

Molecular Detection of Some *Anaplasma* Species in Blood of Dogs in Baghdad Province, Iraq

Suha S. Ahmed Al –Obaidi*, Jenna M. Khalaf Al-Ani, and Naseir B. Al-Shammari

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

ABSTRACT

A total of 150 blood samples were collected from dogs and examined by the polymerase chain reaction (PCR) technique, which was used to detect the *16S RNA* gene of *Anaplasma platys* and *Anaplasma phagocytophilum*. Subsequent analysis of the PCR amplicons was achieved by nucleotides sequencing of some positive samples. Totally, the findings show the presence of PCR products (i.e., *Anaplasma* spp. infection) in 12/150 (8.0%) of the dogs under study. While 5/150 (3.33%) of the cases were *A. platys*, 7/150 (4.66%) were *A. phagocytophilum*. Nucleotide sequencing confirmed the identity of the amplified genes whose sequences were compared with other references belong to 15 of *16S rRNA* gene of *A. platys* and 14 references of *16S rRNA* gene of *A. phagocytophilum*, and the isolate sequences of this study were deposited on the Gene Bank. The identity and similarity scores between the isolates of this study and reference strains ranged from 98 to 99%. In conclusion, canine anaplasmosis prevalence in dogs could be underestimated in Iraq, and the phylogenetic tree of the local *A. platys* and *A. phagocytophilum* isolates were found to resemble other worldwide strains of *Anaplasma* spp. with a high degree of similarity.

Keywords: *Anaplasma platys*, *A. phagocytophilum*, PCR, Dogs, Blood, Iraq

Introduction

Dogs are among the most common pets which can be influenced by many tick-borne diseases of parasitic, bacterial, and viral origins resulting in considerable impacts on the health (1-3). *Anaplasma phagocytophilum* and *A. platys*, two bacterial pathogenic agents belonging to *Anaplasma taceae* family of Rickettsia in less order, are known to infect livestock and domestic animals including dogs (4). Canine anaplasmosis caused by *A. phagocytophilum* is characterized primarily by granulocytic vacuoles (canine granulocytic anaplasmosis); whereas, *A. platys* specifically infects the platelets resulting in cyclic thrombocytopenia (canine thrombocytopenic anaplasmosis) (5, 6).

Recently, many molecular assays are available commercially for diagnosis of canine anaplasmosis such as polymerase chain reaction (PCR) that demonstrated a high efficacy in diagnosis of acute as well as chronic infection (7, 8). Nucleic acid (DNA or RNA) based sequencing methods are greatly effective tools, which were broadly used for both diagnosis and characterization of these bacteria (9, 10). This study aimed to detect the incidence of *A. platys* and *A. phagocytophilum* in blood of dogs in Baghdad province by PCR technique in Iraq where neither molecular nor sequencing data are available on these bacteria in dogs.

Materials and Methods

Blood Sample Collection

Whole blood samples were collected from the cephalic vein of 150 dogs into EDTA anticoagulant tubes. The samples were transferred in a cooling box to the research laboratory at the Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad. The blood samples were used for the molecular diagnosis of *Anaplasma* spp. The study was

*Correspondence: fjdamfs@gmail.com, Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Baghdad, Iraq. Received: 2 December 2019, Accepted: 22 April, 2020, Published: 28 June 2020.

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carried out from October 2018 till April 2019 and approved by the Animal Care and Use Committee.

DNA Extraction

Genomic DNA from the whole 150 blood samples was extracted using a G-spin DNA extraction kit (Cat. No. 17045, Life Science/Korea). This kit was used for DNA extraction from the bacteria in a short time, and company instructions were followed. Concentration of genomic DNA was checked by

using a Nanodrop spectrophotometer. The DNA purity (absorbance at 260/280 nm) was also checked.

Primers Used

The primers used for amplification of the *16S rRNA* gene of *A. platys* and *A. phagocytophilum* from blood samples of dog are shown in Table 1. They were produced by IDT (Integrated DNA Technologies company, Canada).

