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Molecular detection of *Anaplasma platys* in dogs in Nineveh province, Iraq

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Abstract

The present study was planned to evaluate the infection concerning *Anaplasma platys* in dogs for the first time in Nineveh Province, Iraq, using microscopic examination and conventional polymerase chain reaction (PCR) techniques. Eighty-one blood samples were randomly collected from dogs (31 household dogs and 50 stray dogs). Those samples were drained from dogs introduced to the Veterinary Teaching Hospital at the University of Mosul and private veterinary clinics in Nineveh province. The microscopic examination was used to check the blood films prepared for preliminary detection of morulae of *A. platys* within platelets; furthermore, the molecular technique was used to validate the presence of *A. platys* by targeting the *gltA* gene. In that, PCR yielded an amplification of 690 bp band size products, which was the expected size of the targeted gene. After that, these PCR amplicons were sent for sequencing to verify the presence of the pathogen, and then the sequence results were subsequently aligned with other strains available on the NCBI database. Finally, a phylogenetic tree for the related strains was constructed by Mega-11 software. The outcomes indicated that the overall infection rate of *A. platys* infection in dogs was 6/81 (7.4%) and 11/81 (13.6%) using microscopic examination and molecular techniques, respectively. Additionally, there was no significant difference within the dogs' categories in this study. In conclusion, it has been shown that *A. platys* in dogs were circulating in Nineveh province; therefore, plans for control measures are advised.

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Introduction

The agent of *Anaplasma platys* (*Ehrlichia platys*) is a tick-borne bacterium that infects mainly dogs and causes Infectious Canine Cyclic Thrombocytopenia (ICCT). This organism has a tropism for platelets and may disrupt these cells. However, the disease usually could be asymptomatic or subclinical in animals; however, it may show inappetence, weakness, emaciation, lethargy, and anemia in infected dogs (1,2). Hard ticks of the Ixodidae family are the primary transmitters for this pathogen. *A. platys* may infect other animals (such as cats, sheep, goats, and camels) with different prevalences worldwide (3). Tick-borne diseases pose a significant risk to human and animal health worldwide, as tick vectors could maintain a variety of pathogens, such as protozoal, bacterial, and viral. Many human infections with *A. platys* have been reported, reflecting its zoonotic potential (4,5). The prevalence rate of *A. platys* in dogs varies according to the region, presence of vectors, breed, and the type of diagnostic tools applied (such as serology, blood smear, and PCR). *A. platys* was previously identified in dogs from the United States in 1978 (6). Although the direct microscopic examination could reveal the presence of *A. platys* in a blood smear, this method has a low sensitivity in detection, especially in chronic cases and/or any cases characterized by low bacteremia (7). Several serological techniques, such as ELISA, indirect fluorescent antibody tests, and other available test kits, have been developed to detect animal Anaplasmosis. Cross-reaction with other members of Anaplasmataceae could be a significant drawback for serological detection (8,9). With the advancement of molecular techniques, many are used to

detect pathogens. Those tools are usually robust, susceptible, and accurate methods mainly used for species identification based on their specific identified genes, such as *gltA*, *groESL*, and *16S ribosomal* RNA (10,11). The susceptibility of dogs to infection with *A. platys* might have a substantial role in the epidemiology and transmission of this agent to other animals and/or humans. As companion animals to humans, dogs threaten public health by transmitting infectious agents, especially tick-borne organisms, such as Lyme borreliosis, Rickettsiosis, and Anaplasmosis (12,13). The latter has been reported as a human pathogen in the United States (14), and human infections have increased during the last two decades, according to the Centers for Disease Control and Prevention (CDC).

Therefore, *Anaplasma* species are considered an essential pathogen with many host preferences. Therefore, this scientific study aimed to investigate the presence of *A. platys* infection in dogs in Nineveh province, Iraq.

Materials and methods

Ethical approval

The study has been approved under the title code (UM.VET.2022.081) by the scientific research committee of the College of Veterinary Medicine, University of Mosul, Mosul, Iraq.

