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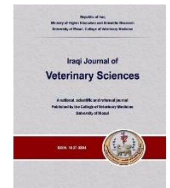

Molecular characterization and phylogenic analysis of *Anaplasma* spp. in small ruminants from Sulaymaniyah governorate, Iraq

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Abstract

Anaplasma spp. are significant arthropod-borne bacteria globally, but documented information about anaplasmosis in small ruminants in the north of Iraq is insufficient. Hence, this study was conducted to determine the prevalence of *Anaplasma* spp. and identify sheep and goat tick vector populations in Sulaymaniyah Governorate, north Iraq. The study population consisted of 470 sheep and 145 goats from 45 livestock farms in 10 geographical locations of Sulaymaniyah Governorate. The study was accomplished from April to December 2017. Blood samples were taken from the jugular vein and used for DNA extraction. Polymerase chain reaction (PCR) was conducted using primers based on the 16S rRNA of *Anaplasma* spp. Fragments of PCR products were sequenced for phylogenetic analysis. The prevalence of *Anaplasma* spp. was 58.9% based on the PCR results. Furthermore, 58.9% of sheep and 57.9% of goats were positive for anaplasmosis. The sequences represented 100% identity with previously documented GenBank isolates of *A. ovis* from Iran, the Netherlands, China, and Mongolia. Altogether, 150 Ixodid ticks were picked from small ruminants within the same flocks and were identified based on morphological features. Various infestation rates were observed; about 40% of the Ixodid ticks belonged to *Rhipicephalus sanguineus*, 34% belonged to *Rhipicephalus turanicus*, 18% were *Hyalomma anatolicum*, and 8% were *Boophilus microplus* (*Rhipicephalus microplus*). The present report is the first molecular study of Anaplasma species in small ruminants from Sulaymaniyah Governorate in northern Iraq to the best of our knowledge. The study concluded that anaplasmosis was endemic in small ruminants from the investigated areas.

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Introduction

Anaplasmosis is a widespread tick-borne disease that results in health problems negatively impacting the benefit of livestock (1). The genus *Anaplasma* includes obligate intracellular bacterial species that result in anaplasmosis in many animals and humans. Among *Anaplasma* spp. that impacted small ruminant health, *A. bovis*, *A. marginale*, *A. ovis,* and *A. phagocytophilum* were identified (2,3). Different *Anaplasma* spp. have diverse cell and host predilections. For example, *A. platys* infects canines' thrombocytes, while *A. bovis* largely parasitizes ruminants' monocytes (4). *Anaplasma centrale*, *A. marginale,* and *A. ovis* attack erythrocytes. Also, *A. phagocytophilum* infects human granulocytes (5).

Ovine and caprine anaplasmosis by *A. ovis* is widely distributed in different regions globally (6). *Anaplasma ovis* might result in chronic infection and, in some cases, hemolytic anemia with paleness and jaundice, with the absence of hemoglobinuria. Clinical signs include anorexia,

a decrease in milk production, abortion, fever, and fatigue. However, the mortality rate is low (7). *Anaplasma ovis* has also been reported in tick vectors *Rhipicephalus, Hyalomma*, *Dermacentor*, and *Ixodes* (8). *Anaplasma phagocytophilum* is another cause of anaplasmosis in small ruminants that leads to tick-borne fever.

This species also infects other domestic animals such as cattle, horses, dogs, and cats (9). *Ixodes* spp., especially *I. ricinus*, are considered the primary tick vector for *A. phagocytophilum*. However, it was so detected in *Dermacentor* spp. (10). In ruminants, *A. phagocytophilum* causes inappetence, high fever, decreased milk yield, miscarriage, and immunosuppression, contributing to the development of secondary infections (11). *Anaplasma bovis*, *A. centrale*, and *A. marginale* have also been discovered in sheep and goats, indicating that they act as potential reservoirs of these organisms (12).

Most reports on *A. ovis* prevalence were from studies on clinically healthy animals. Nevertheless, *A. ovis* contagion could be severer in stressful conditions or the presence of other diseases (13). The lower productivity of local breeds of sheep could be associated with anaplasmosis, as they are easily exposed to stress and endemic pathogens (14).

Various studies on tick-borne hemoparasitic infections in sheep and goats were accomplished, while data about molecular identification of anaplasmosis is scarce. Hence, this study was conducted to determine the molecular characterization of anaplasmosis in small ruminants from Sulaymaniyah Governorate in the north of Iraq.

Materials and methods

Study area, sample collection, and DNA extraction

The study was carried out from April to December 2017, including 45 small ruminant farms from 10 different districts in Sulaymaniyah, Iraq, namely Arbat, Bakrajo, Bazian, Mawat, Nal Parez, Piramagroon, Sayid Sadiq, Sharazoor, Sitak, and Sulaimani. Six hundred fifteen animals, including 470 sheep and 145 goats, were selected randomly from different age groups older than one year.

