

## Research article

# Molecular survey and phylogeny of *Anaplasma Ovis* in small ruminants in Al-Qadisiyah Province, Iraq

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## Abstract

*Anaplasma ovis* is a one of an important group of the tick-borne pathogen and an obligate intraerythrocytic bacterium, which infects sheep and goats as well as wild ruminants. The phylogenetic study of *A. ovis* in small ruminants has not studied yet in Iraq. In this study, the presence of *A. ovis* was investigated in a total of 80 (40 sheep and 40 goats) obtained from 16 randomly selected small ruminants flocks in AL-Qadisiyah in Iraq. All blood samples tested microscopically, firstly by Diff-quick stained blood smear for the detection of intraerythrocytic pathogens. Total DNA was extracted from a sample and submitted to PCR based on fragment amplified of 16S rRNA gene followed nucleotide sequencing and phylogenetic analysis. Six out of 80 samples, (10%) from sheep and (5%) from goats gave positive results. The results of nucleotides sequencing and multiple alignments revealed related Iraqi isolates had a high identity (99.70% - 97.21%) with isolates of other countries, the phylogenetic analysis demonstrated that Iraqi isolates of *A. ovis* fell one clade near to Russian and Sweden strains and shared 99.7 %-98.36 % with them. In conclusions: this work indicates to detect *A. ovis* at a low rate in sheep and goat in Iraq and it had a high genetic similarity to world strains.

**Key word:** *Anaplasma ovis*, Iraq, PCR, phylogenetic.

## Introduction

Ovine anaplasmosis is a tick-transmitted rickettsia disease that is caused by *A. ovis* infected sheep, goats and wide range of ruminants. The pathogen is highly endemic throughout tropical and subtropical area (1). There are two among six species of the *Anaplasma* genus infect the red blood cells of sheep and goat, *A. ovis* and *A. marginale* (2, 3). *A. ovis* is a gram-negative, non-motile and intraerythrocytic bacterium have emerged as zoonotic importance (4). In spite of, there is a variety of ways to transmission of Rickettsia pathogen; ticks and biting flies considerable important two vectors are responsible for local cases of infection. In addition, geography and climate are determining

which vectors (7, 8). Although, *A. ovis* considered to be has moderate pathogenicity and usually induce mostly mild clinical signs in infected animals (5, 6), the infection in sheep and goat is usually recognized as such, and may cause debility, depression, weight loss, decreased milk production, severe anemia, and jaundice and sometime abortion in tropical and subtropical area also it can predispose hosts to other infections lead to severe illness and finally death, (9). Diagnosis of *A. ovis* is performed routinely by morphological identification based on location of inclusion bodies marginally within the erythrocytes (10). Blood smears staining with Giemsa stain can be indeed

used to detect Anaplasma agents in the animals clinically suspected to acute anaplasmosis, however(11). However, the present study was conducted to determine prevalence *A. ovis* of sheep and goat in different pasture in middle of Iraq based on microscopy and molecular tests, and determined the genetic identity and phylogeny of *A. ovis*.

## Materials and Methods

### Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 289

### Collection of blood samples:

From 2016 to 2017 and during the tick activating seasons, ethylene diamine tetraacetic acid, (EDTA) blood samples were collected from jugular vein of 80 asymptomatic domestic small ruminants (40 sheep and 40 goats) farmed in different areas of Al-Diwaniyah governorate. Thin blood smears were prepared for preliminary diagnosis by microscope and remaining blood was stored at -20 C° for DNA extraction.

### Direct examination:

Examination of blood smear by light microscopy for a month before conducting animal experiments, thin blood smears were prepared by fixed blood smears with methanol were stained with Diff-quick stain (Syrbio, Switzerland) according company instructions examined under oil immersion lens (x100) for detection Anaplasma inclusion bodies.

### DNA extraction:

DNA was extracted from all blood samples by using a genomic DNA extraction kit (Geneaid DNA extraction kit, Korea) according the manufacturer's instructions for blood extraction. DNA yields were measured by Nano droop 2000 (The Thermo Scientific, German) and typical concentration values ranged from 15- 24 ng/μl. The extracted

DNA was stored at -20 C until use for PCR analysis.

