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Molecular prevalence of *Anaplasma phagocytophilum* in sheep from Iraq

Karrar Jasim Hamzah[1*] and Saleem Amin Hasso[2]

[1]*Department of Veterinary Internal and Preventive Medicine, College of Veterinary Medicine, Al-Qasim Green University, Babylon, Iraq*
[2]*Department of Veterinary Internal and Preventive Medicine, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq*

Abstract
Background: Tick-borne diseases are widely distributed among animal populations and are responsible for significant economic losses. However, little attention has been offered for screening such infections world widely. *Anaplasma phagocytophilum* is among those neglected tick-borne pathogens, particularly in the developing countries.
Aim: This study was conducted to detect *A. phagocytophilum* infection among sheep in three governorates of Iraq (Babylon, Wasit, and Missan) and try to identify the potential tick vector responsible for *A. phagocytophilum* transmission among sheep in these analyzed regions.
Methods: A total of 297 blood samples and 103 ticks were collected and examined for *A. phagocytophilum* by polymerase chain reaction using specific primers amplifying partial sequence for *msp4* gene.
Results: The results showed that about 14 out of 297 tested sheep were positive for *A. phagocytophilum*. There was no difference between *A. phygocytophilum* prevalence according to animal gender, age, and sampling period. Furthermore, our analysis showed that the main vectors of *A. phagocytophilum*: *Ixodes scapularis*, *I. pacificus*, or *I. ricinus* were not identified in three regions of Iraq (*Rhipicephalus turanicus*, *Hyalomma anatolicum*, and *Hyalomma turanicum* were identified).
Conclusion: These results highlight the importance of the survey of the tick-borne bacterial infections in Iraq and in the Middle East region in general.
Keywords: *Anaplasma phagocytophilum*, Iraq, Prevalence, Sheep, Ticks.

Introduction

Anaplasma phagocytophilum infection is recorded mainly in sheep and a wide range of domestic animals. *Anaplasma phagocytophilum* also considered as a zoonotic for humans as well. *Anaplasma phagocytophilum* infection is a tick-borne transmitted disease mainly caused by ixodid tick species (Ogden *et al.*, 2003; Ben Said *et al.*, 2018). The number of clinical cases of human granulocytic anaplasmosis is increasing in Europe and Asia (Park *et al.*, 2003; Ruscio and Cinco, 2003; Ohashi *et al.*, 2005). However, the distribution of *A. phagocytophilum* is determined by the population of tick vectors and reservoir host species (Stuen *et al.*, 2013). In North America, the common vectors are *Ixodes scapularis* and *I. pacificus* (Teglas and Foley, 2006). However, in Europe, the *I. ricinus* is the main tick vector for *A. phagocytophilum* (Woldehiwet, 2006). In the Middle East, the *Ixodes ricinus* is reported as the main possible tick vector for transmitting *A. phagocytophilum* in Iran and —Turkey (Gokce *et al.*, 2008; Noaman and Shayan, 2009). The detection of *A. phagocytophilum* using molecular methods like the polymerase chain reaction (PCR) is considering as an effective and sensitive method (Smrdel *et al.*, 2015; Stuen, 2016; Yang *et al.*, 2016).

Tick-borne fever (TBF) caused by the bacterium *A. phagocytophilum* transmitted by the tick *I. ricinus* is one of the main challenges for sheep farming industry during the grazing season (Grøva *et al.*, 2011). The infection with *A. phagocytophilum* is widespread in the USA (Foley *et al.*, 2008), in Europe (Woldehiwet, 2006), and in Asia (Jilintai *et al.*, 2009; Yang *et al.*, 2013). Anaplasma infection has been also reported in some Middle East countries like Turkey (Gokce *et al.*, 2008) and in Iran (Noaman and Shayan, 2009).

This study was aimed to detect *A. phagocytophilum* in sheep located from three regions of Iraq (Babylon, Wasit, and Missan). Furthermore, this study tried to detect and identify the tick vector responsible for *A. phagocytophilum* transmission in these three areas.

Materials and Methods

Animals and data collection

About 400 samples divided into 297 sheep and 103 ticks were involved in this study. Sheep were mixed in gender and the ages were ranging from less than 1 year to more than 5 years. They were randomly selected from three different areas of Iraq (Babylon, Wasit, and Missan) during the period from February to October 2018.