Table 1. The primers sequences

Name of primer	Sequence	Size of product(bp)	Ref.
EC9 Forward	5'-TACCTTGTTACGACTT-3'	1500 bp	(11)
EC12 Reverse	5'-TGATCCTGGCTCAGAACGAACG-3'		

PCR Reaction

The PCR reaction mixture involved the use of iNTRONs Maxime PCR PerMix kit. The components of the kit included: i-Taq DNA Polymerase 5U/μl, dNTP mixture 2.5 mM, reaction buffer 1X, gel loading buffer 1X. The PCR working solution in a final volume of 25 μl included: 5 μl of Taq PCR PreMix, 10 picomoles/μl (1 μl) of each of forward and reverse primers, 1.5 μl of the DNA templates well as 16.5 μl of distilled water.

PCR Conditions

The conventional PCR thermocycler was used in this study, and the conditions of the reaction included initial denaturation at 94 °C for 8 min and 1 cycle, followed by the second denaturation at 94 °C for 45 sec, annealing at 58 °C for 45 sec and extension at 72 °C for 45 sec, and these three steps were repeated for 35 cycles to end with the final extension at 72 °C for 5 min.

Agarose Gel Electrophoresis of DNA

The PCR products were analyzed by agarose gel electrophoresis. The agarose gel was made in 1.5% concentration by melting 1.5 g of agarose in 100 ml of previously made TBE buffer. The gel was heated to boil, then left to cool down to 45-50°C. Afterwards, the gel was poured in its tray,

a comb was placed into its suitable place to make holes that would hold the samples, and finally the gel was left to solidify at room temperature for approximately 30 min. Later, the comb was removed gently from the solid gel, and the gel tray was fixed inside electrophoresis tank filled with TBE buffer that covered the gel surface.

Preparation of Samples

Before loading the DNA samples into the agarose gel, 3 μl of the processor loading buffer (Intron/Korea) were mixed with 5 μl of each DNA sample to be electrophoresed. Following mixing, the samples were loaded into the holes of the gel. Then, the electrophoresis was run at an electric current of 7 V/c2 for 1-2 h. The gel was tested by a source of UV light after placing the gel in pool containing 3 μl Red safe nucleic acid staining solution to make DNA bands visible.

Sequencing and Sequence Alignment

Sequencing of the gene was performed by National Instrumentation Center for Environmental Management. After obtaining the DNA sequences by email, they were treated manually for homology search using Basic Local Alignment Search Tool (BLAST) program, which is available at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>)

Results

The DNA concentration of the samples ranged between 1.6-1.9 ng/L. All isolates from which the DNA was extracted showed bands of approximately 1500 bp of the proposed *16S rRNA* gene amplicon on the agarose gel (Figure 1). Figure 2 shows the results of the PCR amplification to the *16S rRNA* gene of *A. platys*.

Phylogenetic of *A. phagocytophilum*

The phylogenetic tree analysis based on the partial sequence of the *16SrRNA* gene in the local *A. phagocytophilum* of dogs isolates analyzed the genetic relationship of this *Anaplasma* spp.. When comparing between *A. phagocytophilum* isolated from dogs of this study with other strains from different sources, including: (ID:

MK814406.1, CP015376.1, KP276588.1, KP745629.1, CP006618.1, JX173652.1, JX173651.1, HQ872464.1, AY527213.1, LC334014.1, KR611718.1, KY114936.1, HM366584.1, GQ450276.1), the degree of identify and similarity among our isolates with reference strain ranged from 99% to 100% (Figure 3).