### *Anaplasma ovis* PCR reaction and condition:

In order to scrutinize the presence of *A. ovis* in Iraqi domestic sheep and goat, 80 blood DNA extractions were initially tested with 16S rRNA by using two conserved primers: sense primer 8F (5-AGAGTTTGATCMTGGCTCAG-3) and antisense 1492R (5-CGGTTACCTTGTTACGACTT-3) which described previously by (12) The amplification was carried out in a thermocycler (Bioneer, Korea) using the PCR program consist from 31 cycles of (94 C for 60 sec; 54C for 60sec; 72 C, 2.5 min) then 72C for 10 min extension for the last cycle, and the initial denaturation was 95C / 5 min. PCR mixture containing was 20μl contained: 10 mM Tris/ HCl, pH 8.3; 2.5 mM MgCl<sub>2</sub>; 50 mM KCl; 200 mM each of deoxynucleoside triphosphates; 30 pM for primer each; and 2U Taq polymerase. DNA of samples were 5μl then the volume was completed to 50 μl with deionized water. The PCR products were electrophoresed via 1.25 % agarose gel, and then visualized by Gel documentary (Bioneer, Korea). The anticipate PCR product for Anaplasma pp. were 686 bp.

**DNA sequencing and phylogenetic analysis:** When we sequenced the positive 16S rRNA PCR amplicons, obtained clean sequencing data for 6 of the samples (4) from sheep and (2) from goats subjected to nucleotides sequencing in Bioneer, (South Korea). The sequences in the both animals (sheep and goat) were most commonly identical to those of *A. ovis* with positive animals province studied. The representative sequences submitted in GenBank with accession numbers, "MF579750.1 and MF579751.1 for *A. ovis* were detected from sheep and goats respectively.

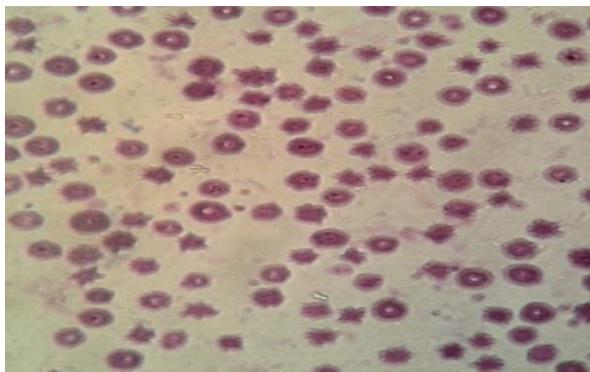
**Results**

During one year, sheep and goats from number of Al-Diwaniyah flocks examined for the presence of *A. ovis*. The flocks selected randomly. Twenty-four cases 30% gave positive result in microscopic examination for presence inclusion bodies within erythrocytes, Anaplasma like structure, Figure (1) Out of 24 specimens that contain erythrocytes inclusion bodies, all samples submitted to PCR test, only six gave positive results for PCR detection using Anaplasma spp primer (figure 2).The PCR assays detected in total of 7.5% (6/80) tested small ruminants, and separately 10% (4/40) of sheep and 5% (2/40) of goat were

determined to be positive for *A. ovis* (Table 1). All positive samples by amplifying of 16S rRNA gene, showed the expected 668 bp. The sequencing result for two of PCR amplicons submitted to GenBank with accession number MF579750 and MF579751. All positive amplicons of *A. ovis* were of the expected sizes of 686 bp (Fig. 3).The percentage identity between the 2-nucleotide sequences of 16S rRNA gene 100%. In addition, these sequences shared 97.21-99.7% sequence identity with previously published sequences worldwide Table (2).

