq	•••••							
MN309737, Sheepq6, Iraq	•••••				•••••			
MN309738, Sheepq7, Iraq	•••••				•••••	•••••	••••••	
MN309739, Sheep8, Iraq								
MN309740,Sheep10,Iraq								
MN309741, Sheep12, Iraq								
	310	320	330	340	350	360	370	3
KY283960, Sheep, Turkey	TATGGTCGCAAGTCT	AAACTTAAA	GGAATTGAC	GAAGGGCAC	ACCAGGCGTG	GAGCCTGCG	CTTAATTTGAC	TCA
MN309736 Sheepg1 Trag								
MN309737 Sheeng6 Trag								
MI200729 Shoopq7 Iraq								
Misos730, Sheepq7, Iraq								
MN309739, Sheep8, Iraq								
MN309740, Sheep10, Iraq	•••••							
MN309741, Sheep12, Iraq	•••••					•••••		• • •
	410	420	430	440	450	460	470	4
	· · · · [ · · · · ] · · · · ] ·			• • • •   • • • •				
KY283960, Sheep, Turkey	CCAGACAGAGGAAGG/	ATTGACAGA	TGATTGCTC	<b>TTTCTTGATT</b>	CTTTGGGTAGT	GGTGCATGG	CCGTTCTTAGTT	rggt
MN309736, Sheepq1, Iraq								
MN309737, Sheepq6, Iraq								
MN309738, Sheepq7, Iraq								
MN309739, Sheep8, Irag								
MN309740, Sheep10, Irag								
MN309741, Sheep12, Trag								
	510	520	530	540				
TV092060 65		1						
kize3960, Sneep, Turkey	TCCGTTAACGAACGA	ACCTTAACO	TGCTATTTA	JUUGGUGUTA	JUCGA			
MN309736, Sheepq1, Iraq	•••••	• • • • • • • • • • •						
MN309737, Sheepq6, Iraq	•••••	• • • • • • • • • • •		•••				
MN309738, Sheepq7, Iraq	•••••							
MN309739, Sheep8, Iraq	•••••		G					
MN309740, Sheep10, Trag								
incost ic, shoopic, itaq								

Figure 1: Parts of the sequence results of cloned *Babesia ovis* positive samples in sheep no. 1, 6, 7, 8, 10, and 12 were compared with published sequences available in the NCBI database (accession no. KY283960)

Hassan

KY581552,Goat,Iran MN309742,Goat1,Iraq MN309743,Goat3,Iraq MN309744,Goat6,Iraq MN309745,Goat8,Iraq	10 TGGGCAGGACCTTGC	20  . TTCTTCTTTGT	30 TGGTTTTGTA	40 	50 GGTTAATAGO	60     3AACGGTTGO	70 J.J.J.J.GGGGCATTCGTA	80 I. 
KY581552, Goat, Iran MN309742, Goat1, Iraq MN309743, Goat3, Iraq MN309744, Goat6, Iraq MN309745, Goat8, Iraq	110 TAGATTTGTTAAAG/ A1	120	130    GAAAGCATTT	140    GCCAAGGACC	150    TTTCCATTAJ	160 II ATCAAGAACO	170    GAAAGTTAGGGG	180  . ATCGA
KY581552,Goat,Iran MN309742,Goat1,Iraq MN309743,Goat3,Iraq MN309744,Goat6,Iraq MN309745,Goat8,Iraq	210 TAGTCCTAACCTTAA A A	220 I.I.I.I.ACCATGCCGAC	230   . TAGGGATTGC	240 	250 	260	270 IIIII TGAGAGAAATC	280  . :AAAGT
KY581552,Goat,Iran MN309742,Goat1,Iraq MN309743,Goat3,Iraq MN309744,Goat6,Iraq MN309745,Goat8,Iraq	310	320 	330   . GAATTGACGC	340 	350 	360    3AGCCTGCGC	370	380  . TCAAC 
KY581552,Goat,Iran MN309742,Goat1,Iraq MN309743,Goat3,Iraq MN309744,Goat6,Iraq MN309745,Goat8,Iraq	410 	420 	430    GATTGCTCTT	440  . TCTTGATTCT	450    TTGGGTAGT(	460     GGTGCATGGC	470 	480  . 'GGTGG
KY581552,Goat,Iran MN309742,Goat1,Iraq MN309743,Goat3,Iraq MN309744,Goat6,Iraq MN309745,Goat8,Iraq	510	520 II.	530 	540 	CGG			