***Corresponding Author:** Karrar J. Hamzah. College of Veterinary Medicine, Al-Qasim Green University, Babylon, Iraq.
Email: *karraraljanabi@vet.uoqasim.edu.iq; dr.karraraljanabi41@gmail.com*

Collection of blood samples

The blood samples were collected from the jugular vein of sheep using EDTA-vacutainer tube. All samples were transferred using cooling boxes. All the laboratory tests were performed at the laboratories of the College of Veterinary Medicine—Al-Qasim Green University.

Collection of ticks

Ticks were collected during the period from February to October 2018. A total of 103 ticks were collected and isolated from the same sheep that were involved in this study from the three aforementioned areas. After collection, the ticks were stored separately in 70% ethanol for classification purposes and for the molecular detection of *A. phagocytophilum* carriage. A stereomicroscope was used to identify the ticks based on their morphological features, such as mouthparts, scutum, color of legs, festoons, interstitial punctuations, presence or absence of adanal shields, posterior groove, and marginal spots (Hoogstraal, 1956; Hosseini-Chegeni *et al.*, 2013).

DNA extraction

The genomic DNA of *A. phagocytophilum* from sheep blood samples and ticks was extracted using a DNA mini extraction kit (Geneaid Biotech Ltd, Taiwan) according to the company's instructions. Extracted genomic DNA of *A. phagocytophilum* was analyzed for a given concentration range of 15–60 ng/µl. The purity of extracted DNA was analyzed using a Nanodrop spectrophotometer at (260/280 nm).

Polymerase chain reaction (PCR)

The PCR technique was performed for the detection of *A. phagocytophilum* in the blood samples and ticks according to the method described by Yang *et al.* (2013). The two versions of extracted DNA (from the sheep blood or ticks) were tested for the presence of *A. phagocytophilum* using PCR by the amplification of the major surface protein 4 coding gene (*msp4*). Specific primers were designed and used: the forward primer is F: 5′-ATGCGTCTGATGTTAGCGGT-3′ and the reverse primer: R: 5′-AAAACTCGCCCCTAACCCAG-3′. The product size is about 503 bp.

DNA sequencing

In order to confirm that the positive PCR products (503-bp band) belong to the *A. phagocytophilum* genome and to test the specificity of the primers for detecting *A. phagocytophilum* in sheep blood and ticks, the products of three positive PCRs were sequenced using Sanger's sequencing method at Macrogen, Korea. The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA (MEGA 6.0 version).

Statistical analysis

The results were analyzed using SPSS statistical software. A Chi-square test was used to assess the association between the variable results. *p*-value of ≤0.05 was considered to be significant.

Results

Detection of A. phagocytophilum DNA in sheep blood

PCR results showed that 14 out of 297 samples (4.71%) were positive to *A. phagocytophilum* (Fig. 1). Furthermore, the infection rate was higher in Wasit and Missan (7.81% (5/64) and 7.04% (5/71), respectively) compared to that in Babylon region (2.46% (4/162) (Table 1 and Fig. 2).

However, the results also showed that the infection rate of *A. phagocytophilum* was not affected by the gender of the examined sheep (Table 2). Indeed, the infection rate was barely equal in both males (2.08%) and females (5.22%) of sheep (Table 2).

Interestingly, results showed that the highest rate of infection was recorded in older sheep (aged 5 years or above) with a rate of 6.25% (Table 3). In contrast, the lowest infection rate was recorded at age less than 2 years (3.57%, Table 3).

Seasonal variation analysis showed that the highest infection rate of *Anaplasma phagocytophilum* was recorded during the summer season of Iraq (May, June, and July: 3/45 (6.66%), 3/48 (6.25 %), and 4/51 (7.84%), respectively) (Table 4 and Fig. 3). However, there were non-significant variations among these months. On the other hand, all samples that were collected during February, March, and October were negative for *A. phagocytophilum* (Table 4 and Fig. 3).

Fig. 1. Agarose gel electrophoresis image showing PCR product analysis of the major surface protein (*MSP4*) gene in *A. phagocytophilum* positive isolates. Where: M: marker (100–2,000 bp), lane (1–13) positive *A. phagocytophilum* at (503) bp PCR product, NC negative control.

