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Molecular and phylogenetic analysis of *Anaplasma phagocytophilum* in equids in Mosul city, Iraq

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Abstract

Anaplasma phagocytophilum is a bacterium that causes Equine Granulocytic Anaplasmosis (EGA) disease in equids (ponies, horses, mules, and donkeys), which was worldwide distribution. The current study is aimed recording the prevalence of *A. phagocytophilum* in equids in Mosul city, Iraq using the PCR technique, determining some of the related factors linked to the prevalence of *A. phagocytophilum*, and exploring the phylogenetic analysis of the bacteria detected in this study. One hundred blood samples were obtained from equids (41 donkeys and 59 horses) of different ages, genders, and management practices. The prevalence of *A. phagocytophilum* in equids in Mosul city was 32%. A higher prevalence of the EGA was linked with the risk factors that include donkeys, more than 3 years, female equids, out-of stables animals, and ticks found on the equids. The individual analysis of 30 sequences of the 16S rRNA gene, which were extracted from equid's blood, confirmed *A. phagocytophilum* bacteria species. These four sequences were deposited to the American GenBank and assigned accession numbers (PQ594492.1, PQ594493.1, PQ594494.1, and PQ594495.1). These sequences were 99.46% - 100% identical to these sequences previously deposited to the NCBI GenBank includes: (KC422267.1) in North Korea, (KC916734.1, MG869512.1, KC916737.1) in China, (AB454076.1, AB196721.1) in Japan, and (MT754315.1, MT754351.1, MT754365.1) in South Korea. In conclusion according to the results, *A. phagocytophilum* is common among equines in Mosul, Iraq, and these results may be useful for future research and beneficial management of the *A. phagocytophilum* in the study region.

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Introduction

Horses (*Equus caballus*) that have been domesticated have special value among farm animals because of their distinctive qualities; this has increased interest in horses and improved their welfare (1). The history of human-equine contact dates back over 5,000 years, which allowed numerous infections to spread to both species (2). The extensive presence of equine hemoparasites, their detrimental effects on equine health and possible mortality, the impediments to equestrian trade (3), limitations on equestrian travel between endemic and non-endemic areas during international sporting events, and the decreased welfare of race horses due to their declining performance, as well as the expense of medication, side effects, and control programs in the absence of a vaccine (4), as well as the zoonotic characteristics of *A. phagocytophilum* and its spread to numerous animal species in addition to humans, as well as the similarity in *Trypanosoma evansi*, that heighten the risk of infection, all emphasize the significance of these hemoparasites (5,6). *Anaplasma phagocytophilum* is a gram-negative bacteria caused Equine Granulocytic Anaplasmosis (EGA), previously called equine granulocytic ehrlichiosis (EGE) (7), that was first documented in horses in the United States of America by Gribble (8). It is one of the obligate intracellular bacteria that infects leukocytes, especially

neutrophils (9), as well as lymphocytes, monocytes, basophils, and eosinophils (10,11). This bacterium infects various types of animals, including equids (12), camels (13), sheep and goats (14), cattle (15), dogs and cats (16,17), as well as humans (18). The Ixodidae ticks is the main mechanical transmitters of EGA, including the genus of Hyalomma, Dermacentor, and Rhipicephalus (19), It can also be transmitted through contaminated instruments and blood transfusions (20), and intrauterine transmission (21). There are 39 countries in the world where the disease is endemic (6), involving countries in Asia, Africa, Europe, and America (22-26). Based on microscopic examination, *Anaplasma phagocytophilum* was initially discovered in horses in Nineveh Province, Iraq, in 2018 (27). Both Saleem *et al*. (10) and Sim *et al*. (28) indicated the existence of many risk factors such as equine type, age, sex, breed, health condition, pregnancy, management, geographic areas, and seasons, that are linked to the prevalence of EGA in equine. The disease also causes a high morbidity and mortality rates in equids naturally infected with *A. phagocytophilum* (12,29). Moreover, the acute form of the EGA is clinically characterized by 39-40°C of fever, mucous membrane pallor, petechial bleeding in the third eyelid, anemia, respiratory distress, lower limb edema, neurological symptoms, and ultimately the animal's death (30,31). Because the EGA clinical symptoms are often mistaken for other blood diseases, such as trypanosomiasis (32), Plasmodium spp. (33), Theileriosis (34), Babesiosis (35), and another disease caused by *Anaplasma platys* (36). Therefore, the diagnosis of the disease must be confirmed by laboratory testing, which includes microscopic examination of blood smears (12,35), serological tests (37), and molecular procedures like nested PCR technique (38).