Phylogenetic Tree of *A. platys*

The phylogenetic tree based on the partial sequence of the *16SrRNA* gene in the local *A. platys* of dogs isolates revealed the genetic relationship of this *Anaplasma* spp. When comparing between *A. platys* isolates of this study with different sources present on GenBank, including (ID: MK814421.1, MK121782.1, KY594914.1, LC269822.1, KX180944.1,

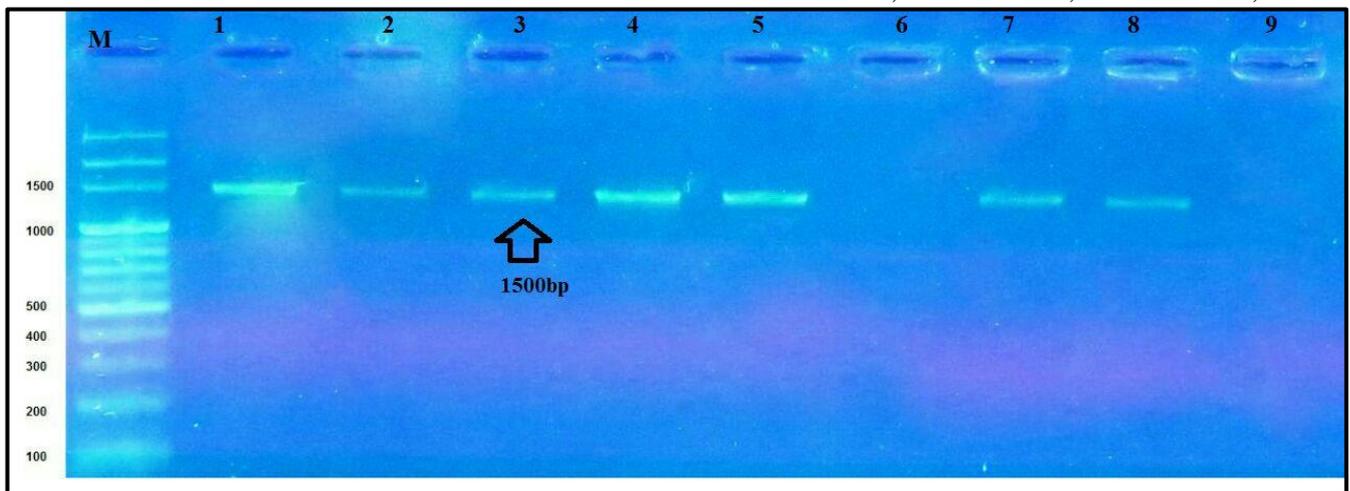


Figure 1. Agarose gel electrophoresis for 16S rRNA gene (1500bp) of *A. phago cytophilum*. Bands were fractionated by electrophoresis on a 1.5% agarose gel for 2 hrs at 5 volt/cm² and visualized under UV light after staining with red stain. Lane M: DNA ladder (100 bp)

