Molecular diagnosis of *Anaplasma phagocytophilum* in ticks infesting cattle in Iraq

A.K. Mahmood, B.K. Ajel, N.M. Al-Maaly and N.M. Badawi

Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

**Article information**

**Article history:**
Received 17 May, 2023
Accepted 12 November, 2023
Available online 08 December, 2023

**Keywords:**
Molecular
Ticks
Anaplasma
Cattle
Iraq

**Correspondence:**
N.M. Badawi
naseir.badawi78@gmail.com

**DOI:** 10.33899/ijvs.2023.140482.3057, ©Authors, 2023, College of Veterinary Medicine, University of Mosul.

This is an open access article under the CC BY 4.0 license (http://creativecommons.org/licenses/by/4.0/).

**Introduction**

Ticks are hematophagous arthropods of the *Arachnidae* family. Ticks can cause irritation of the skin as well as anemia when they feed on the blood meal of the host. Ticks are also a major vector of pathogens to animals worldwide, such as *Anaplasma* spp., *Babesia*, and *Theileria* (1,2). Ticks are important medical and veterinary obligate ectoparasites of mammals, reptiles, and birds (3). Tick bites are unpleasant and can lead to secondary infections; certain species can paralyze small children and animals, and ticks are carriers of various illnesses that affect humans and animals (4,5). These parasites are the most economically valuable ectoparasites in cattle. *Anaplasma* causes problems like decreased productivity, diseases, decreased fertility, and even death (6). These closely related bacteria have several characteristics in common, like concurrent infection coexistence in ticks and reservoirs in wild ruminant hosts and domestic hosts in the same geographic area (7). Ticks take all *Anaplasma phagocytophilum* stages-adult, nymph, and larva-and transfer them to mammals through the next meal of blood (8,9). Ticks are significant and the most common ectoparasites of birds, reptiles, and mammals worldwide (10). Ticks also have more opposite effects on livestock than any other group of arthropods, parasitizing a wide variety of vertebrate hosts and transmitting a wide range of pathogens (11). Tick prevention and disease transmission remained a threat to the animal husbandry sector in subtropical and tropical areas of the world, and due to the economic and veterinary value of ticks, many countries in those regions were concerned (12). The effects of tick-borne diseases and ticks on the national economy and individuals require...
by applying proper strategies for tick control on a priority basis (13).

The study aimed to detect *Anaplasma phagocytophilum* in ticks that infected cattle in Iraq, classify these ticks, and the phylogenetic analysis of the positive samples.

**Materials and methods**

**Ethical approve**

This study approved by Committee of University of Baghdad No. 16 in 6 June 2022, and the number of university order 2872 in 12 June 2022.

**Study area**

The study was conducted in Baghdad governorate, Iraq. All 50 cattle from various herds throughout the study region were chosen randomly. Ticks were obtained at seven separate intervals. From July, June to August 2020.

**Ticks’ collection and identification**

Ticks were collected from various locations on the cattle’s body. To avoid contamination, the ticks were put in Eppendorf tubes with a 70 percent ethanol content of 1.5 mL. They were transferred to the laboratory, examined, and identified using a binocular stereomicroscope down to the species level. The case history included the date, site, animal number, age, and sex of the cattle. The specimens were washed twice with distilled water before being dried on blanched pulp. Accordingly, they were described at the species level (14,15). The most critical identification characteristics are the color, scale, shape, and punctuation of the mouthparts, the anal groove, the scutum, the festoon, and the legs of ticks. Identified and sterilized ticks were stored before DNA extraction to detect *A. phagocytophilum* PCR.

**Molecular assay**

The ticks were crushed into small pieces before DNA extraction. The crushing ticks were mixed with tissue lysis buffer using a micro pestle, and then 30 μl proteinase K was added and mixed by vortex before being incubated for 1 hour at 60°C. Genomic DNA was extracted from tick species according to the instructions of the company (gSYAN DNA mini extraction kit, insect sample protocol, Geneaid, Twian) in elution steps of 100 μl for the best DNA quantitative result. The template DNA tick genome was quantified using a NanoDrop spectrophotometer (THERMO, USA), measuring each DNA sample’s DNA purity by measuring absorbance at 260/280 nm from 1.80 to 1.95. Finally, the extracted DNA samples were kept at -20°C until they were used for polymerase chain reaction tests. The 16S rRNA gene of *Anaplasma phagocytophilum* was detected using nested PCR, and two sets of primers (16) for amplification of 926 bp fragments. The first sets of primers used in the first round TCC TGG CTC AGA ACG AAC GCT GGC GGC and AGT CAC TGA CCC AAC CTT AAA TGG CTG (1433 bp), while the second sets of primers used in the second round: GTC GAA CGG ATT ATT CTT TAT AGC TCG and CCC TCT CGT TAA GAA GGA TCT AAT CTCC (926 bp). The total volume of the PCR reaction was 25 μl including the master mix (12.5 μl) according to the instructions from Promega (USA), one μl of each forward and reverse primer, three μl template DNA, and 7.5 μl nucleus-free water.