Table 1. Infection rate of *A. phagocytophilum* by PCR technique.

Governorate	Number of blood samples	Infected	(%)
Babylon	162	4	2.46[A]
Misan	71	5	7.04[A]
Wasit	64	5	7.81[A]
Total	297	14	4.71[A]

X2: chi-square value (X2 = 5.223).
Similar letters denote the non-significant differences at $p < 0.05$

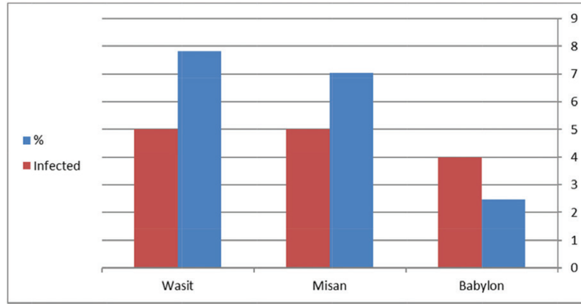

Fig. 2. Prevalence of *A. phagocytophilum* according to regions. Babylon 4/162, Misan 5/71, and Wasit 5/64. Red color representative numbers of infected sheep with *A. phagocytophilum*, % representative the percentages of infection.

Table 2. Infection and gender relationship of blood samples examined by PCR technique.

Sex	Number of blood samples	Infected	(%)
Male	48	1	2.08^{A}
Female	249	13	5.22^{A}
Total	297	14	4.71

X2: chi square value (X2 = 0.799).
Similar letters denote the non-significant differences at $p < 0.05$.

Table 3. Infection rates of *A. phagocytophilum* and age of sheep examined by PCR technique.

Age/ years	Number of blood samples	Infected	(%)
<2	84	3	3.57^{A}
2–5	117	5	4.27^{A}
>5	96	6	6.25^{A}
Total	297	14	4.71

X2: chi square value (X2 = 2.426).
Similar letters denote the non-significant differences at $p < 0.05$.

Survey of A. phagocytophilum in ticks

PCR result indicated that collected ticks were identified as *Rhipicephalus turanicus*, *Hyalomma anatolicum*, and *Hyalomma turanicum* and none of these examined ticks (total 103) was carrying *A. phagocytophilum* (Table 5).

Confirmation of PCR products by sequencing

The results of *A. phagocytophilum* sequencing were analyzed and annotated according to *A. phagocytophilum* genomes that banked in NCBI GenBank database using Basic Local Alignment Search Tool (BLAST).

Phylogenetic analysis of A. phagocytophilum

To investigate the possible genetic relationship between *A. phagocytophilum* strains detected in the current study and *A. phagocytophilum* available in GenBank database at the NCBI, a simple phylogenetic tree was generated based on the partial sequences of major surface protein 4 coding gene (*msp4*). The partial sequences of *msp4* gene of three positive *A. phagocytophilum* samples (GenBank accession numbers MK423169, MK423170, and MK423171) were shown to be closely related to *A. phagocytophilum* isolates found in GenBank (Table 6 and Fig. 4).

Table 4. Infection rate of *A. phagocytophilum* according to months of study by PCR examination.

Months	Number of blood samples	Infected	(%)
February	12	0	0^{A}
March	14	0	0^{A}
April	40	1	2.50^{A}
May	45	3	6.66^{A}
June	48	3	6.25^{A}
July	51	4	7.84^{A}
August	37	2	5.40^{A}
September	24	1	4.16^{A}
October	26	0	0^{A}
Total	297	14	4.71

X2: chi square value (X2 = 4.810).
Similar letters denote the non-significant differences at $p < 0.05$.