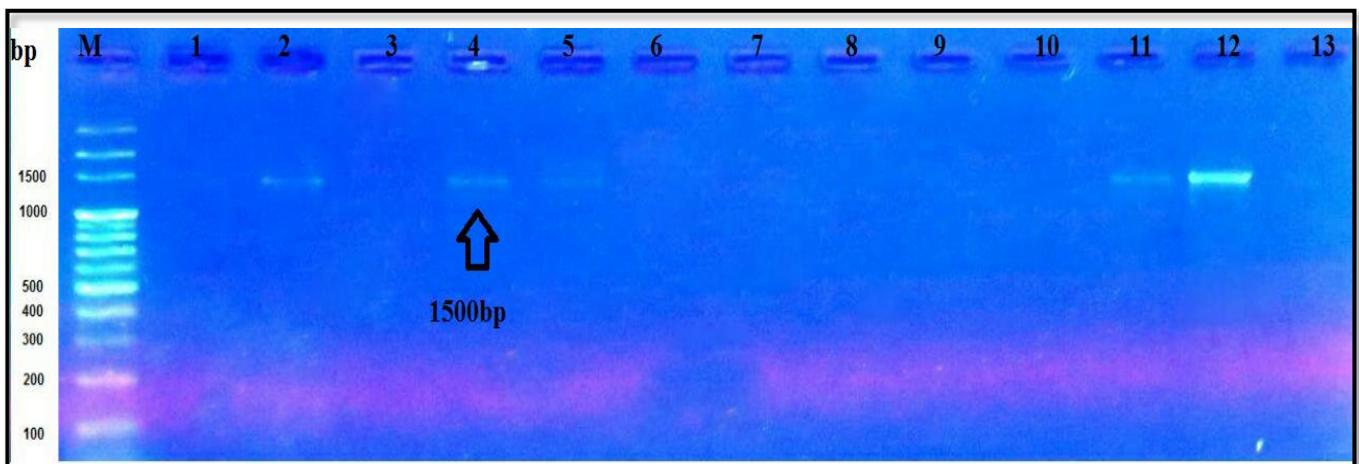


Figure 2. Agarose gel electrophoresis of PCR products for 16S rRNA gene. The amplified fragment is expected to have a molecular size of 1500 bp. The product was electrophoresed on 1.5% agarose for 1hr. M=DNA ladder (100 bp)

KY114935.1, KX447504.1, KJ659044.1, AY530806.1), the degree of identify ranged from 99% to 100% as seen in Figure 4.
 JQ396431.1, EU439943.1, EF139459.1,
 AF536828.1, AF303467.1, AF287153.1,