**Table (1): Numbers and percentages of *Anaplasma ovis* in sheep and goat tested by microscopic and molecular assays**

Animals	Samples	+ve microscopic exam.	%	+ve PCR	%
Sheep	40	17	42.5%	4	10%
Goat	40	7	17.5%	2	5%
Total	80	24	30%	6	7.5%



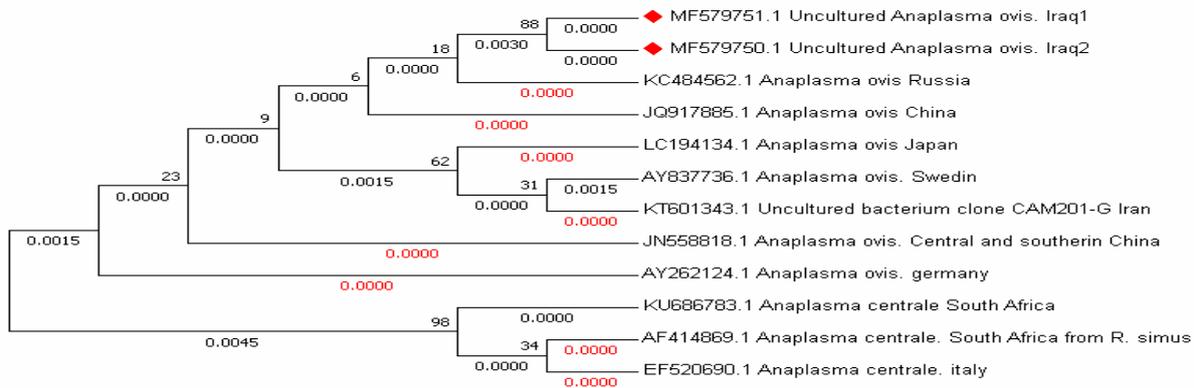
**Figure (1): Blood smear of sheep show the *Anaplasma spp.* stained by Diff-quick stain (100×)**



**Figure (2): Electrophoresis of 686 bp 16s rRNA gene *Anaplasma ovis* detected from sheep and goats in Iraq.**

**Table (2): Table. Estimates of Evolutionary Divergence between Sequences of Anaplasma spp. based on 16SrRNA PCR that detected from sheep and goat in Iraq. Analyses was conducted by the Maximum Composite Likelihood model. The differences in the bias composition among sequences were considered in evolutionary comparisons. The analysis involved 2 partial gene sequences of Iraqi isolates (Itaqi1 and Itaqi2) aligned with 10 GenBank sequences. All positions containing gaps and missing data were eliminated. There were a total of 679 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.**

	1	2	3	4	5	6	7	8	9	10	11	12
1. MF579751.1 Uncultured Anaplasma ovis, Iraq1		0.000	0.004	0.002	0.003	0.004	0.002	0.002	0.002	0.002	0.004	0.002
2. MF579750.1 Uncultured Anaplasma ovis, Iraq2	0.000		0.004	0.002	0.003	0.004	0.002	0.002	0.002	0.002	0.004	0.002
3. AF414869.1 Anaplasma centrale, South Africa from R. simus	0.009	0.009		0.003	0.004	0.000	0.003	0.003	0.003	0.003	0.000	0.003
4. AY262124.1 Anaplasma ovis, germany	0.003	0.003	0.006		0.002	0.003	0.000	0.000	0.000	0.001	0.003	0.001
5. AY837736.1 Anaplasma ovis, Swedin	0.006	0.006	0.009	0.003		0.004	0.002	0.002	0.002	0.001	0.004	0.001
6. EF520690.1 Anaplasma centrale, italy	0.009	0.009	0.000	0.006	0.009		0.003	0.003	0.003	0.003	0.000	0.003
7. JN558818.1 Anaplasma ovis, Central and southerin China	0.003	0.003	0.006	0.000	0.003	0.006		0.000	0.000	0.001	0.003	0.001
8. JQ917885.1 Anaplasma ovis China	0.003	0.003	0.006	0.000	0.003	0.006	0.000		0.000	0.001	0.003	0.001
9. KC484562.1 Anaplasma ovis Russia	0.003	0.003	0.006	0.000	0.003	0.006	0.000	0.000		0.001	0.003	0.001
10. KT601343.1 Uncultured bacterium clone CAM201-G Iran	0.004	0.004	0.007	0.001	0.001	0.007	0.001	0.001	0.001		0.003	0.000
11. KJ686783.1 Anaplasma centrale South Africa	0.009	0.009	0.000	0.006	0.009	0.000	0.006	0.006	0.006	0.007		0.003
12. LC194134.1 Anaplasma ovis Japan	0.004	0.004	0.007	0.001	0.001	0.007	0.001	0.001	0.001	0.000	0.007	



