Research Article

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Molecular detection and phylogenetic analysis of *Anaplasma phagocytophilum* bacteria in cows and it is infested ticks in Mosul city, Iraq

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Abstract

The present study was conducted to molecular detection of *Anaplasma phagocytophilum* in infected cows and it is infested ticks in Mosul, Iraq using nested polymerase chain reaction technique (N-PCR) and to investigate the phylogenetic analysis of *A. phagocytophilum* diagnosed in this study. A total of 50 blood samples (3 ml) were collected from cows in various areas of Mosul city. Additionally, 169 hard ticks were collected from different parts of infected cows. Results revealed that the infection rate of *A. phagocytophilum* in cows at Mosul city was 72% and in engorged female ticks was 81.2% using N-PCR technique. The infestation rate hard ticks on cows were 46% and four species of hard ticks were microscopically identified and classified includes *Hyalomma anatolicum anatolicum* (39.64%), *Rhipicephalus turanicus* (21.89%), *Rh. sanguineu*s (23.07%) and *Boophillus annulatus* (15.38%) with significantly predominant *Hyalomma anatolicum anatolicum.* The individual sequencing analysis for five sequences of 16S rRNA gene includes: one extracted from cow blood and four extracted from engorgement female ticks. The sequences of *A. phagocytophilum* were recorded in the NCBI Genbank under the accession numbers (OR002120.1, OR002121.1, OR002122.1, OR002123.1 and OR002124.1). These sequences were highly identity (100%) to those sequences recorded in the NCBI Genbank such as (MT221233.1, MT221234.1) in Norway, (MK239930.1, MK239931.1) in Southern Korea, (MN170722.1, MN170722.1) in Spain, (MK814411.1, MK814412.1) in South Africa, (LC435049.1, LC435050.1) in Japan and (MH122889.1, OQ727069.1) in Poland, and Malawi respectively. This finding might be advantageous for the future studies and strategically control of this bacterium in the study area.

Keywords: *Anaplasma phagocytophilum*, Phylogenetic analysis, Mosul.

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Introduction

Anaplasma phagocytophilum is an important pathogen of emerging infectious blood diseases (1, 2), infects different types of animals including cattle, sheep, goats, horses, dogs, cats, camels, deers, rodents, wild mammals, and birds (3-6), as well as humans (7).*Anaplasma phagocytophilum* causing tick-born fever (TBF) disease or called pasture fever disease in ruminants (8,9,10). It is an obligate intracellular, gram-negative polymorphic bacterium, belongs to the order Rickettsiales of the family Anaplasmataceae and genus Anaplasma (11).

Studies indicate that there are many genes can be used to determine the strains of *A. phagocytophilum* bacteria, which registered in the NCBI Genbank, including 16S rRNA, msp4, msp2, groEL (12), GroEL, HSP70 (2), MLST, anKA (13), GltA (6). There are six sub-serotypes of *A. phagocytophilum* including *A. phagocytophilum* 1, *A. phagocytophilum* 3, *A. phagocytophilum* 5, *A. phagocytophilum* 7, *A. phagocytophilum* A-HE, A. *phagocytophilum* A-D-HE (14).

Tick-born fever disease is mainly transmitted by Ixodidae ticks such as *Ixodes ricinus*, *I. scapularis*, *I. pacificus*, *I. spinipalpis*, *I. Persulcatus* (15), *Hyalomma anatolicum anatolicum*, *H. Anatolicum excavatum, H. detritum, H. turanicum, H. Scupense, H. marginatum, Boophilus annulatus, Rhipicephalus turanicus, Rh. Sanguineus, Rh. pusillus, Haemaphysalis punctate* (16-19) and *Dermacenter albipictus* (20). Additionally, the disease can be mechanically transmission and/or transplacental or intrauterine transmission (8,21,22). *Anaplasma phagocytophilum* infection results in significant economic losses due to high mortality rates, decreased milk production, stillbirths, and abortions, as well as the costs associated with treating infected animals and eradicating the agent that spreads the infection (18,23). Furthermore, about 42 nations where the disease is endemic, including those in Asia, Europe, Africa, and America (24-27), and the case fatality of infected animals reaches to 5% (28).