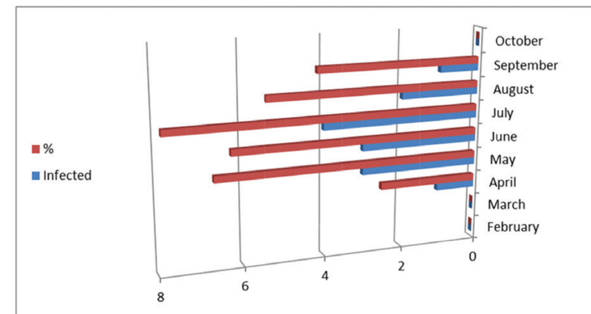

Fig. 3. Infection rate according to months. Red color representative numbers of infected sheep with *A. phagocytophilum*, % representative the percentages of infection. February 0/12, March 0/14, April 1/40, May 3/45, June 3/48, July 4/51, August 2/37, September 1/24, and October 0/26.

Table 5. Relationships between infection of *A. phagocytophilum* with tick infestation.

Governorate	Number of infected sheep with *A. phagocytophilum*	Number of infested sheep with ticks
Babylon	4	54
Misan	5	25
Wasit	5	24
Total	14	103

Table 6. NCBI-BLAST homology sequence identity (%) for *msp4* gene in local *A. phagocytophilum* and NCBI-Blast *A. phagocytophilum.*

NCBI BLAST Isolate	Accession No	Country	Host	NCBI-BLAST Homology sequence identity (%)
A. phagocytophilum	MK423171.1	Iraq	Sheep	100
A. phagocytophilum	MK423169.1	Iraq	Sheep	100
A. phagocytophilum	MF974860.1	Hungary	Capreolus capreolus	100
A. phagocytophilum	MF974859.1	Hungary	Capreolus capreolus	100
A. phagocytophilum	KM205434.1	Slovenia	Capreolus capreolus	100
A. phagocytophilum	EU180060.1	Slovakia	Roe deer	100
A. phagocytophilum	EU180058.1	Slovakia	Roe deer	99.801
A. phagocytophilum	MF974848.1	Hungary	Cervus elaphus	99.600
A. phagocytophilum	MF974852.1	Hungary	Dama dama	99.602
A. phagocytophilum	KM205426.1	Slovenia	Cervus elaphus	99.205
A. phagocytophilum	KM205420.1	Slovenia	Rupicapra rupicapra	99.205
A. phagocytophilum	EU180062.1	Slovakia	Roe deer	98.807
A. phagocytophilum	AY706391.1	Slovakia	Roe deer	98.807
A. phagocytophilum	MK423170.1	Iraq	Sheep	99.391
A. phagocytophilum	KP861636.1	Spain	Ixodes sp	98.41
A. phagocytophilum	EU857668.1	France	Cow	98.41
A. phagocytophilum	KM205427.1	Slovenia	Cervus elaphus	98.211
A. phagocytophilum	KM205429.1	Slovenia	Cervus elaphus	98.16
A. phagocytophilum	AY706391.1	Norway	Ovine	98.014
A. phagocytophilum	KM205439.1	Slovenia	*Ixodes ricinus* (tick)	98.012
A. phagocytophilum	KM205435.1	Slovenia	Capreolus capreolus	98.012
A. phagocytophilum	KM205433.1	Slovenia	Capreolus capreolus	98.012
A. phagocytophilum	KM205431.1	Slovenia	Capreolus capreolus	98.012
A. phagocytophilum	KM205428.1	Slovenia	Capreolus capreolus	98.012
A. phagocytophilum	JX841250.1	France	Rangifer tarandus (reindeer)	98.012
A. phagocytophilum	HQ661160.1	Slovak Republic	*Ixodes ricinus*	98.012
A. ovis	HQ456350.1	China	Sheep	65.30
A. ovis	KY283958.1	Turkey	Sheep	65.30
A. ovis	FJ460452.1	Cyprus	Sheep	65.35
A. ovis	EF190513.1	Hungary	Sheep	63.50
A. ovis	LC412081.1	Mongolia	Sheep	63.50
A. ovis	DQ674246.1	USA	Sheep	63.50
A. ovis	KU497699.1	Sudan	Sheep	63.50
A. ovis	LC141078.1	Mongolia	Cattle	65.35
A. ovis	MH790274.1	Iran	Sheep	65.35
A. ovis	MG283274.1	China	Sheep	65.30

Discussion

This study targeted the detection of *A. phagocytophilum* in sheep blood and infesting ticks by amplifying the major surface protein 4 coding gene (*msp4*). This gene is reported to be conserved for *A. phagocytophilum* (de la Fuente *et al.*, 2005a; Silaghi *et al.*, 2011; Öter *et al.*, 2015). This consistent infection rates result with what has been found in previous studies conducted by Derdáková *et al.* (2011)