Blood was taken from the jugular vein, collected in tubes with anticoagulants, and kept at -20°C till DNA extraction. DNA was extracted using a specific commercial Kit from Genet Bio (South Korea) and kept at -20°C till use.

Polymerase chain reaction

All DNA samples were tested using PCR with primer sets used in studies published previously. The forward primer 5′-TACCTTGTTACGACTT-3′ and reverse primer 5′-TGATCCTGGCTCAGAACGAACG-3′ were applied for amplification of the 1462 bp portion of the 16S rRNA gene of *Anaplasma* spp. (15,16).

PCR reactions took place with a green master mix (2X) from GeNet Bio (South Korea) in a total volume of 20.0µL. The content included a 10.0µL master mix, 5.0µL DNA template, and 10.0 pmol from each sense and antisense primers. The final volume was completed with 3.0µL nuclear-free water, and amplification was done in a programmable thermal cycler (Prime, UK) with an initial denaturation of 5 min at 94°C, followed by a three-step program. The program included 40-sec denaturation at 94°C, 35-sec annealing at 55°C, and 1.5 min extension at 72°C for a total of 40 cycles.

Gel electrophoresis

Following PCR amplification, the 1462-bp amplification product was loaded on a 1% agarose gel and stained with ethidium bromide (17). The electrophoresis was run at an electric current of 85 Volt for 65 min, and a UV transilluminator projected them (Figure 1).

DNA sequencing

Five PCR products were randomly selected, three from sheep and two from goats, for sequencing, conducted by Sanger DNA Sequencing System in South Korea. The nucleotide sequence identities and resemblances were checked applying BLASTn analysis.

Nucleotide sequence accession numbers

The sequences were submitted in the GenBank database of NCBI (National Center for Biotechnology Information) by direct submission. The sequences were allotted accession numbers MT645478 to MT645482 for *Anaplasma* spp.

Phylogenetic analysis

The survey's sequences were equated with GenBank sequences of *Anaplasma* by phylogenetic analysis employing MEGA X (18). Maximum likelihood was applied to build phylogenetic trees for *Anaplasma* species-bootstrap analysis with a thousand replications to calculate the confidence of the nodes and branches of the trees.

Tick collection and identification

One hundred fifty ticks were detached carefully from different parts of the animal body, including adult and nymphal stages, using blunt forceps. Collected ticks were put in 70% ethanol (19), then labeled and brought to the laboratory. Adult ticks were morphologically distinguished to the species level under 40X magnification of a dissecting microscope. The identification of the tick genera was made according to Walker's keys and descriptions (20).

Statistical analysis

A Chi-squared test was applied to find an association between variables of animal spp. with *Anaplasma* infection. A probability value of ≤ 0.05 was regarded as statistically significant. Statistical Package for Social Sciences (SPSS) version 19.0 (IBM, USA) was used.

Results

Analysis of 615 blood samples of small ruminants with PCR revealed that 58.7% (n=361) were infected with *Anaplasma* spp. The prevalence rates of anaplasmosis were as follows: 58.9% of the total 470 examined sheep were positive for *Anaplasma* spp., whereas 84 of 145 goats 57.9% were positive for anaplasmosis. No statistically significant difference in the rates of anaplasmosis occurrence was present between sheep and goats (P>0.05).

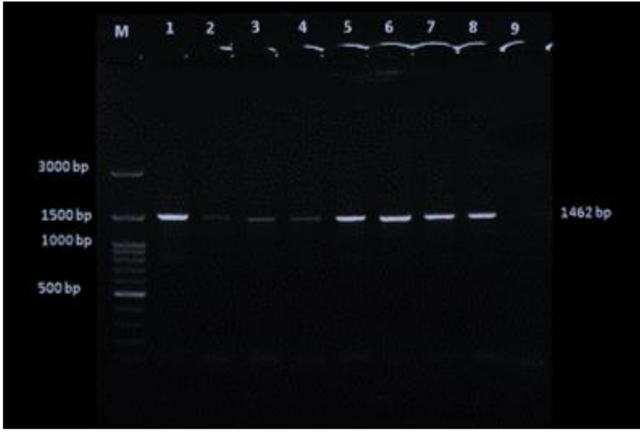

Figure 1: Agarose gel electrophoresis of amplified products of *Anaplasma* spp. stained with ethidium bromide. Lane M revealed a 100-bp DNA ladder. Lanes 1-8 were the positive samples for *Anaplasma* species, and lane 9 is the negative control. The large size of the bands may be related to the presence of large amounts of DNA.