Clinical signs are rarely help in diagnosis of the disease, as it is associated with other blood diseases in cattle, such as Theileriosis (29), Babesiosis (30) and Trypanosomiasis (31). Therefore, a variety of laboratory tests are carried out to confirm the diagnosis of *A. phagocytophilum* such as microscopic examination of the blood smears stained with Giemsa for detection morulae of *A. phagocytophilum* (32), serological tests like indirect enzyme-linked immunosorbent assay, Indirect fluorescent antibody test (12), and the rapid SNAP 4Dx Plus test (33), Furthermore, molecular techniques such as nested polymerase chain reaction (PCR) techniques (34), and quantitative real- time PCR technique (18).

The presence of *A. phagocytophilum* in cows has been documented in several Iraqi provinces, including Nineveh (35), Al-Qadisiyah, Al-Najaf Al-Ashraf, and Babylon (3,32). Due to few research on detecting *A. phagocytophilum* in cows in Mosul city of Iraq using recent laboratory methods. Therefore, the present study aimed to detection of *A. phagocytophilum* in cows and it is associated ticks for the first time in Mosul city-Iraq using N-PCR technique, and to investigate the phylogenetic analysis of *A. phagocytophilum* diagnosed in the current study

Materials And Methods

Ethical approval

The present study was ethically permitted by the animal ethics committee of the college of veterinary medicine, university of Mosul, (UD.VET. 2022.031) on the 1st of August 2022.

Animals and samples collections

This study was included 50 cows from both sexes, and different ages (1-5years), breeds, and management practices obtained from various regions of Mosul city, which was clinically suspected infected with *A. phagocytophilum.* During the period from September 2022 to April 2023, 50 blood samples (3ml of blood) were drawn from all cows via jugular vein then kept in tubes with anticoagulant Ethylene diamine aceticacid (EDTA), which were stored at -20°C until tested using N-PCR technique. Furthermore, 169 hard ticks -Ixodid ticks- (60 males and 109 females) were collected from different parts of body, then the males and non-engorgement females' ticks were kept in formalin (10%) until microscopic identification. While, other engorgement females' ticks were kept in ethanol (70%) at 4°C until microscopic identification and detection of *A. phagocytophilum* inside them using N-PCR technique.

Identification and classification of ticks

Based on morphological characterization using a stereoscopic microscope and in accordance with taxonomic keys (36,37,38), the identification and classification of hard ticks (n=169) at the genus and species levels was done.

DNA extraction and amplification for N-PCR technique

The DNA were extracted from cows' blood samples (n=50) and from engorgement female ticks (n=16) fed naturally on cows' blood using the AddPrep Genomic DNA Extraction Kit (Add Bio, Korea). The process was performed as mentioned by the manufacturer. Using the Nanophotometer (BioDrop, Germany), regarding to wavelength 260nm the concentration of extracted DNA was ranged between 80.9 - 370.5 ng/ µl. Additionally, the purity of extracted DNA, calculated by ratio of (A260 nm to A280 nm), which was between 1.7 - 1.9.

To amplify the highly conserved 16S rRNA gene of *A. phagocytophilum* using N-PCR technique. The DNA extracted from clinically and laboratory positive cow for *A. phagocytophilum* was used as a positive control. Furthermore, the extracted DNA from healthy and add all PCR competent except DNA used as negative control. The oligonucleotides of specific primers were designed by Yousefi *et al*. (39). These primers were supplied by (Macrogen Inc. South Korea), which comprising for the first round (1st R.) primer P1 (5’-AGAGTTTGATCCTGGCTCAG-3’) and primer P2 (5’-AGCACTCATCGTTTACAGCG-3’) and for the second round (2nd R.) primer P4 (5’-GTTAAGCCCTGGTATTTCAC-3’) and primer P6 (5’-CTTTATAGCTTG CTATAAAGAA-3’). To identify the positive cows and positive ticks for Anaplasma spp. using the primers (P1 and P2), were in approximately band size at 781 bp. in the 1st R., and to identify the positive cows and positive ticks for *Anaplasma phagocytophilum* using the primers (P4 and P6), were in approximately band size at 509 bp. in the 2nd R.