Animals and samples collection

The study examined 81 dogs; part of those samples was retrieved from the Veterinary Teaching Clinic of the College of Veterinary Medicine, University of Mosul, Iraq, and the others were from private veterinary stations in Nineveh province. The clinical examinations were applied to all animals. From March 2023 till November 2023, the blood samples were collected from the cephalic vein in dogs via the vacutainer tubes, and thin blood smears were made and stained with Giemsa stain for direct microscopic examination (15).

Microscopic examination of blood smears

In this topic, stained blood smears were prepared and examined under a light microscope (100X Oil immersion lens) to detect *A. platys* inclusion bodies within platelets.

PCR and sequencing

DNA was extracted from the EDTA-processed blood samples using a commercial extraction kit (Roche Ltd., Switzerland). Then, DNA concentration was evaluated with the aid of Nanodrop (Biochrom, UK). The polymerase chain reaction (PCR) was applied for all included samples (n=81) to confirm the results of direct microscopic findings. The *gltA* gene (a citrate synthase-responsible gene) was used as a molecular marker for detecting *A. platys* in DNA samples. For this purpose, the primers targeted by this gene in the current study were used (16), which included (Forward primer: ATGCTGTTTTGATGTGCGGG and reverse primer: CCGCACGGTCGCTGTT); these primers were provided by Macrogen Company, which is located in South Korea. The PCR conditions were as follows: initial denaturation at 95°C for 2 min, then 95°C for 30 s with 40 cycles for denaturation, annealing at 58°C for 30 s, followed by 72°C for 30 s for the extension, and the reaction was ended at 72°C for 5 min for a final extension step. The PCR products were visualized after running on 1% agarose via a UV transillumination system (Chemi-Doc System, Carlsbad, USA). Following checking the appropriate band size, the PCR products with forward primer were sent for sequencing by the Sanger method (Macrogen Co., South Korea). By aligning the recovered sequences with other strains listed on the NCBI website, the local *A. platys* sequences were identified. Moreover, the sequence of the *gltA* gene for the obtained strain of *A. platys* was deposited in the NCBI GenBank database. A neighbor-joining approach was selected for constructing a phylogenetic tree using Mega-11 software.

Results

The current work revealed that the overall infection rate of *A. platys* in dogs in Nineveh province was 6/81 (7.40%) based on the findings of microscopic examination (ME) of the blood films, and it was 11/81 (13.6%) with PCR technique. The clinical examination of suspected dogs showed loss of appetite, fever (39.5°C - 40°C), depression, weakness, pale mucus membranes, petechial hemorrhage on different parts of the body, nasal discharges, and coughing. However, some suspected dogs showed only signs of anemia, such as weakness and/or paleness of mucus membranes.

In this study, the infection rate of *A. platys* was 8.0% and 14.0% in stray dogs, whereas in household dogs, its prevalence was 6.45% and 12.9 %, according to the findings of ME and PCR techniques, respectively. Although more positive animals were detected in the stray dogs group, no significant statistical difference was observed between the two groups. The results based on the ME of 81 thin blood smears showed the presence of inclusion bodies of *A. platys* inside the platelets (Figure 1). In molecular analysis, the concentration and purity of the obtained DNA ranged between 55.3 - 285.9 ng/µl and between 1.8-1.9, respectively. In addition, the PCR screening for 81 DNA samples revealed the presence of amplified fragments of *gltA* gene with a band size in approximately 690 bp (Figure 2).

By comparing the retrieved local sequence OR194151.1 of the *gltA* gene of *A. platys* genotype to the available database in GenBank, it was possible to show that the local sequence was closely related 100% identity to those of Argentine MN725733.1, France AB058782.1, Thailand OP270645.1, China KR011928.1, Philippines JN121381.1, Italy DQ525687.1, Spain AY530807.1, and Brazil EU516387.1, as shown in (Tables 1 and 2).