The study objectives are to identify the prevalence of *A. phagocytophilum* in equids in Mosul City, Iraq, using molecular techniques, to explore some risk factors linked with bacteria prevalence, and to explore the phylogenetic analysis of the bacteria detected in this study.

Materials and Methods

Ethical approval

The University of Mosul, College of Veterinary Medicine's Animal Ethics Committee permits this work on April 30, 2023 (UM. VET. 2023. 088130).

Animals and sample size

The study involved 100 equids in all, comprising 59 horses and 41 donkeys of both genders, different ages, and management practices. All equids obtained from different areas in Mosul City, Iraq. Animal were examined for gastrointestinal parasites (39), and the clinical signs of animals were recording, including fever, anorexia, anemia, respiratory disturbance, edema in the lower limbs, recumbency, nervous signs and the presence of ticks on various body regions, with various frequency. Moreover, according to Charan and Biswas (40), the sample size was determined in the current study by using the suspected prevalence of EGA in Mosul City, which was 5%, with a 95% confidence level and an absolute error of 0.05. For this study, a minimum of 73 equids were needed. However, 100 samples were collected.

Samples obtained

From December 2023 to July 2024, 100 blood samples (10 milliliters) were collected from equids via a jugular vein puncture using serial syringes, which were placed in tubes with anticoagulant EDTA (ethylene diamine tetraacetic acid) and then kept in the deepfreeze at -20ºC until the polymerase chain reaction (PCR) technique done.

PCR technique and sequencing

According to the manufacturer instructions of the AddPrep Genomic DNA Extraction Kit (Add Bio, Korea), the DNA of *A. phagocytophilum* were extracted from all equids blood samples. The concentration and purity of the extracted DNA were estimated according to Hassan *et al*. (41,42). Using PCR technique, 16S rRNA gene of Anaplasma spp. were amplified. As a positive control, the DNA was taken from a clinically infected horse. Furthermore, the negative control was to contain all components of the reaction except the DNA. Kawahara *et al*. (43) created the universal oligonucleotide primers, which were provided by Macrogen Inc. in South Korea, includes the forward primer EC9 (TACCTTGTT ACGACTT) and the reverse primer EC12A (TGATCCTGGCTCAGAACGAACG). Anaplasma species were detected in a band size of around 1462 bp. The PCR reaction and program were performed without any change, according to Alani and Yousif, (44). After loading the final PCR results onto a 1.5% agarose gel and staining them with Safe-RedTM dye, the bands were visible when exposed to UV transillumination (BIO-RAD/USA). The positive PCR products for Anaplasma spp. were sent to Macrogen Inc., South Korea for sequencing. Phylogenetic trees were created using neighbor-joining (NJ) and MEGA11 software tools (45), with the sequence of *Anaplasma platys* (OR194151.1) in Iraq used as an outgroup.

Statistical analysis

Using Epi-InfoTM (version 7), the odds ratio for (type of equids, ages, gender, management, and presence of ticks) was analyzed using the X^2 *test* and Fisher Exact; If the P value was below 0.05, the data was taken as significant.

Results

The results of this study showed that the prevalence of *A. phagocytophilum* in equids was 32% (32/100) using PCR technique. Based on molecular technique results

demonstrated that the prevalence of *A. phagocytophilum* was significantly (P<0.0012) greater in donkeys 51.2% (OR: 4.58 times, CI:1.868-11.233), compared to horses 18.6% (Table 1). The prevalence of *A. phagocytophilum* was significantly (P<0.0016) greater in 2 years to over 3 years old equids 55.3% (OR: 9.90 times, CI: 2.043-48.014) compared to less than 1years and 1-2 years old were 11.1% and 11.4%, respectively (Table 1). Concerning the gender, the prevalence of *A. phagocytophilum* was significantly (P<0.0227) higher among females 42.1% (OR: 3.18 times, CI: 1.254-+68.071) compared to males 18.6% (Table 1). Furthermore, the prevalence of *A. phagocytophilum* was significantly (P<0.0134) higher among animals in grazing 38.2% (OR: 3.71 times, CI: 1.375-10.026), compared to animals in stable 14.2% (Table 1). Significantly higher prevalence (P<0.0000) of *A. phagocytophilum* in equids infested with ticks 76.4**%** (OR: 10.94 times, CI: 3.193-37.533), compared to equids non- infested with ticks 22.8% (Table 1).