**Figure (3) :** Phylogenetic tree of show evolutionary relationships of *A. ovis* based on the 16S rRNA partial gene sequence. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N. and Nei M. 1987). The bootstrap consensus tree inferred from 1050 replicates (Felsenstein J. 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1050 replicates) are shown above the branches. (below the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura K., Nei M., and Kumar S. 2004) and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 679 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar S., Stecher G., and Tamura K. 2016).

## Discussion

Although, the small ruminants (Sheep and goats) are highly important agricultural animals in most developing countries, and the ruminants considered as important hosts and reservoirs for different *Anaplasma* species in the subtropical and Mediterranean areas (13), *A. ovis* was relatively frequent detected in sheep and goats (14). In Iraq, the investigations of *A. ovis* infections in sheep and goat have been studied recently, since then, only few reports concerning the infections were carry out (15). The importance of microscopic examination in detection of haemoparasites in blood smears as the most inexpensive and quickest is well considered as laboratory test, but also the least sensitive, and is highly dependent on experience of examiners (16, 17). Although, the most common diagnostic method to be used for detection of *Anaplasma spp.* is the microscopic examination with Giemsa stained blood smears or other differentiated stains, But in carrier animals due to that the number of *Anaplasma spp.* fluctuate in the host over time and this variability in bacteremia levels may have been below the

detection threshold. Other reason, the difficulty to differentiate among *Anaplasma* organism and other hemoparasites group and structural blood cells abnormalities like Howell-Jolly bodies, Heinz bodies, or staining technique artifacts, which are often seen in stained blood smears this method is not sufficient for the characterization of persistently infected cattle (18, 19). For differentiation of *A. ovis* from other *Anaplasma spp.* infection, the PCR amplicons from all positive PCR samples was sequencing and alignment with global previously published sequences at NCBI database. The infection of *A. ovis* was detected in the surveyed area of Al-Diwaniyah governorate in this study, and in general, the infection rate was low, but the high infection rate 10% in sheep compared with goats 5%, that indicates the limited distribution of this pathogen in the area of study. In this survey was conducted in the middle region of Iraq *A. ovis* was genetic identified and phylogenetic study for the first time in this region, but their overall prevalence 7.5% was lower than reported by

other authors. For instance, our results were in agreement with demonstrated the lower prevalence of *A. ovis* in sheep (10.00%) than the results reported by (20, 21) who recorded 20.8% and 43.08% respectively. In contrast to this study, the present study demonstrated the *A. ovis* in sheep was higher than other percentage detected in of sheep in west Iran 5.00% by PCR (19). However, more samples may be needed to define the prevalence of infection in the small ruminant of Al-Diwaniyah governorate. In contrast to this study, a high frequency of *A. ovis* was detected in the small ruminants may be correlated with variety of predominant of transmitted vectors among regions (22). Additionally, *A. ovis* infection in goat 5.00 % was lower than the results reported by (18).

This variation may have been the result of different sampling methods, geographical diversity and climate differences and presence of mechanical and biological transmitting vectors. According to previous study in neighboring areas (17), level of parasitemia, which is a higher level of sensitivity than the 0.1 to 0.2% obtained by light microscopic examination of blood smears (23). The differences in the prevalence of small ruminants on the farms we studied were most likely due to differing husbandry practices and tick exposure (23, 24)

### Conclusion

The study showed the low prevalence of *A. ovis* in the blood of healthy sheep and goat in Al-Qadisiyah Province.

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