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ORIGINAL ARTICLE

Molecular identification of *Anaplasma platys* in cattle by nested PCR

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ABSTRACT

Background and Objectives: Anaplasmosis is a zoonotic disease caused by Gram- negative bacterium from Anaplasmataceae family. Anaplasma causes high economic losses worldwide. 16S rRNA analysis was used to diagnose *Anaplasma platys* in Cattle. Phylogenetic tree and estimation of evolutionary divergence between *A. platys* isolates were performed.
Materials and Methods: A total of 60 blood samples were collected from a cattle farm in AL- Diwaniyah province. 16S rRNA gene was identified using nested PCR. Overall, 40% of cattle that were chosen to collect the blood were identified to be infected with *A. platys.*
Results: The results have shown presence of targeting partial region of 16S rRNA gene in 24 samples out of 60. Sequencing results of 10 samples have revealed that the phylogenetic tree was divided in to two separate clades. Five isolates of *A. platys*- Iraq (accession no. OP646782, OP646783, OP646784, OP646790, and OP646791) were located in one clade with the *A. platys*- China (accession no. MN193068.1). While, five isolates (accession no. OP646785, OP646786, OP646787, OP646788, OP646789) were in different clade with two isolates of *A. platys*- Africa and *A. platys*- Zambia in distinct branches, close to the Rickettsiales.
Conclusion: The phylogenetic study of *A. platys* sequences indicated that the isolates were collected from a cattle farm in Al-Dewaniyah were similar and close related to *A. platys*- China, *A. platys*- Zambia and *A. platys*- Africa). This study suggests that cattle can be considered a reservoir of *A. platys*.

Keywords: *Anaplasma platys*; Cattle diseases; Tick-borne diseases; 16S rRNA

INTRODUCTION

Anaplasmosis is a tick- borne disease that infects humans and animals. It causes by Gram- negative bacteria affect blood cells (1, 2). Anaplasmosis is widely distributed and it is considered as an endemic disease in 43 countries around the world (3). Different species of *Anaplasma* such as *A. platys, A. marginale, A. centrale, A. bovis, A. ovis, A. caudatum,* and *A. phagocy*- tophilum can cause infection (1). *Anaplasma* spp. naturally need vector as ticks and vertebral host to survive (4). Ixodidae ticks are the biological vectors that have a key role in the proliferation and transport of *Anaplasma* (5).

First observation of *A. platys* (previously *Ehrlichia platys*) in animals was in 1978 in the USA (6-8), while in human the first case was reported in 1992 (9). *A. platys* mainly affects dogs, but it also infects

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human and other animals as cattle, cats, and camels causing thrombocytopenia due to platelets damage (10-13).

Clinically, infection with *Anaplasma* causes fever, anorexia, loss of weight, paleness of mucus membranes, nasal discharge, bilateral uveitis and lymphadenomegaly (6, 12). Non- septic blood equipment or the contamination during blood transformation and the flies' bite are the main ways that cause the infection. Also, the pathogen may transfer from the mother to the new born vertically (14).

Although their efficiency may be varied between different labs, it was noticed that molecular methods have an important role in the diagnosis of Anaplasmosis by PCR technique (15, 16). However, serological diagnosis methods were also very effective in *A. platys* diagnosis in cats and dogs and were increased from 0.4% to 87.5% effective diagnostic methods (17). Furthermore, Molecular Evolutionary Genetics Analysis (MEGA) software has improved and enabled sequence analysis by alignments, phylogeny and study sequence divergence (18). In this study, PCR assay was used to identify and recognise *A. platys* and focusing was on the genetic analysis and evolutionary divergence between different isolates of this organism in cattle.

MATERIALS AND METHODS

Samples collection and DNA extraction. Blood samples were collected from cattle stock in AL- Diwaniyah province aseptically then genomic DNA was extracted using kit from AddBIO (Korea) following the manufacturer's recommendation as 20 µl of Proteinase K solution (20 mg/ml) was added to 200 µl of whole blood then an equal amount of binding buffer was added to the mixture followed by incubation at 56ºC for 10 minutes followed by adding 200 µl of ethanol. The generated lysate was transferred into the provided spin column and then centrifuged at 13000 rpm for 1 minute. The flow-through was removed and the membrane of the spin column was washed twice using washing buffer 1 and 2 by adding 500 µl and centrifuged at 13000 rpm for 1 minute. The membrane was then dried out by additional centrifugation meanwhile the DNA was eluted with 50 µl of elution buffer.