Table 1: Risk factors Liked with the prevalence of *Anaplasma phagocytophilum* determined by PCR

	Category	Examined / Positive n (%)
Type of equids	Horses	59/11 (18.6) [a]
	Donkeys	41/21 (51.2) [b]
Ages	< 1 year	18/2 (11.1) [a]
	1 - 2 years	35/4 (11.4) [a]
	2 - >3 years	47/26 (55.3) [b]
Gender	Males	43/8 (18.6) [a]
	Females	57/24 (42.1) [b]
Management	In stable	42/6 (14.2) [a]
	In grazing	68/26 (38.2) [a]
Presence of ticks	No	83/19 (22.8) [a]
	Yes	17/13 (76.4) [b]

Different letters indicating significant differences at P<0.05.

In the current study, using online Blastn program for separately sequences analysis for sequences of the 16SrRNA gene including two extracted from horses' blood and also two extracted from Donkeys blood, *A. phagocytophilum* sequences were deposited in the American NCBI GenBank, where they were assigned accession numbers PQ594492.1, PQ594493.1, PQ594494.1, and PQ594495.1 (Figure 1 and Table 2). The sequences that obtained were highly identical 99.46%- 100% with those previously sequences recorded in the Genbank like KC422267.1 in North Korea, KC916734.1, MG869512.1, KC916737.1 in China AB454076.1+8798AB19672165/87.1 in Japan, and MT754365.1, MT754315.1, MT754335.1 in South Korea (Table 3). Furthermore, MEGA11 software's maximum likelihood method was used for constructing phylogenetic tree, it showed that the sequences obtained of *A. phagocytophilum* were closely linked 99.46-100% identity to the sequences that were accessible in the GenBank database, as was previously reported. The tree was rooted with OR194151.1-*Anaplasma platys*-Iraq utilized as an outgroup (Figure 2).

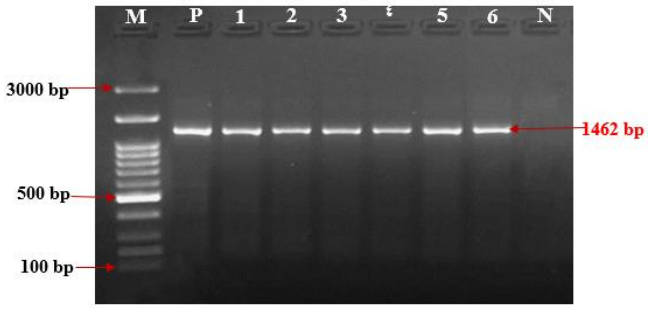

Figure 1: Electrophoresis gel was observed to have positive equids blood samples for anaplasma spp. with around band size at 1462bp.

Table 2: The American NCBI GenBank accession numbers of *Anaplasma phagocytophilum* sequences in equids

Accession numbers of 16S rRNA gene	Host
PQ594492.1	Horse
PQ594493.1	Horse
PQ594494.1	Donkey
PQ594495.1	Donkey

Table 3: Similarity between the sequences obtained of Anaplasma phagocytophilum and the sequences of the same bacteria in GenBank utilizing the BLASTn program

Local accession Number	Query Cover %	% of Identic	references accession numbers	Country
	100	100	KC422267.1	North Korea
	100	99.78	KC916734.1	China
PQ594492.1	100	99.57	AB454076.1	Japan
PQ594493.1	100	99.57	AB196721.1	Japan
PQ594494.1	100	99.57	MT754365.1	South Korea
PQ594495.1	100	99.57	MT754315.1	South Korea
	100	99.57	MT754335.1	South Korea
	100	99.57	MG869512.1	China
	100	99.46	KC916737.1	China