Thermal conditions of the polymerase chain reaction assay were used to amplify 926 bp of the 16S rRNA gene according to the following: initial denaturation for 10 minutes at 95 °C for each PCR round, followed by 38 cycles (95 °C for 40 seconds, 65°C for 40 seconds for the first round, 61°C for 40 seconds for the second round, and 72 °C for 40 seconds), and the final extension of 72 °C for 7 minutes for each PCR round furthermore, the PCR products were visualized by a UV transilluminator, loaded in 5 μl DNA products on a 1 % agarose gel with Ethidium Bromide, and electrophoresed at 80 volts and 100 amperes for 50 minutes. The size marker of a 100-bp ladder was used in this study.

**Sequence analysis**

The PCR products of one sample were submitted to Macrogen (Korea) for sequence analysis for the 16S rRNA gene. The phylogenetic tree was made by the Molecular Evolutionary Genetics Analysis version (MEGA 6.0).

**Results**

**Ticks’ classification and molecular results**

This study showed that the ticks were 23 males and 77 females out of 100 species collected from 50 cattle in different regions of Baghdad city. The ticks were classified as *Hyalomma anatolicum* (Figure 1, A) as the most prevalent tick species 33.9%, *Hyalomma uranum* 28.5% (Figure 1, B), *Hyalomma marginatum* 20.7% (Figure 1, C), and *Hyalomma excavatum* 16.9% (Figure 1, D). The results revealed non-significant differences between males and females according to infestation rate (Table 1). At the same time, the infection by *Anaplasma phagocytophilum* was present in two species of *Hyalomma marginatum* after nested PCR examination to detect a fragment of the 16S rRNA gene of *Anaplasma phagocytophilum* about 926 bp (Figure 2).

![Figure 1](image-url)
Some studies in Iraq found a high prevalence of Anaplasma spp. strains worldwide with a high degree of pathogenicity and reservoirs that may differ from human strains. Anaplasma phagocytophilum DNA was detected in Ixodes ricinus and Hyalomma (Hy). detritum, and Hy. marginatum in North Africa (Tunisia, Algeria, and Morocco). DNA of Anaplasma phagocytophilum has been observed in Haemaphysalis longicornis, Haemaphysalis concinna, Dermacentor silvarum, and I. persulcatus in China (19,24). Anaplasma phagocytophilum is widespread in H. qinghaiensis, found in Gannan Tibetan Autonomous Prefecture (25). Anaplasma phagocytophilum has been observed by a polymerase chain reaction in Haemaphysalis longicornis in Asia (26). Rhipicephalus turanicus, Hyalomma marginatum, and Boophilus kohlsi had a role in transmission of Anaplasma spp., while Rhipicephalus sanguinus and Hyalomma marginatum may be essential Anaplasma spp. vector ticks. Furthermore, 20 species of ticks, such as Rhipicephalus spp., Hyalomma spp., Ixodes spp., Boophilus spp., and Dermacentor spp., have been identified as vectors for Anaplasma spp. around the world (27). Additionally, the prevalence reported by Yang et al. (26) was lower than the 2% infection rate of Anaplasma phagocytophilum ticks in the current study. These findings are consistent for Ybañez et al. (28) of the ticks detected 2.4% in Japan out of all ticks collected. Anaplasma can be spread through the body by vectors from the genera Boophilus, Rhipicephalus, and Hyalomma (29).

The results of this study may help us learn more about how ticks spread in Iraq. The prevalence of anaplasmosis in dogs in Iraq may be underestimated, and the tree of phylogenetic local Anaplasma platys and Anaplasma phagocytophilum isolates were found to mimic other Anaplasma spp. strains worldwide with a high degree of similarity (30). Summer infection rates are much higher (31). The prevalence of anaplasmosis in ruminants may be underestimated, and the tree of phylogenetic local Anaplasma platys and Anaplasma phagocytophilum isolates were found to mimic other Anaplasma spp. strains worldwide with a high degree of similarity (30). Summer infection rates are much higher (31).

Some studies in Iraq found a high prevalence of Anaplasma phagocytophilum in ruminants, around 58.9% (32). Therefore, further studies on tick prevalence are also suggested in other areas of Iraq to clarify the knowledge of tick distribution in cattle. The present research work was the first study to define the incidence of Anaplasma phagocytophilum infestation in ticks using PCR in Iraq.