PCR primers and reaction. In this study, primers designed by Kim et al. (19) were used including nested PCR approach. In the first round, 0,05 µM of each primer including EE-1 and EE-2 as follows 5' TCCTGGCTCAGAACGAACGCTGGCGGC 3' and 5' AGTCACTGACCCAACCTTAAATGGCTG3' with 10 µl of Hot start Taq master mix (AddBIO, Korea) and 2 µl of template DNA in a total volume 20 µl. Thermal conditions were initial denaturation at 95ºC for 10 minutes followed by 35 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 50 seconds. Finally, 72ºC for 5 minutes.

Similar conditions were used in the second round using 1 µl from the product of the first round and internal primers targeting the 16S rRNA gene to amplify 926 bp region including EE-3 5' GTCGAACGGATTATTCTTTATAGCTTGC 3' and EE-4 5' CCCTTCCGTTAAGAAGGATCTAATCTCC 3' (19). The generated PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide. These products were sent to Macrogen (Korea) for Sanger sequencing.

Sequencing preparation. Approximately 10 *A. platys* positive samples were selected and scaled up to 25 µL of PCR product for Sanger sequencing. The PCR products were purified using the GeneJET™ PCR Purification Kit (Fermentas), according to the manufacturer's instructions. One volume of binding buffer was added to the PCR product, the re-suspension solution was then transferred to the GeneJET™ purification column which was then micro-centrifuged for 60 s. A 700 µl volume of wash buffer was added and the column was then micro-centrifuged again for 60 s. The column was micro- centrifuged for 1 min, then 30-50 µl of elution buffer or autoclaved qH_2O was added and the column was centrifuged for 3 min. All micro- centrifugation was at 13,000 rpm.

Sequencing sending. To sequence PCR products, pure DNA samples of 15 µl and 50 ng in the sterile Eppendorf tubes were submitted with 15 µl of suitable sequencing primers. The results of sequencing then were analysed using NCBI BLAST.

Bioinformatic analysis. Phylogenetic tree of the targeting partial region within 16S rRNA gene in *A. platys* was constructed depending on the lowest Bayesian Information Criterion score by the maximum likelihood (ML) method as implemented in the MEGA X software using Bootstrap analysis with 1000 replicates and rooted with *Rickettsiales* bacterium (20).

RESULTS

PCR reaction. Positive results of the nested PCR for *A. platys* were found in 24 isolates with primer sets EE-3 5' and EE-4 5' to give a 926 bp of the targeting partial region of 16S rRNA gene in *A. platys.* This reaction was performed using PCR products of primer sets EE-1 and EE-2 as a template (Fig. 1). Gel electrophoresis results have shown that the 16S rRNA gene exists in 24 samples. This identification enables confirmation of the organism as *A. platys* according to Bakken (19).

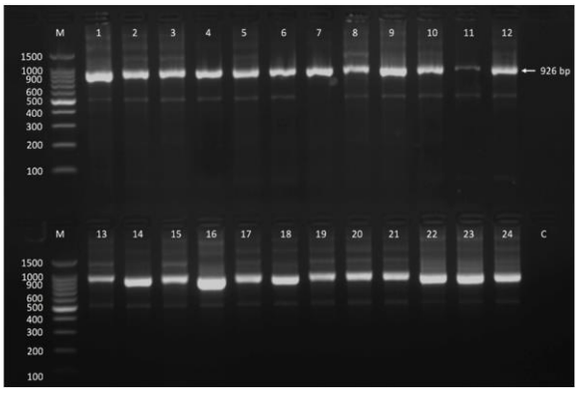

Fig. 1. Gel electrophoresis image (1.5%) agarose shows the PCR product (1-24) of *Anaplasma platys* targeting partial region within 16S rRNA gene (size = 926 bp). M: is molecular marker (Promega, USA); C: is control negative in which similar PCR components were used except H_2O was added instead of template DNA.