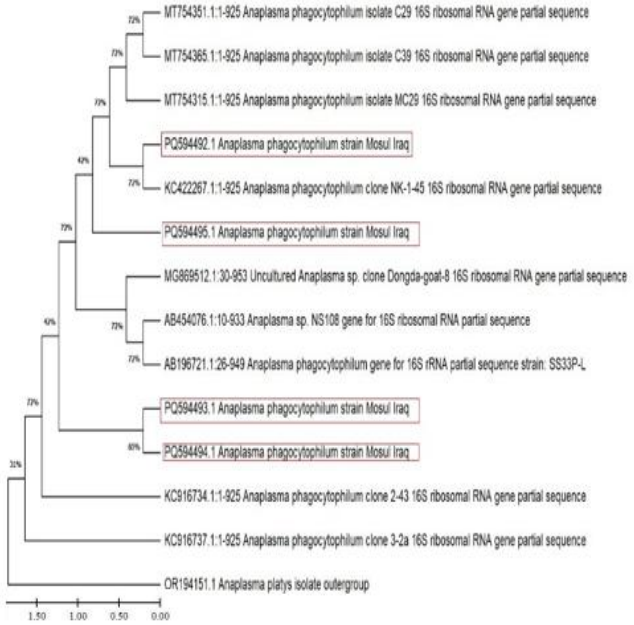

Figure 2: Phylogenetic tree of obtained sequences of the *Anaplasma phagocytophilum* in equids from Mosul City, Iraq (red rectangular), and the OR194151.1- *Anaplasma platys* -Iraq utilized as an outgroup.

Discussion

In the present study, the prevalence of *A. phagocytophilum* in equids was 32% in Mosul City, Iraq, using the PCR technique. This finding was greater or lower than the prevalence documented in earlier research in Iraq. Al-Jwari and Al-Obaidi, (12) and Al-Badrani and Al-Iraq, (27) observed that the prevalence of *A. phagocytophilum* in equids in Nineveh Governorate was 46.1% and 28.8% utilizing indirect ELISA and microscopic examination of blood smears, respectively. In Baghdad Governorate, the prevalence of *A. phagocytophilum* in the horse was 13.12% and 6.87% using conventional PCR technique and microscopic examination, respectively (44). Furthermore, Mohand and Saleem, (46) found that the prevalence of EGA in Erbil Governorate was 16%, and in Duhok Governorate it was 12% using the nested PCR technique. Several studies have documented the prevalence of *A. phagocytophilum* in horses in various countries, such as in Egypt, where it was 11.1% in horses using the snap-4dX ELISA test (47), in Turkey, it was 8.6% and 6.4% in horses utilizing indirect ELISA and multiplex PCR technique, respectively (19), In Korea was 0.2 in horses using nested PCR technique (38), in Germany, it was 15.2% in horses using the real time PCR technique (17), and in Southwest Virginia, it was 35%% in horses employing the conventional PCR technique (48). Because of management practices, variations in the sensitivity and accuracy of diagnostic tests used, sample size, tick presence on horses and in stables, equine susceptibility, seasonal variations, climatic conditions, and vector control programs, the prevalence of *A. phagocytophilum* in equids differs from nation to nation (10,12,44,48).

Based on the PCR technique, donkeys had a significantly greater prevalence of *A. phagocytophilum* than infected horses. This finding concurred with that of de Oliveira *et al*. (49) who reported that donkeys had a considerably greater frequency of the EGA than mules. This might be because, compared to mules and horses, donkeys are more likely to be employed, which puts them under continual stress, lowers their immunity, and exposes them to ticks. Conversely, there is no discernible variation in the prevalence of *A. phagocytophilum* across horses, donkeys, and mules (46). According to the findings, the prevalence of *A. phagocytophilum* rose as the equids' ages increased. This result was in agreement with Oğuz, (19), Laamari *et al*. (37), and Alani and Yousif (44). Because foals obtain temporary protection from their mares through colostrum, which contains antibodies and will protect them from the disease in the first months after birth, they may be less susceptible to the disease after birth (21). Another study reported that the prevalence of *A. phagocytophilum* was non-significantly altered as the horse aged (50). Furthermore, this study observed that there was significant greater prevalence of *A. phagocytophilum* in female equines than male equines. This finding concurred with that of Hinson *et al*. (47). Suggesting that the causes may be related to physiological factors like estrus, pregnancy, and lactation period in females, which are regarded as stress factors and reduce the animal's immunity (51,52). However, no relationship between sex and the prevalence of EGA in horses was indicated by Laamari *et al*. (37) and Dražovská *et al*. (50). Additionally, equines outside stables had a significantly higher frequency of *A. phagocytophilum* than equines inside stables. This outcome aligns with the findings of Saleem *et al*. (10). The horses in stables receive better care than draft horses, so the latter are more susceptible to ticks, stress, and weakened immune systems (2). In the current study, equids with tick infestations had a significantly greater prevalence of *A. phagocytophilum* than equids without tick infestations. These outcomes were consistent with those of Alali *et al*. (53). One possible explanation for this could be that ticks are the primary *A. phagocytophilum* vector (15).