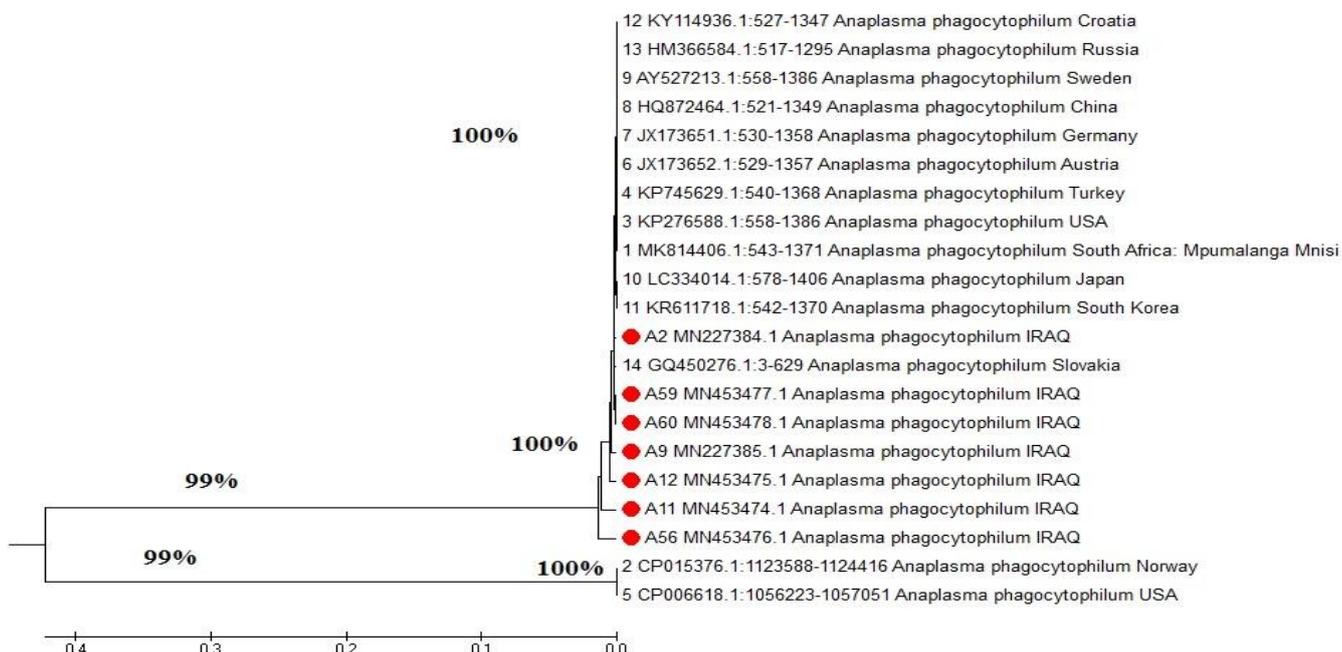


Figure 3. Neighbor-joining tree of *A. phagocytophilum* using the 16S rRNA gene. The above gene of *A. phagocytophilum* in the Iraqi isolates is represented by red circles compared with that of the 15 related strains deposited in the Genbank

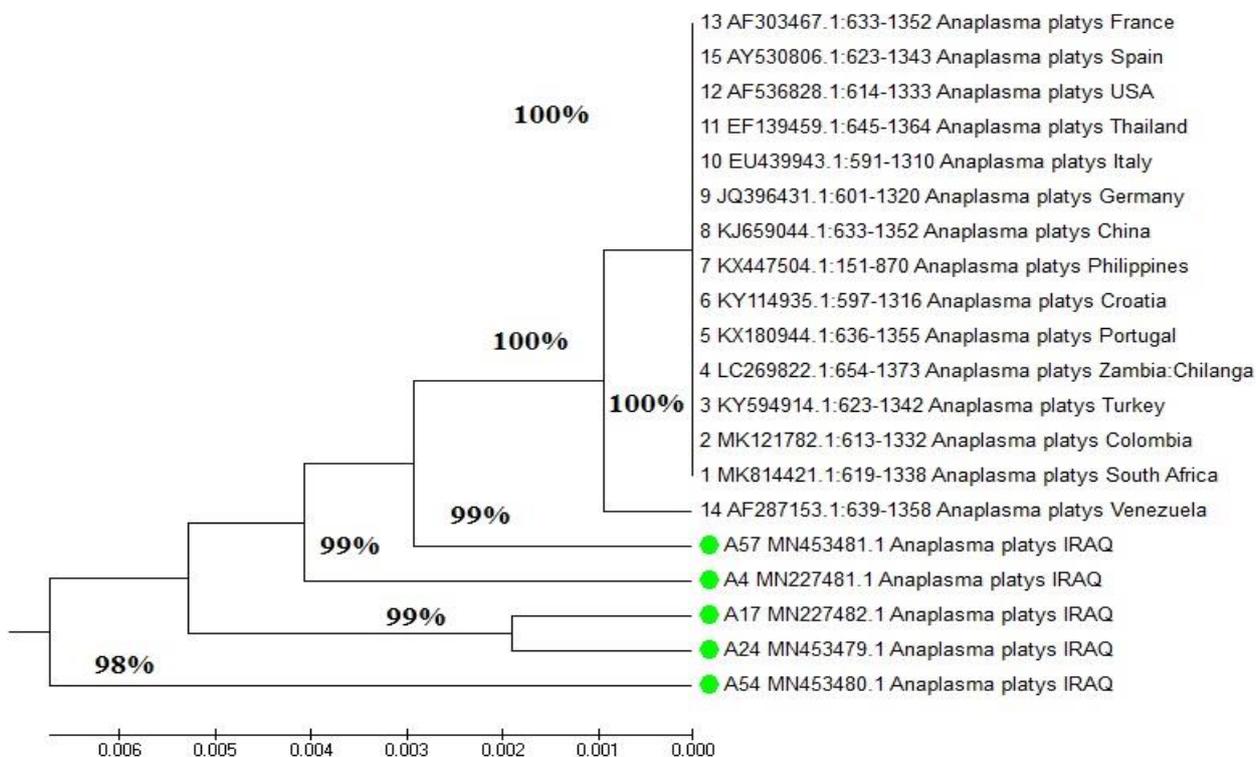


Figure 4. Neighbor-joining tree of *A. platys* using the 16S rRNA gene. The above gene of *A. platys* in the Iraqi isolates is represented by green circles compared with that of the 15 related strains deposited in the Genbank