Moreover, the 1st R. and 2nd R. of the N-PCR technique were performed with a total volume of 25µl for each, including (2X) master mix 12.5µl, each primer (P1 and P2), (P4 and P6) 1µl (10 pmol) respectively, template DNA 2µl for 1st R. and PCR product 1µl from 1st R. as template DNA for 2nd R., and complete the total volume with nuclease-free water 8.5µl and 9.5µl respectively. The program setting for the thermocycler (BIO-RAD/ USA) for the 1st R. and 2nd R. were as follows: predenaturation step at 95°C for 5min (1 cycle), denaturation step at 94°C for 30s, annealing step at 58°C for 1min (1st R.), at 57°C for 1min (2 nd R.) and extension step at 72°C for 45s (35 cycles), with a final extension step at 72°C for 5 min (1 cycle), as described previously (39), with some steps modification. The final PCR products were loaded in a agarose gel (1.5%) that was stained with Safe-Red™ dye, and the resulting bands were visualized under UV transillumination (BIO-RAD/ USA).

Sequencing of cDNA

For purification and sequencing five of PCR amplicons composing (one from cow and four from engorgement female ticks), which were tested positive to *A. phagocytophilum* using Nested- PCR technique, which sent to Macrogen Company (South Korea). The sequences of 16S rRNA were analyzed using multiple sequence alignment with online tool (CLUSTALW) GenomeNet and then compared with other available BDV sequences in GenBank by NCBI BLAST (BLASTn) from NCBI (http://www.ncbi.nlm.nih.gov). The Likelihood method on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings (40). Furthermore, the 16S rRNA gene sequence of MT052416-*Anaplasma capra* -Tick- South Korea were employed as an outgroup in the create phylogenetic tree.

Statistical analysis

X^2- test and Kappa value were used by IBM-SPSS Version 22 (Inc., Chicago, USA), to analyze the data in the present study. Statistically significant data was determined at the P value ≤ 0.05.

Results

The current study was revealed that the infection rate of *A. phagocytophilum* in cows at Mosul city was 72% (36 out of 50) and in engorged female ticks was 81.2% (13 out of 16) using N-PCR technique (Table1). Furthermore, the amplified DNA fragments of the 16S rRNA gene for *A. phagocytophilum* in 50 blood samples from cows using N-PCR technique observed in the 1st R. the positive cows for Anaplasma spp. in approximately band size at 781 bp. and the positive cows for *A. phagocytophilum* in approximately band size at 509 bp. in the 2nd R. (Figure 1).

The results of the study were also revealed that the infestation rate hard ticks on cows was 46% (23 out of 50) and four species of hard ticks were microscopically identified and classified, were including *Hyalomma anatolicum anatolicum (*39.64%), *Rhipicephalus turanicus* (21.89%), *Rh.*

sanguineus (23.07%) and *Boophillus annulatus* (15.38%) with significantly (P<0.05) predominant *Hyalomma anatolicum anatolicum compared with other species* (Figure 2,3). Moreover, the amplified DNA fragments of the 16S rRNA gene for *A. phagocytophilum* in 16 engorged female ticks using N-PCR technique observed in the 1st R. the positive ticks for Anaplasma spp. in approximately band size at 781 bp. and the positive ticks for *A. phagocytophilum* in approximately band size at 509 bp. in the 2nd R. (Table 2),(Figure 4).

In the present study, the individual sequencing analysis (BLASTn) for five sequences of 16S rRNA gene includes: one extracted from cow blood and four extracted from engorgement female ticks. The sequences of *A. phagocytophilum* were recorded in the NCBI Genbank under the accession numbers (OR002120.1, OR002121.1, OR002122.1, OR002123.1 and OR002124) (Table 3). These sequences were highly related (100%) to those sequences has been recorded in the Genbank such (MT221233.1, MT221234.1) in Norway, (MK239930.1, MK239931.1) in Southern Korea, (MN170722.1, MN170722.1) in Spain, (MK814411.1, MK814412.1) in South Africa, (LC435049.1, LC435050.1) in Japan and (MH122889.1, OQ727069.1) in Poland, and Malawi, respectively (Table 4).