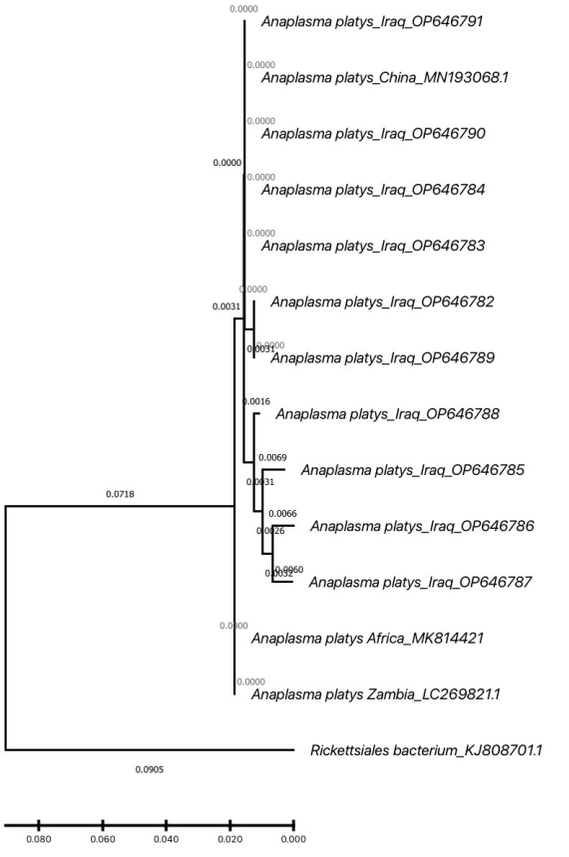

Fig. 2. Phylogram of the identified sequences targeting partial region within 16S rRNA gene of *Anaplasma platys*. this was carried out by MEGA X using Bootstrap analysis with 1000 replicates and rooted with *Rickettsiales* bacterium (20).

Phylogenetic tree analysis. The analysed sequences were deposited in the NCBI GenBank database meanwhile accession numbers were obtained as follows (OP646782, OP646783, OP646784, OP646785, OP646786, OP646787, OP646788, OP646789, OP646790, and OP646791) (Fig. 2).

The ten sequences of *A. platys* isolates were used to create the phylogenetic tree by the MEGA X using bootstrap analysis with 1000 replicates and *Rickettsiales* bacterium was as an outgroup. It has been found that the maximum likelihood tree of *A. platys* constructed with the 16S rRNA sequences is clearly divided into two different clads (Fig. 2).

Five isolates of *A. platys*- Iraq (accession no. OP646782, OP646783, OP646784, OP646790, and OP646791) were close to each other and located in the same clade with the *A. platys*- China (accession no. MN193068.1). However, the other five isolates of *A. platys*- Iraq (accession no. OP646785, OP646786, OP646787, OP646788, OP646789) were in another clade of the tree with two strains of *A. platys*- Africa (accession no. mk814421) and *A. platys*- Zambia (accession no. LC269821.1) and closer to the *Rickettsiales* bacterium (accession no. KJ808701.1). Importantly, it was noticed that *A. platys* sequences in this clade (accession no. OP646785, OP646786, OP646787, OP646788) were in distinct branches separate from each other.

Estimation of evolutionary divergence between *A. platys* sequences. The genetic relation among the *A. platys* sequences was determined by estimating the evolutionary divergence between sequences of the different isolates and comparing with the *Rickettsiales* bacterium sequence. The results have revealed that *A. platys* 16S rRNA gene showed a range of difference

from 0.0- 0.015 between all *A. platys* isolates. While *Rickettsiales* bacterium 16S rRNA was slightly more different with an evolutionary divergence between 0.14- 0.15 compared to the *A. platys* 16S rRNA sequences (Table 1).

Table 1. Estimation of evolutionary divergence of the 16S rRNA sequences was performed to find out the relation between *A. platys* isolates and *Rickettsiales* bacterium

	OP646782	OP646783	OP646784	OP646785	OP646786	OP646787	OP646788	OP646789	OP646790	OP646791	MN193068.1	MK814421	LC269821.1
Anaplasma_platys_Iraq_OP646782													
Anaplasma_platys_Iraq_OP646783	0.003146823												
Anaplasma_platys_Iraq_OP646784	0.003146823	0											
Anaplasma_platys_Iraq_OP646785	0.016017481	0.012784558	0.012784558										
Anaplasma_platys_Iraq_OP646786	0.012717348	0.011115026	0.011115026	0.014399171									
Anaplasma_platys_Iraq_OP646787	0.012717348	0.011115026	0.011115026	0.014399171	0.012717348								
Anaplasma_platys_Iraq_OP646788	0.007907494	0.004733747	0.004733747	0.011154029	0.012695103	0.012739672							
Anaplasma_platys_Iraq_OP646789	0	0.003146823	0.003146823	0.016017481	0.012717348	0.012717348	0.007907494						
Anaplasma_platys_Iraq_OP646790	0.003146823	0	0	0.012784558	0.011115026	0.011115026	0.004733747	0.003146823					
Anaplasma_platys_Iraq_OP646791	0.003146823	0	0	0.012784558	0.011115026	0.011115026	0.004733747	0.003146823	0				
Anaplasma_platys_China_MN193068.1	0.003146823	0	0	0.012784558	0.011115026	0.011115026	0.004733747	0.003146823	0	0			
Anaplasma platys Africa MK814421	0.006318825	0.003152254	0.003152254	0.016045878	0.014348502	0.014348502	0.004741949	0.006318825	0.003152254	0.003152254	0.003152254		
Anaplasma_platys_Zambia_LC269821.1	0.006318825	0.003152254	0.003152254	0.016045878	0.014348502	0.014348502	0.004741949	0.006318825	0.003152254	0.003152254	0.003152254	0	
Rickettsiales_bacterium_KJ808701.1	0.225822312	0.22058252	0.22058252	0.243481285	0.240455213	0.237783614	0.223854705	0.225822312	0.22058252	0.22058252	0.22058252	0.219395004	0.219395004