Figure 2: Parts of the sequence results of cloned *Babesia ovis* positive samples in goats no. 1, 3, 6, and 8 were compared with published sequences available in the NCBI database (accession no. KY581552)

difference in climatic conditions that affect the vector activity, examined regions are neighboring countries and experimental methods might explain the difference in the results. In goat, of the 45 blood smears examined, 5 (11.11%) samples were positive for *Babesia* spp., this agreed with Hassan, 2011, who revealed that the rate of infection was 13.5% from a total 205 goats in Erbil Province and Abdul-Hassan and Ali (2016) revealed that 8/68 (11.7%) cases of examined goats were given positive result in AL-Qadisiya province. Esmaeilnejad et al., 2015, showed that 12.2% (15/122) were infected with *B. ovis* in goat. As well as, our result partially agreed with Sulaiman et al. (2010) in Mosul, who showed that the rate of infection was 15.4% from a total 175 goat and Iqbal et al. (2011) revealed that the infection rate 16 (24%). However, disagreed with Abdullah and Mohammed (2014), who showed that, the rate of infection 16/25 (64%) from a total of 25 goats in Sulaimani city. Furthermore, Naderi et al. (2017), in Iran, explained from a total of 51 sample 9 (2.3%) goats were infected.

Classically microscopic examination of Giemsa-stained blood smears is used to detect *Babesia* infections (Abdullah and Mohammed, 2014 and Esmaeilnejad et al., 2015). However, this method has a low sensitivity and requires expertise because these parasites have similar morphology and therefore, different species may be confused (Bilgic et al., 2017 and Gholamreza et al., 2017). In this study, Hassan





molecular epidemiology was used to diagnose this disease in sheep and goats from Erbil Province. *B. avis* was detected in the sampling region, suggesting that they may be important tick-borne diseases because the molecular prevalence of *B. avis* in small ruminants is a powerful tool and practical assay since it can detect extremely low parasitemia rates and simultaneously identify *Babesia* species (Chisua et al., 2019); moreover, the microscopic technique does not detect carrier animals with very low parasitemia (Rjeibi et al., 2014).

The rate of infection was higher 20 (20.41%) in sheep by molecular method than goats 8 (9.04%). It was stated that *B. ovis* induces symptoms more frequently in sheep than

goats (Bilgic et al., 2017) and the result agreed with (Aktas et al., 2007 and Iqbal et al., 2011) revealed that prevalence rate of *B. ovis* infection in sheep was higher than in goats in Turkey and Pakistan, respectively. Figure 3 revealed that a phylogenetic analysis of the partial 18S rRNA sequence was performed by aligning the six sequence types obtained in this study in sheep with selected *Babesia* sequences (AY260178, DQ409332, EF194112, JQ867387, KF459964, KP998109- KP998113, KU342694-KU342698, AY349159, KY581550, KR536611, AY533146, KP670199, KF723612, and KF723611) (Habibi et al., 2004; Altay et al., 2007; Rjeibi et al., 2014; Erster et al., 2016; Rjeibi et al., 2016; Ozubek and Aktas, 2017; and Zhou et al., 2017).



Figure 4: Phylogenetic positioning of *Babesia ovis* isolated from goats according to the sequence of 18S *SSU rRNA* constructed in MEGA ver.7 using the neighbor-joining method

Furthermore, in goats [Figure 4] show phylogenetic analysis with the selected sequences in gene bank (HM241887- HM241890, KF459964, KF681515, KY581552, KY581551, AY150058, and DQ287954) (Criado et al., 2006; Rjeibi et al., 2014; Zhou et al., 2017; and Sevinc et al., 2018). The phylogram showed that *B. ovis* from this study was placed in the same clade with all other sequences in this phylogenetic tree. Similar results were reported in Turkey and Iran where small ruminants were only infected by *B. ovis* (Altay et al., 2007 and Esmaeilnejad et al., 2014). As well as, the long branch length revealed that *B. ovis* obtained in this study has an increased degree of genetic divergence concerning the other selected sequences obtain in a gene bank, which suggests that geographical location could be the reason for this difference.

## CONCLUSION

This study has detected the presence of *B. ovis* in small ruminants in Erbil provinces. The sheep are more sensitive than goats to infection by using microscopic and PCR. A second method is a powerful tool, high sensitivity, and practical assay since it can detect extremely low parasitemia rates and simultaneously identify *Babesia* species than the first one. Sequencing and phylogenetic analysis showed that *B. ovis* in Erbil was not different from the pathogens in other animals from geographically distinct regions. These data provide important information about the incidence of *B. ovis* in sheep and goat and its benefits for managing and controlling programs of the disease.

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