Additionally, the analysis of phylogenetic tree using maximum likelihood method in MEGA11 software revealed that the local sequences of *A. phagocytophilum* was closely related (100% identity) to those available sequences of *A. phagocytophilum* in the GenBank database that mentioned above. The tree was rooted with MT052416-*Anaplasma capra* -Tick- South Korea as an outgroup (Figure 5).

Table 1: Infection rate of *Anaplasma phagocytophilum* in cows' blood and engorgement female ticks using N-PCR technique.

Type of samples	No. of tested cows	No. of positive	Infection rate %
Cows' blood	50	36	72
Engorgement female ticks	16	13	81.2

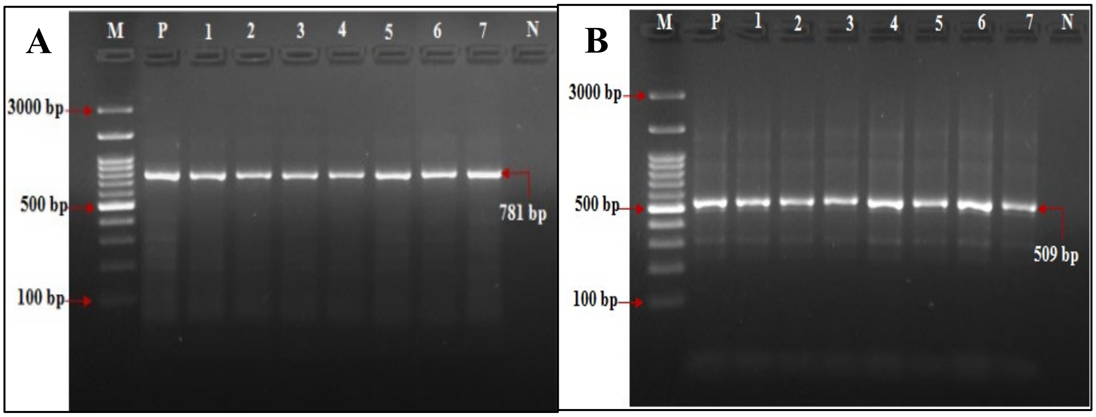

Figure 1: Representing A: the 1st R. of N-PCR, Lanes 1-7) The positive cows for Anaplasma spp. in approximately band size 781bp.; B: the 2st R. of N-PCR. Lanes 1-7) The positive cows for *A. phagocytophilum* in approximately band size 509 bp. Lane M: Exact Mark 100-3000bp DNA ladder; Lane P) DNA extracted from infected cow used as positive control for *A. phagocytophilum*; Lane N) add all PCR competent except DNA used as negative control.

