

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

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Abstract

Ticks are a major vector of many pathogens associated with some diseases of various animals worldwide, such as anaplasmosis, babesiosis, and theileriosis. For identification and classification, 100 hard ticks of different species were taken from 50 cattle in other regions of Baghdad province. Surveillance was carried out from June, July, and August 2020 to identify ticks with *A. phagocytophilum* in cattle. Ticks were classified as *Hyalomma anatolicum* (n = 36), *Hyalomma turanicum* (n = 30), *Hyalomma excavatum* (n = 22), and *Hyalomma marginatum* (n = 12). The amplification of a 926-bp fragment of the 16S rRNA gene of *A. phagocytophilum* using nested polymerase chain reactions with two sets of primers. The results showed two positive cases of *A. phagocytophilum* in *Hyalomma marginatum*. A phylogenetic tree based on partial sequences of 16S rRNA from one sample of *A. phagocytophilum* was submitted to the NCBI under accession MW422836.1 with a matching percentage of 97% with isolates from South Korea MF582329.1 and MK814412.1, China MH722235.1, and Estonia HQ629923.1. The study concluded that the *Hyalomma marginatum* ticks had a role in *A. phagocytophilum* transmission. This study was the first polymerase chain reaction used in Iraq to study the functions of ticks in *A. phagocytophilum* infection in cattle in Iraq and classify the ticks that infected the cattle in Iraq.

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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

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 blached 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 cruising 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 TTG C and CCC TTC 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: The ticks were classified as *Hyalomma anatolicum* (A), *Hyalomma turanicum* (B), *Hyalomma marginatum* (C), and *Hyalomma excavatum* (D).

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

Species for ticks	Ticks number	Female	PCR positive	Males	PCR Positive
<i>Hyalomma anatolicum</i>	36	27	0	9	0
<i>Hyalomma uranum</i>	30	24	0	6	0
<i>Hyalomma marginatum</i>	22	17	2	5	0
<i>Hyalomma excavatum</i>	12	9	0	3	0

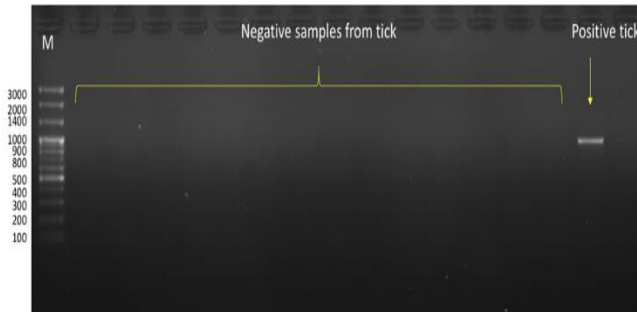


Figure 2: Partial fragments of the 18S rRNA gene 926 bp of *Anaplasma phagocytophilum* in *Hyalomma marginatum*-infested cattle. Eighty volts and 100 amperes for 50 minutes.

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).

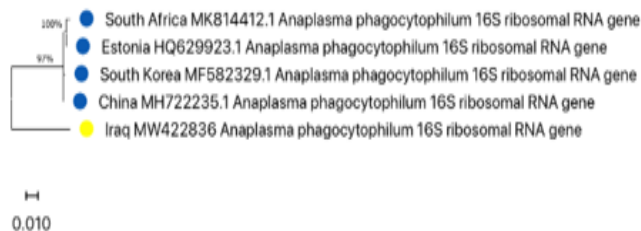


Figure 3: A phylogenetic tree was carried out using MEGA6 to analyze partial 16S rRNA gene sequences of *Anaplasma phagocytophilum* infecting *Hyalomma marginatum* in cattle.

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

Hyalomma is a common cow parasite in Iraq, but *Rhipicephalus turanicus*, *Boophilus annulatus*, *H. anatolicum excavatum*, *H. marginatum turanicum*, *H. anatolicum*, and *H. asiaticum* are less common (21,22). Massung *et al.* (23) suggested that *Anaplasma phagocytophilum* strains in ruminants may share some common features involving pathogenicity and reservoirs that may differ from human strains.

Anaplasma phagocytophilum DNA was detected in *Ioxdes 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). 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.

Conclusion

The study concluded that the *Hyalomma marginatum* ticks had a role in *A. 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.

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Conflict of interest

The authors declare that there is no conflict of interest.

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التشخيص الجزيئي للانابلازما فاكستوفيلم في القراد المصيب للماشية في العراق

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الخلاصة

يعد القراد هو الناقل الرئيسي للعديد من المسببات المرضية التي ترتبط ببعض الأمراض في الحيوانات المختلفة حول العالم مثل الانابلازما والبايزيا والتاليريا. ولغرض التعرف على اهم أنواع القراد الذي يصيب الماشية في العراق وتصنيفها تم أخذ عدد ١٠٠ من القراد الصلب بمختلف الأنواع من ٥٠ رأس من الماشية في مناطق مختلفة من محافظة بغداد. تم إجراء مراقبة خلال شهر حزيران وتموز وأب لسنة ٢٠٢٠ للتعرف على القراد الناقل للمسبب المرضي انابلازما فاجوسيتوفيلم في الماشية. تم تصنيف أعداد القراد الى ٣٦ من نوع زجاجي العين الأناضولي و ٣٠ قرادة من نوع زجاجي العين الاقطع و ٢٢ قرادة من نوع زجاجي العين المقعر و ١٢ قرادة من نوع زجاجي العين مميز الحواف. تم إجراء فحص تفاعل البلمرة المتسلسل لتضخيم جزء حجمه ٩٢٦ قاعدة نيوكليدية من جين ١٦ أس الحمض النووي الرايبيريبيوزي للمسبب المرضي انابلازما فاجوسيتوفيلم بطريقة تفاعل البلمرة المتسلسل العشي مع مجموعتين من البادئات. أظهرت النتائج حالتين إيجابيتين لمسبب انابلازما فاجوسيتوفيلم في القراد من نوع زجاجي العين مميز الحواف. تم عمل شجرة النشوء والتطور بناءً على التسلسل الجزيئي لجين جين ١٦ أس الحمض النووي الرايبيريبيوزي من عينة واحدة، وجدت الدراسة إن هناك نسبة تطابق يصل الى ٩٧% مع عزلتين من كوريا الجنوبية وعزلة واحدة لكل من الصين وإستونيا. خلصت الدراسة إلى أن قراد زجاجي العين مميز الحواف كان له دور في انتقال انابلازما فاجوسيتوفيلم وكانت 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.