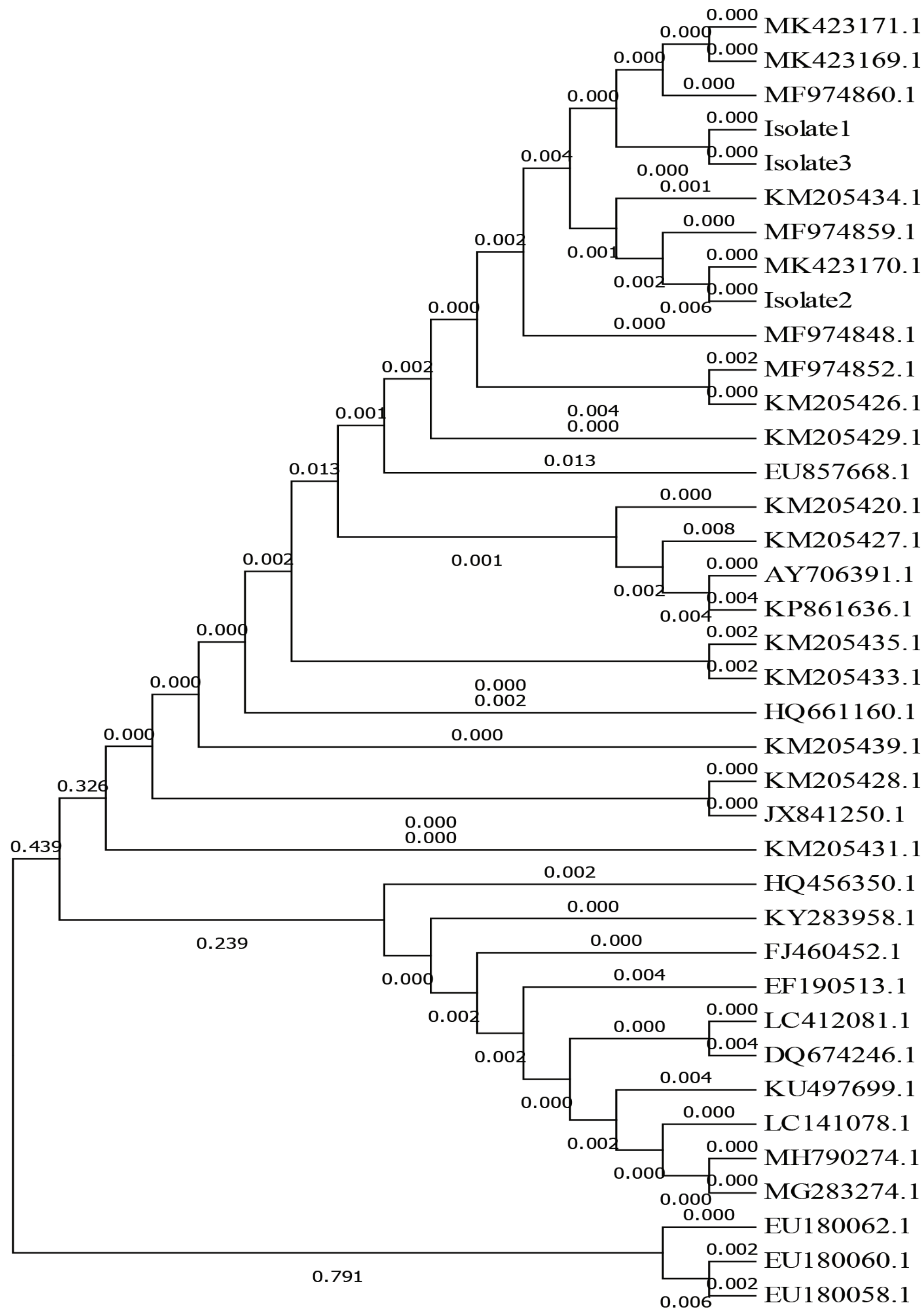

Fig. 4. Phylogenetic tree of *A. phagocytophilum* by Maximum Likelihood method based on partial sequence major surface protein 4 (*MSP4*) and relating the local strain of *A. phagocytophilum* Gene Bank accession numbers sequence MK423169, MK423170, and MK423171 to other isolated strains, the Gene Bank accession numbers of EF190513.1, LC141078.1, KU497699.1, MH790274.1, LC412081.1, MG283274.1, KY283958.1, HQ456350.1, FJ460452.1, and DQ674246.1 were related to *Anaplasma ovis*.