All DNA sequences showed high similarity with previously registered *Anaplasma* spp. in the GenBank database (Figure 2). The sequences showed 100% identity with the *A. ovis* isolates with accession no. JF514506 and JF514512 from Iran, also AF318945, KX579073, MG869525, and LC194134 from the Netherlands, China, and Mongolia, respectively. Also, the identity of *Anaplasma* spp. new isolates with *A. phagocytophilum* reported isolates were 96.0% similar with isolates MN611755 and MN611756 from Turkey; 96.8% and 97.3% with isolates KJ782387 and KX272641 from China, respectively.

The phylogenetic tree constructed based on the 16S rRNA gene of *Anaplasma* species revealed that the isolates from Sulaymaniyah Governorate were clustered together. They represented a close resemblance and laid in a clade with previously reported *A. ovis* isolates from sheep and goats in Iraq, Iran, China, Mongolia, and the Netherlands. Additionally, *Anaplasma* new isolates were clustered separately with *A. ovis* isolates KC778788 and KC778789 from northern Iraq, with homologies between 99.6% and 100.0% (Figure 2).

The identified collected ticks belonged to *Hyalomma anatolicum*, *Rhipicephalus sanguineus, Rhipicephalus turanicus,* and *Boophilus microplus (Rhipicephalus microplus),* with various infestation rates. Of the 150 collected Ixodid ticks, 60 tick samples (40%) were identified as *Rhipicephalus sanguineus*, and 51 samples (34%) were *Hayaloma anatolicum*. Also, 27 samples (18%) were identified as *Rhipicephalus turanicus,* and 12 ticks (8%) belonged to *Boophilus microplus (Rhipicephalus microplus)*. The dataset represented a high level of infestation of small ruminants with *Rhipicephalus* spp. ticks.

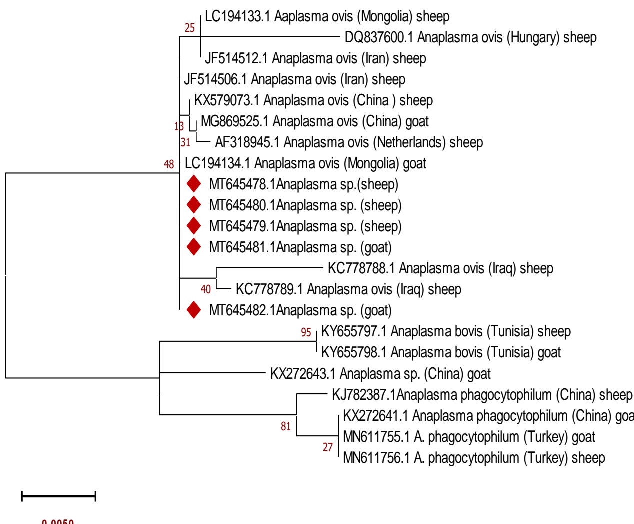

Figure 2: Phylogenetic tree constructed using the 16S rRNA gene of *Anaplasma* spp. The tree was constructed with the maximum likelihood method Kimura 2-parameter model and bootstrap test of 1000 replications using MEGA X. The study's sequences are marked with red diamonds.

Discussion

Anaplasmosis is a significant problem for small ruminants that frequently occurs in tropical and subtropical regions (21). About 58.7% (n=361) of examined samples were positive for *Anaplasma* spp. by PCR assay. Following our findings, high prevalence rates of small ruminant anaplasmoses were also reported by PCR in Iran (22), Turkey (23), and Saudi Arabia (24). However, lower prevalence rates of 40.8% and 29.3% were reported in Kenya (25) and Pakistan (26).

The present study is consistent with previous reports, in which a higher rate of anaplasmosis was reported in sheep than in goats. Shabana *et al.* (24) found that the prevalence was 25.3% in sheep and 15.5% in goats, using PCR assay. Also, prevalence rates of 32.0% and 25.3% were reported in sheep and goats, respectively, by Ghaffar *et al.* (26). However, Eisawi *et al.* (27) found a higher prevalence of

35.9% among goats than sheep, 32.5%. Also, prevalence rates of 34.7% from goat and 21.8% from sheep were reported by Yousefi *et al.* (3).

Based on previous studies, *A. ovis* was the most prevalent species among small ruminants (28,29). Furthermore, *A. phagocytophilum* was reported at various prevalence rates in different geographical areas. A prevalence of 4.7% was reported in the middle region of Iraq by Hamzah and Hasso (30). In Turkey, prevalence rates of 8.51% and 1.08% were also reported (31,32). However, infection rates as high as 28.8% and 66.7% were reported from other regions such as China and Turkey (33,34).