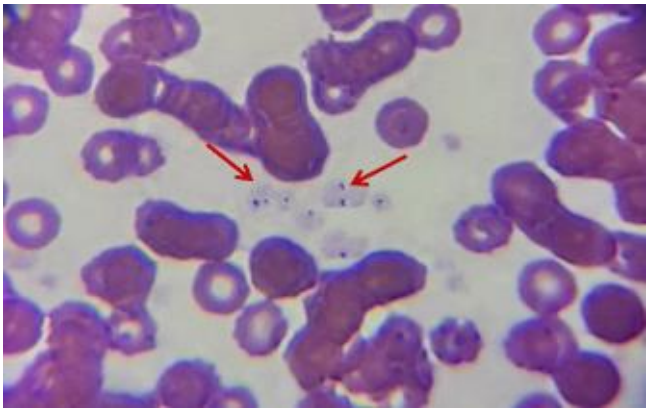

Figure 1: A blood smear stained with Giemsa stain showed inclusion bodies (morulae) of *A. platys* in platelets of an infected dog, examined under a light microscope with an oil immersion at (1000X).

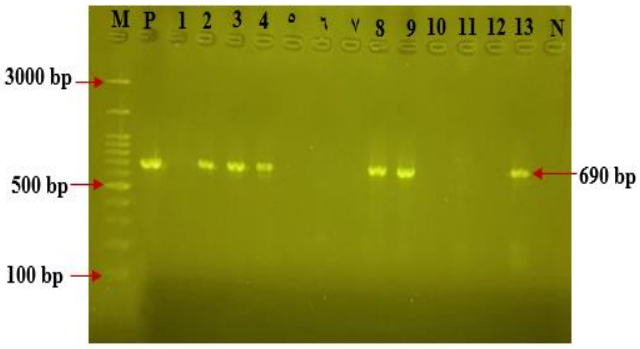

Figure 2: PCR amplicons loaded on 1% agarose gel. Lane M: DNA ladder 100-3000bp; Lane P: A positive control for *A. platys* (DNA sample from an infected dog); Lanes (2, 3, 4, 8, 9, and 13) showed the expected band size of about 690 bp; Lane N: considered as negative control.

Table 1: The nucleotide sequence of *gltA* gene for the local *Anaplasma platys* isolate WSHM1

Local genotype	Gene	Sequence	Accession No.
WSHM1	*gltA*	ACTGATGGGATACAATTGATAGACATCACTACGTTGTACAGAGAC CATAAGGTCTTGACCTACGATCCGGGATTCATGTCTACCGCGGCA TGCAGCTCTGAGATAACCTTTATCGACGGAGAAAAGGGTATACTG CGCCACCGCGGGCTTGACATTGCAGACCTAATAGGAATTAAAGGT GGCTTTTGTAGTGTGGCCCATTTACTGCTCTATGGGGTTTTGCCAT CAGACACAGTGTTCGAGCAATTTTCGGCAGCTATAGGGGCGCAGC ATGCTCTGTCATCCGACGTTTTAGGCGTGATTTCATCCTTCAGGAG AGATGCTCATCCCATGGCAATATTGATGGCATGCTTTTCAACTTTG GCTGCGAAGTATCATGGGGATAATAGGGGAAATGAAGAGC	OR194151.1

Table 2: Similarities between the local strain of *Anaplasma platys* (OR194151.1) and other genotypes on the GenBank