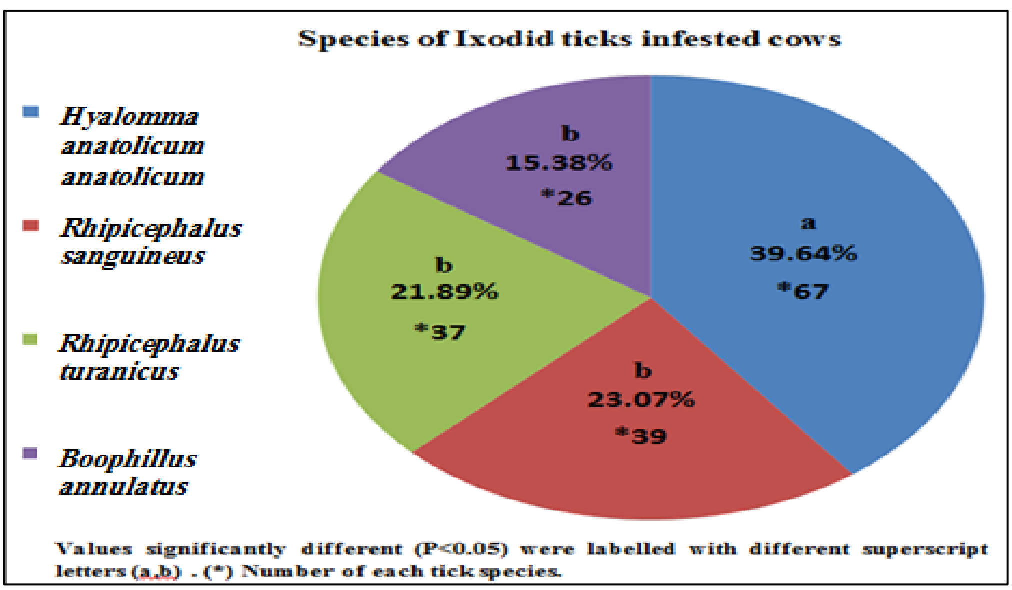

Figure 2: The species and infestation rate of hard ticks infested cows (n=169)

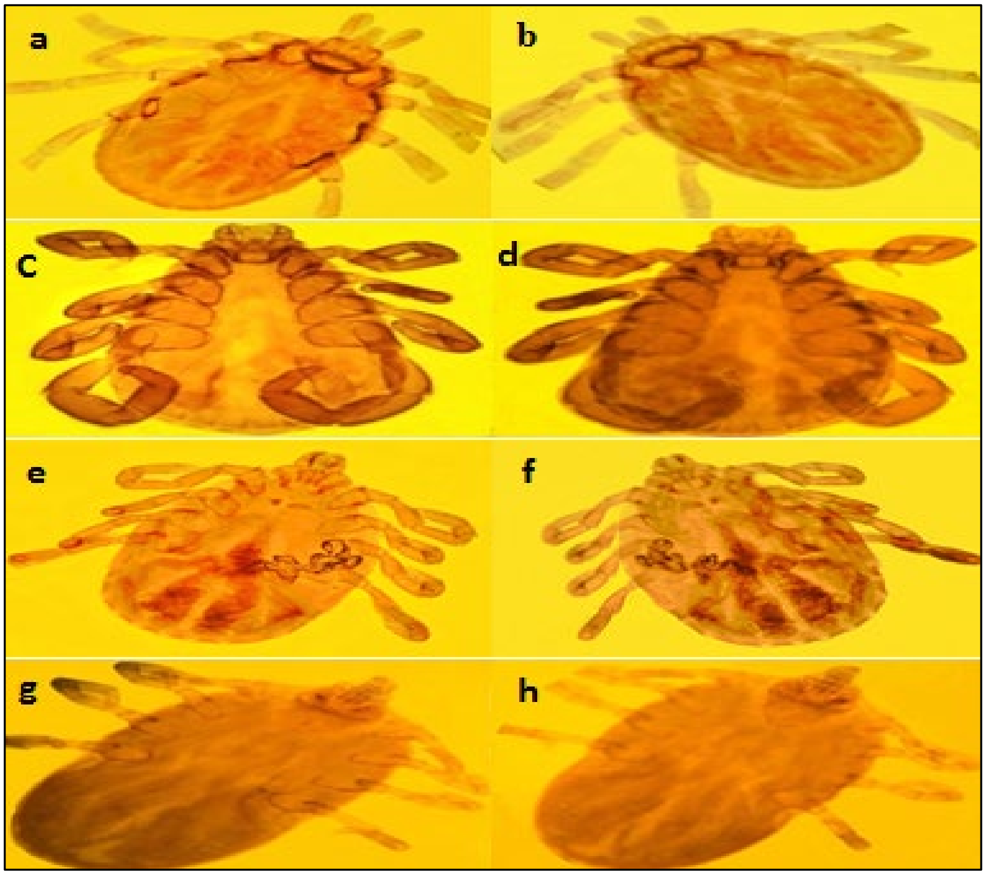

Figure 3: a&b) *Hyalomma anatolicum anatolicum* dorsal & ventral view female; c&d); *Rhipicephalus sanguineous* dorsal & ventral view male; e&f) *Rhipicephalus turanicus* dorsal & ventral view female; g&h) *Boophilus annulatus* dorsal & ventral view female.