Discussion

The results of the present study reported low anaplasmosis infection rate (12/150; 8.0%) in dogs of Baghdad city using PCR. While the cases infected with *A. platys* were 5/150 (3.33%), 7/150 (4.66%) of the cases were infected with *A. phagocytophilum*. When comparing the results with previous studies carried out in other countries, the overall prevalence of canine anaplasmosis obtained from the present study was compatible with many other studies, such as that of Turkey where the infection rates obtained using the PCR were 4.0% for *A. phagocytophilum* and 6.0% for *A. platys* (11). In Colombia, *A. platys* registered 2.2%, and *A. phagocytophilum* accounted for 3.3% out of 91 sampled dogs (12). The infection rates of anaplasmosis in dog population in different areas of the world have been investigated. These studies have reported higher prevalence of *A. phagocytophilum* in the world than that observed in Iraq, in which the infection rate was 2.62% (12, 13). In Italy, 1.25% of the samples were positive for *A. phagocytophilum* using PCR (13, 14). In China, the prevalence of this bacterium in stray dogs was 0.4% (15).

Concerning *A. platys*, the rate of infection in Qatar was 1.6% (16). It was found that 4.0% of dogs in Sicily, Italy were infected with *A. platys* (17). The variations in worldwide prevalence of anaplasmosis can be associated with the infection status, differences in climate and ecology, distribution of vectors, socioeconomic factors, and the tests used to evaluate infection, such as nested PCR or Real time PCR (18). Therefore, it is expected that the source of anaplasmosis in dogs might come from importing animals from neighboring and non-neighboring countries of Iraq as a high degree of homology was observed between the sequences described in the literature and those determined in the present study. In addition, the phylogenetic analysis of *Anaplasma* spp. by using the *16S rRNA* gene indicated that all of these strains might have similar genealogical species relationships, and these findings are in agreement with (19, 20).

In conclusion, canine anaplasmosis prevalence in dogs could be underestimated in Iraq and the phylogenetic tree of the local *A. platys* and *A. phagocytophilum* isolates resemble other worldwide strains of *Anaplasma* spp. with a high degree of similarity.

Conflict of Interest

All authors declare that there is no conflict of interest.

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الكشف الجزيئي لبعض انواع جراثيم Anaplasma في محافظة بغداد /العراق

سهى شهاب احمد العبيدي، جنان محمود خلف العاني ونصير بدوي الشمري

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

الخلاصة

من مجموع 150 عينة دم تم جمعها من الكلاب في محافظة بغداد فحصت عينات الدم بصبغها بصبغه الكيمزا وجرى تفاعل سلسلة خميرة البلمرة باستخدام بواقي Primer خاص بجين (S rRNA16) للمسبب المرضي وبعدها تسلسل القواعد النيروجينية لبعض العينات المصابة. تم التشخيص التاكيدي بواسطة الحمض النووي في الدم وكان معدل الاصابة الكلي 150/12 (8%)، ظهرت النتائج 5 عينة من 12 عينة دم مصابة بال 41.67% (A.platys) وبلغ اجمالي الكلاب المصابة بال *A.phagocytophilum* مع معدل اصابه 12/7 (58.33%). تم اجراء الفحص التاكيدي بواسطة تسلسل القواعد النيروجينية باستخدام خمسة عشر مراجع من الجينات S rRNA16 من *A.platys* واربعه عشر مراجع من الجينات S rRNA16 من *A.phagocytophilum* المسجلة في بنك الجينات، تراوحت درجة التطابق والتشابه بين النتائج في عزلاتنا مع السلالات المرجعية من 98% الى 99%.

الكلمات المفتاحية:- *A.platys*, PCR, *A.phagocytophilum*, كلاب, الدم, العراق