Name of strains	GenBank	Query	Sequence	Country
A. platys isolate CA citrate synthase (*gltA*) gene	MN725733.1	100%	403/403 (100%)	Argentine
A. platys RDC citrate synthase (*gltA*) gene	AF478130.1	100%	403/403 (100%)	Congo
A. platys isolate AG049-Saraburi citrate synthase (*gltA*) gene	OP270645.1	100%	402/403 (99.75%)	Thailand
A. platys isolate WSti2f citrate synthase (*gltA*) gene	KR011928.1	100%	402/403 (99.75%)	China
A. platys isolate DSE citrate synthase (*gltA*) gene	JN121381.1	100%	402/403 (99.75%)	Philippines
A. platys strain Dog 4 Sicily citrate synthase (*gltA*) gene	DQ525687.1	100%	402/403 (99.75%)	Italy
A.platys citrate synthase (*gltA*) gene	AY530807.1	100%	402/403 (99.75%)	Spain
A. platys (*gltA*) gene for citrate synthase	AB058782.1	100%	402/403 (99.75%)	France
A. platys strain RP citrate synthase (*gltA*) gene	EU516387.1	100%	400/403 (99.50%)	Brazil

Furthermore, the analysis of a phylogenetic tree using the maximum likelihood method in MEGA-11 software revealed that the local sequences of *A. platys* were closely related 99.50 - 100% identity to those available strains of *A. platys* in the GenBank database. The tree was rooted with *Anaplasma phagocytophilum* GQ412342.1/China, regarded as an outgroup (Figure 3).

Discussion

The tick-borne infection caused by *A. platys* is prevalent in tropical and subtropical areas. With the expansion of tick vectors due to climate change, the risk of introducing pathogens into new regions is potentially high. In this study, *A. platys* was reported for the first time in the northern part of Iraq. The microscopic examination of blood smears has a low sensitivity in detecting *A. platys*. However, it is a quick

and cheap method used routinely in laboratory diagnosis (7,17). The difficulties in examining blood film due to misleading artifacts, nuclear debris, and/or cytoplasmic aggregations may require validating the microscopic findings with more specific diagnostic tools such as serological tests and/or molecular methods such as PCR (18). The serological tests may indicate exposure to pathogens, whereas molecular tools could be more robust regarding diagnostic sensitivity and specificity (19-21). Moreover, the serological assays could result in false positive findings from cross-reaction with other genotypes (9,22).

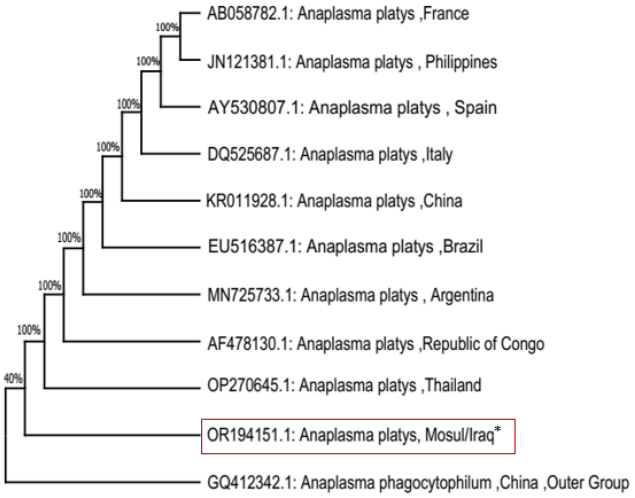

Figure 3: Phylogenetic hierarchy, built by the Tamura-Nei model using the Maximum Likelihood approach, of the partial sequence of *gltA* gene for the local isolate of *A. platys*. The bootstrap analysis with 1000 replications was used. This tree was used *Anaplasma phagocytophilum* (GQ412342.), China as an outgroup.

The phylogenetic tree of the local sequences of *A. platys* revealed that they share common phylogenetic characteristics and have a very tight evolutionary relationship with the other sequences of *A. platys* that have been submitted to the NCBI GenBank from different resources including Argentine (23), Congo (24), Thailand (25), China (26), Philippines (27), Italy (10), Spain (28), France (29), and Brazil (30) with the 99.5-100% identity. The Bootstrap analysis created the ancestor tree based on 1000 re-sampling and the Likelihood method on the Tamura-Nei model by MEGA-11 software (31,32).