Table 2: The infestation rate of *Anaplasma phagocytophilum* in engorged females Ixodid ticks using N-PCR technique.

Tick species	No. of engorged female ticks	No. of positive	Pathogen %
Hyalomma anatolicum anatolicum	6	5	*Anaplasma phagocytophilum*
Rhipicephalus turanicus	4	3	
Rhipicephalus sanguineus	3	3	
Boophillus annulatus	3	2	
Total	16	13	81.2%

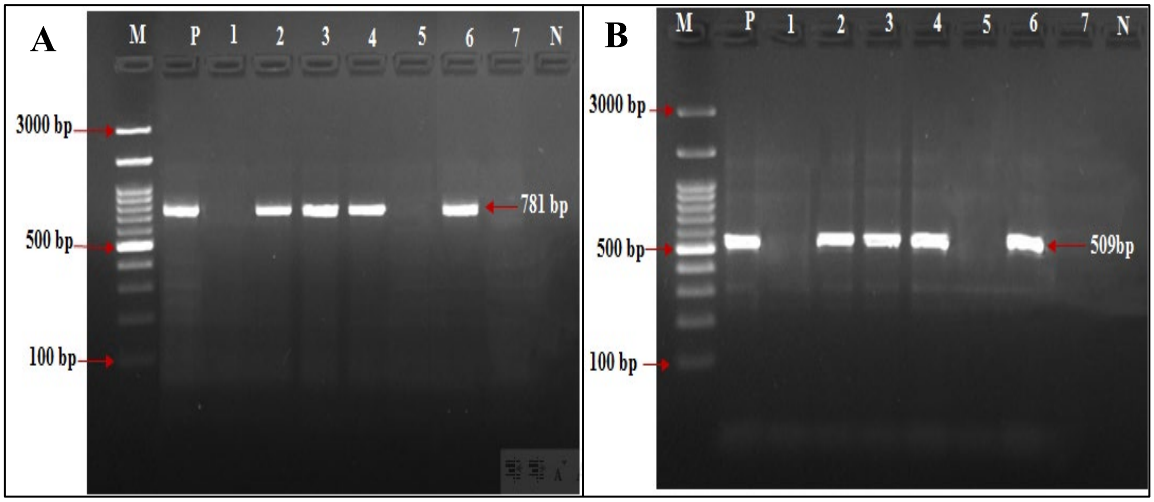

Figure 4: Representing A: the 1st R. of N-PCR, Lanes 2,3,4,6) The positive ticks for *Anaplasma spp*. in approximately band size 781bp.; B: the 2st R. of N-PCR. Lanes 2,3,4,6) The positive ticks for *A. phagocytophilum* in approximately band size 509 bp. Lane M: Exact Mark 100-3000bp DNA ladder; Lane P) DNA extracted from infected cow used as positive control for *A. phagocytophilum*; Lane N) add all PCR competent except DNA used as negative control.

Table 3: The *Anaplasma phagocytophilum* sequences NCBI GenBank accession numbers in cows and engorged female Ixodid ticks.

Accession numbers of 16S rRNA gene	Pathogen	Type of isolate
OR002120.1	*A. phagocytophilum*	*Ticks: Hyalomma anatolicum anatolicum*
OR002121.1	*A. phagocytophilum*	Cow blood
OR002122.1	*A. phagocytophilum*	*Ticks: Rhipicephalus turanicus*
OR002123.1	*A. phagocytophilum*	*Ticks: Rhipicephalus sanguineus*
OR002124.1	*A. phagocytophilum*	*Ticks: Boophillus annulatus*

Table 4: Homology between the local *Anaplasma phagocytophilum* sequences and other sequences of same pathogen in GenBank using NCBI BLASTn.