In this study, the Tamura-Nei model in MEGA11 software was subjected to 1000 replications utilizing bootstrap analysis and the likelihood approach (45), The phylogenetic tree of the local *A. phagocytophilum* sequences indicated that they have a very tight evolutionary relationship 99.46-100% and have common phylogenetic characteristics with the other *A. phagocytophilum* sequences released in the NCBI GenBank for different countries, involving North Korea (54), China (55,56), Japan (43), and South Korea (57).

Conclusions

The outcomes of this study indicate that *A. phagocytophilum* is widely spread and still circulating among equids in Mosul City, Iraq. There is numerous risk factors related to the high prevalence of the *A. phagocytophilum* that were infected equids. Furthermore, a strategically designed control program of ticks must be employed to avoid the distribution of this bacteria.

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Conflict of interest

No potential conflicts of interest are disclosed by the authors.

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التحليل الجزئي وشجرة النشوء للأنابلازما البلعمة في الخيليات في مدينة الموصل، العراق

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الخلاصة

أنابلازما البلعمة هي بكتيريا تسبب مرض الأنابلازما المحبة للخيول في الخيليات (الخيول القزمة والبغال والحمير)، وواسع الانتشار في العالم. تهدف الدراسة الحالية إلى تسجيل مدى انتشار بكتيريا الأنابلازما البلعمية في الخيليات في مدينة الموصل، العراق باستخدام تقنية تفاعل البلمرة المتسلسل، مع تحديد بعض عوامل الخطورة النسبية المرتبطة بانتشار أنابلازما البلعمة، والتحري عن التحليل النشوئي للبكتيريا المشخصة في هذه الدراسة. تم جمع مائة عينة دم من الخيليات (٤١ حمارا و ٥٩ حصانا) من مختلف الأعمار والأجناس والممارسات الإدارية. أظهرت النتائج أن معدل انتشار بكتيريا أنابلازما البلعمة في الخيليات في مدينة الموصل بلغت ٣٢%. لوحظ ارتفاع معنوي في معدل انتشار مرض الأنابلازما المحببة للخيول والمرتبطة بعوامل الخطر كالحمير، والأعمار أكثر من ٣ سنوات، وإناث الخيليات، والحيوانات خارج الإسطبلات، والقراد الموجود على الخيول. أكد التحليل الفردي لـ ٣٠ تسلسلا من جين 16S rRNA، والتي تم استخلاصها من دم الخيليات، وجود أنواع من بكتيريا أنابلازما البلعمة. تم إيداع أربعة من هذه التسلسلات في بنك الجينات بأرقام تسلسلية PQ594492.1 و PQ594493.1و PQ594494.1 وPQ594495.1. وكانت تلك التسلسلات مطابقة بنسبة ٩٩,٤٦% - ١٠٠% لتلك التسلسلات المودعة سابقا في بنك الجينات مثل: KC422267.1 في كوريا الشمالية و KC916734.1، MG869512.1، KC916737.1 في الصين و AB454076.1، AB196721.1 في اليابان و MT754315.1،MT754351.1، MT754365.1 وفي كوريا الجنوبية. استنتج، إن أنابلازما البلعمة منتشر على نطاق واسع بين الخيليات في مدينة الموصل، العراق وقد تساعدنا هذه النتائج في الدراسات المستقبلية السيطرة الاستراتيجية على الأنابلازما البلعمية في منطقة الدراسة.