Furthermore, Ben Said *et al.* (2017) demonstrated that in Tunisia, prevalence rates of strains genetically related to *A. phagocytophilum* in sheep was 3.9%. Using PCR method, Scharf *et al.* (2011) mentioned that the infection rate of *A. phagocytophilum* was 4% in sheep.

However, Kiilerich *et al.* (2009) reported that the infection rates of *A. phagocytophilum* in Denmark, Italy, and Turkey were higher than those of the current study and of Derdáková *et al.* (2011), Ben Said *et al.* (2017), Scharf *et al.* (2011). Indeed, the infection rates have been reported to be variable between countries and could even vary considerably between neighboring farms (Stuen *et al.*, 2002b). The prevalence of *A. phagocytophilum* infection in sheep has been reported to range from 0.51% to 80% worldwide (Stuen and Bergström, 2001; de la Fuente *et al.*, 2005b).

Our results showed that infection rates slightly vary according to the sex and conclude that the female sheep have a higher infection rate than males. This could be due to the fact that females are more exposed to different physiological changes such as stresses caused by pregnancies, parturition, and lactation (Weiss and Wardrop, 2011). The age group of more than 5 years showed the highest infection rates, while the sheep aged from 2 to 5 years recorded the lowest rates.

These results are compatible with Chaudhry *et al.* (2010) who mentioned that the highest rate of infection in adult animals could be due to the chronicity of infection. Ben Said *et al.* (2015) also reported that the infection rate with *A. phagocytophilum*-like strains was higher in adult compared to young sheep. Extensive study by (Egenvall *et al.*, 2000) showed that higher infection rates were significant in older ages. These results also agreed with Radostits *et al.* (2006). Poitout *et al*. (2005) discussed the seasonality and geographical distribution of *A. phagocytophilum* infections and found that this due to the feeding habits of each particular tick vector. Most cases were observed between April and July, with fewer cases observed in October. In Berlin (Germany), Kohn *et al*. (2008) reported that most cases occurred between April and September, and the seasonal distribution of the disease most likely reflects periods of peak tick activities. This finding is in agreement with Beall *et al*. (2008). The common consequence of the infection with *A. phagocytophilum* in sheep is the direct and indirect losses and causes immunosuppression status that may lead to secondary infections. *A. phagocytophilum* infection causes direct losses and mortality rates of 30% lamb in the flock (Stuen and Kjølleberg, 2000). The extent of indirect production loss due to TBF decreases the bodyweight of lamb (Stuen *et al*., 2002a).

Jensen *et al.* (2007) indicated that a high infestation with ticks could increase the incidence of *A. phagocytophilum* infection compared to those with a low rate of tick infestation. Kohn *et al.* (2011) also mentioned that *A. phagocytophilum* infection is higher in the months of tick activity. The present results are also supported by Chvostáč *et al.* (2018) and Grøva (2011) who reported that the seasonal dynamics of tick activity was the highest in April, May, June, and July when correlated with the highest prevalence of *A. phagocytophilum* infection. Overall, this study is a pioneering study regarding molecular detection of *A. phagocytophilum* in local Iraqi sheep from three different regions of Iraq. To our knowledge, this study is the first study that reported *A. phagocytophilum* infection in Iraqi sheep.

Conclusion

PCR method complemented by sequencing step seems to be accurate and confirmative for the detection and the genetic characterization of *A. phagocytophilum* in sheep. Furthermore, our results suggested that other tick species might be responsible for the transmission of *A. phagocytophilum* to Iraqi sheep.

Acknowledgment

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Conflict of interest

We declare no conflicts of interest in relation to this paper.

Author Contributions

Karrar Jasim Hamzah and Saleem Amin Hasso conceived and wrote the study. All authors read and approved the final manuscript.

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