Local sequences Accession No.	Pathogen Identified	Query Cover %	Identic Number %	GenBank Accession Number	Country Identification
		100	100	MT221234	Norway
OR002120.1		100	100	MT221233	Norway
		100	100	MK239931	Southern Korea
OR002121.1		100	100	MK239930	Southern Korea
		100	100	MN170724	Spain
OR002122.1		100	100	MN170722	Spain
	Anaplasma phagocytophilum	100	100	MK814412	South Africa
OR002123.1		100	100	MK814411	South Africa
		100	100	LC435050	Japan
OR002124.1		100	100	LC435049	Japan
		100	100	MH122889	Poland
		100	100	OQ727069	Malawi

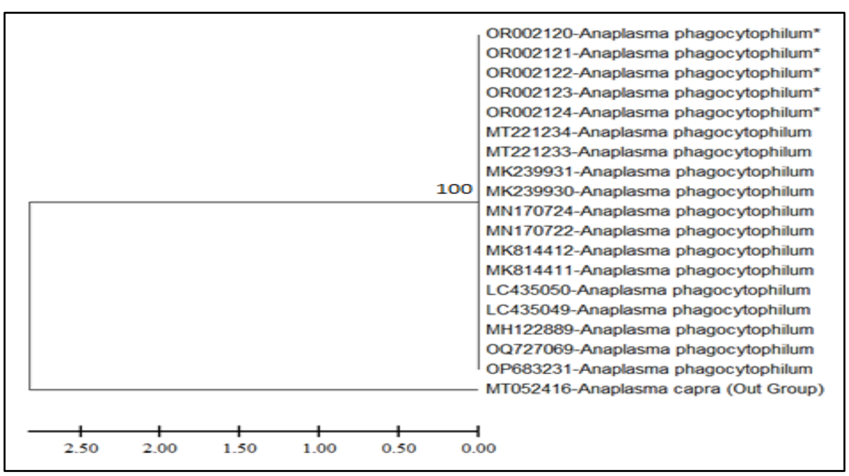

Figure 5: The partial sequences of the 16S rRNA were used to build the evolutionary tree of *Anaplasma phagocytophilum*. The written code with (*) represents the local *Anaplasma phagocytophilum* sequences (Mosul-Iraq), and the MT052416-*Anaplasma capra* was used as an outgroup.

Discussion

In the current study, the infection rate of *A. phagocytophilum* in Mosul city was 72% using N-PCR. This finding is higher compared with the other studies reported *A. phagocytophilum* in Iraq. Infection rate of *A. phagocytophilum* in Al-Najaf Al-Ashraf and Babylon provinces was 6.15% and 4.61%, respectively using N-PCR technique (3). Moreover, the infection rate in Al-Qadisiyah province was 40% using conventional technique (32). There are different studies around the world observed varying in the infection rate of *A. phagocytophilum* in cows using various laboratory techniques such as in Turkey was 30.8% using revers line blot hybridization assay (28), in Iran was 15.5% using N-PCR technique (27), in Egypt was 5.3% using Real time PCR technique (41), in Tunisia was 0.6% using Duplex-PCR technique (39), in Ethiopia was 2.73% using restriction fragment length polymorphism technique (43), and in Northern Germany was 60% using ME of blood smears (13). It has been found that the variations in the infection rate of *A. phagocytophilum* in variety regions and countries were belonged to different management practices, environmental conditions, efficient diagnostics techniques used in various studies, sample size and presence and/or absence of many factors as age, immune status of host, and the presence of ticks in the field and on animals (27, 44, 45).

In the current study, the infestation rate hard ticks on cows were 46% and four species of hard ticks were microscopically identified and classified includes *Hyalomma anatolicum anatolicum*, *Rhipicephalus turanicus*, *Rh. sanguineus* and *Boophillus annulatus*. This finding is agreement with previous studies in which these species of Ixodid ticks reported in different provinces of Iraq (16-19). Furthermore, in the present study a significantly predominant *Hyalomma anatolicum anatolicum* compared with other tick species. This finding might be due to it is ability to endure the dry and severe climate, this result coincided with previous studies (46,47,48). Moreover, the results showed the possibility of the diagnosis of infection with *A. phagocytophilum* in the DNA extracted from the cows' blood and engorged female ticks using N-PCR technique, this finding agreement with previous studies (27,39).