DISCUSSION

Anaplasmosis is clinically clear notable in cattle and could be fatal in some cases (21). The symptom of the disease has specific signs like high temperature, headache, and myalgias (15). PCR assay is considered as one of the important techniques that can be successfully used to diagnose the *A. platys* in blood samples of animals and human (22, 23). The 16S rRNA gene has been used to detect and identify pathogens and to study the phylogenetic relations in many organisms because it is highly conserved (24). Thus, 16S rRNA was used in the present study to identify *A. platys* in cattle blood samples. However, the blood samples were collected at only one time point and it is possible that the result could be different if the samples were collected in more than one time around the year.

Normal and nested PCR were used in this study for diagnosis of *A. platys.* Two sets of primers (EE-1, EE-2, EE-3, and EE-4) were used to detect and amplify the targeting partial region of 16S rRNA (19). Interestingly, these primers were used in previous work to detect *A. Phagocytophiulm* before the deposition of the *A. platys* sequence. However, the sequencing results have shown specific identification of *A. platys.* Therefore, the same primers were used for the diagnosis of both organisms. PCR results have shown that nearly 40 % (24 samples) of the collected samples were positive.

It is important to note that *A. marginale* was more incidence in water buffalo accompanied with the rickettsial pathogens (24). While, *A. platys* is mostly infect in cattle depending on the availability of vector. Similar results were proven in different studies and showed that incidence of *A. platys* infection in cattle, sheep and camels was high and is not restricted in dogs or cats (19, 24). In addition, it was noticed that infection with *A. platys* is high in cattle and human in addition to the dogs (10, 11, 13).

Only 10 of the positive samples were sent for sequencing to study the sequence diversity. It was noticed that the phylogenetic tree was divided into

two different clades. Five isolates of *A. platys*- Iraq (accession no. OP646782, OP646783, OP646784, OP646790, and OP646791) were in one clade with the *A. platys*- China (accession no. MN193068.1). However, *A. platys*- Iraq (accession no. OP646785, OP646786, OP646787, OP646788, OP646789) were in a different clade with the *A. platys*- Africa (accession no. mk814421) and *A. platys*- Zambia (accession no. LC269821.1).

Moreover, results of the evolutionary divergence between sequences of the *A. platys* showed that the *A. platys* 16S rRNA genes were different at 0.0- 0.015 while *Rickettsiales* bacterium 16S rRNA was slightly different in the evolutionary divergence at 0.14-0.15 compared to the *A. platys* 16S rRNA sequences (Table 1). Importantly, the cattle blood samples were collected from a cattle farm in AL- Diwaniyah province where dogs were found which may consider one of the factors in cattle infection due to environmental contamination. Also, *R. sanguineus* (dog tick) was in the farm where the samples were collected and its role in *A. platys* transmission was determined (depending on the presence of *A. platys* DNA in this tick). It was identified that the availability of the natural vector (ticks) is considered a key cause of *A. platys* infection (4, 5). Therefore, one of the important strategies to control and restrict Anaplasmosis in cattle is vector management.

CONCLUSION

The findings of this study suggest that cattle can be considered a reservoir of *A. platys.* The phylogenetic study of *A. platys* sequences indicated that the isolates were collected from a cattle farm in Al- Diwaniyah were similar and close related to other strains from different countries like (*A. platys*- China, *A. platys*- Zambia and *A. platys*- Africa). In this study *A. platys* in cattle were isolated from blood samples using genomic DNA extraction without separation of blood components and also using sets of primers were used to detect *A. Phagocytophiulm*.

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