**Table 1: The infestation rates of ticks and ticks infected by *Anaplasma phagocytophilum* according to PCR**

<table>
<thead>
<tr>
<th>Species for ticks</th>
<th>Ticks number</th>
<th>Female</th>
<th>PCR positive</th>
<th>Males</th>
<th>PCR Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hyalomma anatolicum</em></td>
<td>36</td>
<td>27</td>
<td>0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td><em>Hyalomma uranum</em></td>
<td>30</td>
<td>24</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td><em>Hyalomma marginatum</em></td>
<td>22</td>
<td>17</td>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td><em>Hyalomma excavatum</em></td>
<td>12</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

**Discussion**

Anaplasma phagocytophilum survives in the wild because it is transmitted in cycles between wild animals and ticks (17). The pathogens have been identified in ticks in most countries in Europe, and the rates of infection range from 0.4 to 67% (18). The tick has been described as the primary vector of anaplasmosis transmission. According to recent studies, many ticks, except *I. persulcatus*, have been found to bear *A. phagocytophilum* (19,20). This genus

**Phylogenetic tree of *Anaplasma phagocytophilum***

One positive sample's partial 16S rRNA sequences were used to make a phylogenetic tree, which was sent to the NCBI as accession number MW422836.1 (Figure 3). The sequence of this sample, presented in the individual clade, has 97% similarity with sister isolates from South Korea (MF582329.1 and MK814412.1), China (MH722235.1), and Estonia (HQ629923.1).

**Figure 3:** A phylogenetic tree was carried out using MEGA6 to analyze partial 16S rRNA gene sequences of *Anaplasma phagocytophilum* infecting *Hyalomma marginatum* in cattle.
Conclusion

The study concluded that the *Hyalomma marginatum* ticks had a role in *Anaplasma phagocytophilum* transmission. This study was the first polymerase chain reaction (PCR) used in Iraq to study the roles of ticks in *Anaplasma phagocytophilum* infection in cattle in Iraq and classify the ticks that infected the cattle in Iraq.

Acknowledgments

The researchers thank the staff of the Natural History Museum for the initial diagnosis of ticks, as well as all the workers in the laboratory for confirming the diagnosis.

Conflict of interest

The authors declare that there is no conflict of interest.

References

التشخيص الجزيئي للانابلازما فاجوسيتوفيلم في القراد
المصيب للماشية في العراق

علاء كامل محمود، باسم كريم عجيل، نبيل محمد أبو المعالي ونصير محمد بدوي

فرع الطب البيطري الباطني والوقائي، كلية الطب البيطري، جامعة بغداد، العراق

الخلاص

بعد القراد هو الناقل الرئيسي للعديد من المسببات المرضية التي ترتبط بعض الأمراض في الحيوانات المختلفة حول العالم مثل الانابلازما والبابيزيا والثاليريا. وللفحص التعرف على أنواع القراد الذي يسبب الماصية في العراق وتقييمهما تم أخذ عدد 150 من الفئران البطنية مختلفة الأنواع من رأس من الماصية في مناطق مختلفة من محافظة بغداد. تم إجراء مراقبة خلال شهر حزيران وتموز وآب سنة 2020 للتعرف على القراد الناقل للمسبب المرضي انابلازما فاجوسيتوفيلم في الماشية. تم تصنيف أعداد القراد إلى 36 من نوع زجاجي العين الأراضي و30 قرادة من نوع زجاجي العين الاقطع و22 قرادة من نوع زجاجي العين المفطر و12 قرادة من نوع زجاجي العين مميز الحافة. تم إجراء فحص تفاعل البلمرة المتسلسل لتمييز القراد المصاب بالانابلازما فاجوسيتوفيلم بطرقية تفاعل البلمرة المتسلسل العشي مع مجموعتين من البادئات. أظهرت النتائج حالتين إيجابيتين لسبب انابلازما فاجوسيتوفيلم في القراد من نوع زجاجي العين مميز الحافة. تم عمل شجرة النشوء والتطور بناء على التسلسل الجزيئي لجين 16 أس الحمض النووي الرايبيريبوزي عن طريق تفاعل البلمرة المتسلسل مع عينة واحدة، وجدت الدراسة أن هناك نسبة تثاثير يصل إلى 77% مع عينتين من كوريا الجنوبية وعزلة واحدة لكل من الصين وإستونيا. خلصت الدراسة إلى أن قرادة زجاجي العين مميز الحافة كان له دور في انتقال انابلازما فاجوسيتوفيلم وكان هذه الدراسة هي المرة الأولى التي يستخدم فيها تفاعل البلمرة المتسلسل في العراق لدراسة دور القراد في عدوى انابلازما فاجوسيتوفيلم في الأبقار في العراق وتصنيف أنواع القراد المصيب للماشية في العراق.