The finding of the phylogenetic tree of the local sequences of *A. phagocytophilum* observed that it possesses common phylogenetic properties and extremely close evolutionary relationship with the other sequences of *A. phagocytophilum* recorded in the NCBI GenBank for various countries including Norway (49), Southern Korea (50), Spain (51), South Africa (52), Japan (53), Poland (54), and Malawi (55), with the 100%

Identity, after performing 1000 using Bootstrap analysis and the Likelihood method on the Tamura-Nei model in MEGA11 software (40).

Conclusions

The present study has been concluded that *A. phagocytophilum* is widespread among cows and Ixodid ticks in Mosul city. *Hyalomma anatolicum anatolicum*, *Rhipicephalus turanicus*, *Rh. sanguineus* and *Boophillus annulatus* are the main vectors of this bacteria. A strategically ticks control program should be implemented to prevent spreading of this type of organism.

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Conflict Of Interest

No conflicts of interest exist, according to the authors, with the publishing of this work.

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الكشف الجزيئي وتحليل الشجرة الجينية لجراثيم *Anaplasma phagocytophilum* في الابقار والقراد المخمج لها في مدينة الموصل، العراق

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الخلاصة

أجريت هذه الدراسة للكشف الجزيئي لجراثيم *Anaplasma phagocytophilum* في الأبقار المصابة والقراد المخمج لها ولأول مرة في مدينة الموصل، العراق باستخدام تقنية تفاعل البلمرة المتسلسل المتداخل، وللتحقق من تحليل الشجرة الجينية لجراثيم *A. phagocytophilum* المشخصة في هذه الدراسة. تم جمع 50 عينة دم (3 مل) من الابقار من مناطق مختلفة من مدينة الموصل. فضلا عن، جمع 169 من القراد الصلب من أجزاء مختلفة من الأبقار المصابة. أظهرت النتائج أن نسبة الإصابة بجراثيم *A. phagocytophilum* في أبقار مدينة الموصل بلغت 72٪ وفي أنثى القراد المحتقنة بلغت 81.2٪ باستخدام تقنية تفاعل البلمرة المتسلسل المتداخل. كما بلغت نسبة الإصابة بالقراد الصلب المتطفل على الأبقار 46٪ ، وتم تحديد أربعة أنواع من القراد الصلب مجهريا وتصنيفها اذ تشمل *Hyalomma anatolicum anatolicum* 39.64٪ و *Rhipicephalus turanicus* 21.89٪ و *Rh. sanguineus* 23.07٪ و *Boophillus annulatus* 15.38٪، وكان النوع *Hyalomma anatolicum anatolicum* السائد معنويا. خضعت التسلسلات الجينية للتحليل الفردي (خمسة تسلسلات) لجراثيم *A. phagocytophilum* من الجين 16S rRNA والتي شملت عينة واحدة مستخلصة من الابقار واربعة عينات مستخلصة من اناث القراد المحتقنة، تم تسجيلها في بنك الجينات المركز الوطني لمعلومات التكنولوجيا الحيوية بأرقام تسلسلية (OR002120.1، OR002121.1، OR002122.1، OR002123.1 و OR002124.1)، وكانت هذه التسلسلات عالية التشابه (100٪) بتلك التسلسلات المسجلة في بنك الجينات مثل (MT221233.1، MT221234.1) في النرويج ، (MK239930.1، MK239931.1) في كوريا الجنوبية ، (MN170722.1، MN170722.1) في إسبانيا (MK814411.1، MK814412.1) في جنوب إفريقيا، (LC435049.1، LC435050.1) في اليابان و (MH122889.1، OQ727069.1) في بولندا وملاوي على التوالي. قد تكون هذه النتيجة مفيدة للدراسات المستقبلية ولغرض السيطرة على هذا النوع من الجراثيم في منطقة الدراسة.

الكلمات المفتاحية: *Anaplasma phagocytophilum*,تحليل الشجرة الجينية، الموصل.