The phylogenetic analysis of obtained *Anaplasma* nucleotide sequences from the current study in sheep and goats revealed that the isolates shared 100% identity with GenBank isolates of *Anaplsama ovis* from Iran with accession numbers JF514506 and JF514512, also with isolates AF318945, KX579073, MG869525, and LC194134 from the Netherlands, China and Mongolia, respectively. New *Anaplasma* spp. isolates were clustered separately with the isolate KC778788 and KC778789 from northern Iraq. The new isolates were 96.0% identical with *A. phagocytophilum* isolates MN611755 and MN611756 from Turkey. They were 96.8% and 97.3% identical with isolates KJ782387 and KX272641 from China.

The identified collected tick from examined sheep and goats within the same small ruminant flocks belonged to *Hyalomma anatolicum*, *Rhipicephalus sanguineus, Rhipicephalus turanicus,* and *Boophilus microplus (Rhipicephalus microplus),* with various infestation rates of 18.0%, 40.0%, 34.0%, and 8.0% for each identified tick species respectively. So, the dataset represented a high level of small ruminant's infestation with *Rhipicephalus* spp. ticks.

Rhipicephalus sanguineus was recognized as being the most predominant species collected from small ruminants. *R. sanguineus* is regarded as a worldwide distributed tick and can convey pathogens such as *Anaplasma* spp., *Coxiella burnetii*, *Ehrlichia* spp., and *Rickettsia rickettsii* (35,36).

Rhipicephalus sanguineus is identified as a common transmitter for *Anaplasma* spp.*,* documented from different geographical areas (37). By molecular study, the *A. ovis* genome was detected from *R. sanguineus* ticks. Furthermore, *A. ovis* has also been detected from *R. turanicus* (8) by molecular assay. This study is considered the first molecular investigation of anaplasmosis in north Iraq. A previous study (38) investigated *Anaplasma ovis* prevalence in angora goats in Duhok, north Iraq. However, the researcher used cELISA in the study.

Conclusion

This study disclosed a high infection rate of *Anaplasma spp.* in small ruminants from the Sulaymaniyah governorate of Iraq. However, sampled animals had no apparent clinical signs, indicating that anaplasmosis was endemic in small ruminants with a subclinical pattern. Sequence analysis revealed that new *Anaplasma* sequence isolates were 100% identical with *A. ovis* isolates from the GenBank database. The identified collected ticks from study areas belonged to different species with higher infestation rates with *Rhipicephalus spp.* The findings will be useful as baseline data for further study about different *Anaplasma* species and identifying their tick vectors.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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التوصيف الجزيئي والتحليل النشوئي لأنواع الأنابلازما في المجترات الصغيرة من محافظة السليمانية، العراق

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الخلاصة

الأنابلازما هي بكتيريا كبيرة تنقلها المفصليات على مستوى العالم. نظرا لقلة وجود معلومات موثقة عن الأنابلازما في المجترات الصغيرة، أجريت هذه الدراسة لتحديد مدى انتشار هذه البكتريا وتحديد مجموعات ناقلات قراد الضأن والماعز في محافظة السليمانية في شمال العراق.

شملت الدراسة ٤٧٠ رأسًا من الضأن و ١٤٥ من المعز من ٤٥ مزرعة للماشية في ١٠ مواقع جغرافية في محافظة السليمانية. تمت الدراسة من نيسان إلى كانون الأول ٢٠١٧. أخذت عينات الدم من الوريد الوداجي واستخدمت في استخراج الحمض النووي. تم إجراء تفاعل البلمرة المتسلسل باستخدام بادئات خاصة للجين ١٦ سفيدبيرك للحمض النووي الرايبوزي الرايبوسومي من أنواع الانابلازما. تم تسلسل هذا الجين لتحليل النشوء والتطور. أظهرت النتائج أن الانتشار الكلي للمرض كان ٥٨,٩٪. علاوة على ذلك، كانت ٥٨,٩٪ من الضأن و ٥٧,٩٪ من الماعز موجبة للأنابلازما. تمثل التسلسلات هوية بنسبة ١٠٠٪ مع عزلات بنك الجينات الموثقة سابقاً إلى الانابلازما البقرية من إيران وهولندا والصين ومنغوليا. إجمالاً، تم جمع ١٥٠ قراد اللَّبُودُ من الضأن والماعز من نفس القطعان وتم التعرف عليها بناءً على السمات الشكلية. لوحظت معدلات إصابة مختلفة؛ حوالي ٤٠٪ من العينات تنتمي إلى عائلة قراد الكلب البني، و ٣٤٪ تنتمي إلى قراد مروحية الراس، و ١٨٪ كانت في قراد الجمال الأناضولي، و ٨٪ كانت من نوع اللبوديات عنكبوتية الشكل. حسب معلوماتنا، إن هذا التقرير هو اول دراسة جزيئية لخمج الانابلازما في المجترات الصغيرة في محافظة السليمانية في شمال العراق. خلصت الدراسة إلى أن الأنابلازما كانت متوطنة في المجترات الصغيرة من المناطق التي تم فحصها.