According to the molecular investigation in tested samples, the current study found that the total infection rate of *A. platys* was 13.6%. On the other hand, the microscopic findings showed an infection rate of 7.4% in examined dogs. The molecular technique is susceptible to detecting the presence of *A. platys*, and it can effectively identify the species. However, the microscopic examination is considered a gold standard test for morphological examination of those agents, but it has less value in detecting them during low bacteremia phases and/or in persistently infected cases (33,34).

Regarding the PCR screening, the study outcome was higher than the reported prevalence rate of 3.33% in the examined dogs in Baghdad city (35) since a different number of targeted samples and different geographical areas might affect this observation. In some neighboring countries, it was reported as 6% in Turkey (36) and 3.7% in Iran (37). The prevalence rate in Egypt was 6.4% (38), whereas it was 5.5% in Algeria (39). Moreover, it showed 7.0% in Thailand (40), in Paraguay 10.67% (41), and in the Caribbean area it was 18.7% (42). The differences in the infection rate with *A. platys* in dogs may be attributed to climate conditions, sample size, abundance of ticks, vector control, different applied screening tests, and mode of living, i.e., stray and/or pet animals.

Conclusion

To our knowledge, this investigation reported the detection of *A. platys* in dogs for the first time in northern Iraq. It was preliminarily verified by microscopic evaluation of blood smears and then PCR and DNA sequencing (Molecular techniques). This report may enhance further epidemiological studies of that bacterial agent in different animal species in Iraq in future studies.

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Conflict of interest

There is no competing interest, as stated by the authors.

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الكشف الجزيئي للانابلازما بلاتيس في الكلاب في محافظة نينوى، العراق

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

الخلاصة

الدراسة الحالية صممت لتقييم النسبة المئوية للخمج بجراثيم الانابلازما بلاتيس في الكلاب في مدينة الموصل، العراق باستخدام تقنيات الفحص المجهري وتفاعل البلمرة المتسلسل التقليدي. تم جمع ٨١ عينة دم بشكل عشوائي من الكلاب (٣١ من الكلاب المنزلية و ٥٠ من الكلاب السائبة). جمعت العينات من الحيوانات الواردة للمستشفى التعليمي البيطري التابع لكلية الطب البيطري، جامعة الموصل. وكذلك من العيادات البيطرية الخاصة المتواجدة في محافظة نينوى. تم استخدام الفحص المجهري لفحص المسحات الدموية والتي سبق تحضيرها للكشف الابتدائي عن الأجسام الاشتمالية او التويثة لجراثيم الانابلازما بلاتيس في الصفيحات الدموية، وبعد ذلك تقنية الفحص الجزيئي تم استخدامها لتأكيد تواجد الانابلازما بلاتيس وذلك باستهداف جين *gltA*، تفاعل البلمرة المتسلسل أنتج تضخيم القطعة بحجم ٦٩٠ قاعدة جينية. بعد ذلك تم إرسال نواتج نفاعل البلمرة المتسلسل للتعرف على تسلسل القواعد النيتروجينية للتأكد من تواجد الكائن الممرض، وبعد الحصول على التسلسل تم بعد ذلك عمل مقارنة مع بقية العترات الموجودة ضمن قاعدة بيانات المركز الوطني لمعلومات التقنيات الأحيائية الأمريكية. بعد ذلك تم عمل شجرة النشوء الجيني للعترات المتقاربة عن طريق برنامج ميكا-١١. أشارت النتائج الى أن نسبة الاصابة الكلية بالانابلازما بلاتيس في الكلاب كانت ٨١/٦ (٧،٤%) و ٨١/١١ (١٣،٦%) باستخدام تقنيات الفحص المجهري وتفاعل البلمرة المتسلسل على التوالي. بالإضافة الى عدم وجود فرق معنوي بين مجاميع الكلاب المشمولة بالدراسة. يستنتج من هذه الدراسة أن الانابلازما بلاتيس منتشرة في محافظة نينوى بالعراق، لذلك ينصح بوضع خطط لتدابير مكافحتها.

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