Molecular detection and phylogenetic analysis of *Anaplasma phagocytophilum* in Cattle

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Abstract

Anaplasma phagocytophilum ,obligate intracellular rickettsial pathogen, is transmitted by ticks and impact human and animal health. This study was conducted to determine the presence of *A.phagocytophilum* in cattle in Al-Qadisiyah province using polymerase chain reaction (PCR) assay followed by partial sequencing of the 16S rRNA gene. A total of 130 blood specimens were collected from cattle suffering from tick infestation and anemia, Diff-quick stained blood smear revealed Anaplasma like inclusion bodies in 63.8% (83/130) microscopically. These specimens containing anaplasma like structure tested by PCR and revealed Anaplasma in 28.9% (24/83). Phylogenetic analysis of ten PCR products revealed 4 were *A. phagocytophilum*, 2 *A. marginale* and 4wereAnaplasma sp.

Keywords: *Anaplasmaphagocytophilum*, prevalence, phylogenetic analysis

Introduction

Anaplasmaphagocytophilum is the lately designated name substituting three species of granulocytic bacteria which include the agent of human granulocytic ehrlichiosis, now known as human granulocytic anaplasmosis (HGA), *Ehrlichiaequi* and *E. phagocytophila* (Ybanez and Inokuma, 2016).

A. phagocytophilum is Gram negative obligate intracellular bacterium which multiply mainly in animals and Human neutrophils (Chen et al, 1994). It is the agent of tick-born fever or pasture fever of ruminants

(Woldehiwet, 2006 ; Stuen,2007). HGA was first described in 1993 and it potential threat to human has been increasingly detected in United States and many countries in European (Grzeszczuk et al. 2007).

Ixodid ticks and *Haemaphysalismegaspinosa* play important role in the transmission of *A. phagocytophilum* in animals (Ybanez et al. 2012 ; Yoshimoto et al. 2010). While *H. formosensis* , *H. longicornis* and *Ixodesovatus* associated with HGA in Japan (Ohashi et al. 2013). Anaplasmosis in cattle is characterized by high fever, inclusions in circulating neutrophils, leukopenia, decrease in milk production, reduce fertility and abortion. After experimental infection in cattle the incubation period 4- 9 days and the fever last for 1-13 days. The infection result in mild to moderate clinical signs and rarely fatal unless other diseases complication (Noaman V. and Shayan P 2009).

In diagnosing cases of A. phagocytophilum infection clinical signs and microscobic examination are unreliable as infection can be persistent and subclinical in animal with no indications in the smears (Aktas and Ozubek, 2015). However, detection of Anaplasma using these methods may be not suitable if the organism is present in low level in the blood (Ndung'n et al. 1995). Serological test for diagnosis of anaplasmosis is also hampered by cross-reaction (Torioni de Echaide et al. 1998).

Polymerase chain reaction (PCR) technique have been used to rapid diagnostic and phylogeny studies of anaplasma species based on 16S rDNA and other genes (MSP1, MSP5, glt A and gro EL). *A. phagocytophilum* has relatively small genome consisting of single circular chromosome (Dunning Hotopp et al., 2006). The total genomic sequence of *A. phagocytophilum* estimated at1471282 bp was deposit to GenBank with code NC007797 in 2006. Complete genome sequencing

of *A. phagocytophilum* has largely facilitated research into diversity of this bacterium (Rymaszewska, 2011).

Materials and methods:

Fifty blood samples of cattle suffering from anemia and tick infestation were collected from unorganized farms in Al-Diwaniyah city begning from summer 2014 till the end ~~during summer 2014~~. The samples were classified in two pathways, one of pathway, 2ml of blood was stained by Diff-quick stained blood smears (refernce) and the other pathway, ~~Three~~ three milliliters of blood were collected from the jugular vein of each animal in to sterile anticoagulant tubes and stored at -20° C until further processing for molecular investigation.

Guidelines for the ethical use and care were obtained from Institutional Animal Care/ College of Veterinary Medicine, University of Al-Qadisiyha.

DNA extraction was done for the collected samples by Presto TM Mini g DNA bacteria extraction kit (Geneaid, Korea), according to the manufacturer instruction. The concentration and purity of extracted DNA were estimated by nanodrop/ ~~thermo~~ Thermo Fisher USA. The purified DNA was stored at -20° C until use in molecular analysis.

The amplification of *A. phagocytophilum* DNA was performed using the following primers , forward (5' –GGT ACC YAC AGA AGA AGT CC-3') and reverse (5'-TAG CAC TCA TCG TTT ACA GC-3'),to amplify a 345bp fragment from 16S rRNA gene according to the (~~refernce~~Refernce).The PCR amplification was done in 50µl total volume including: 100-300 ng DNA template, 10 pm of each forward and reverse primer, 25 µl of 2X Taq master mix with dye and 17 µl of nuclease free water, were used to amplified target DNA under the

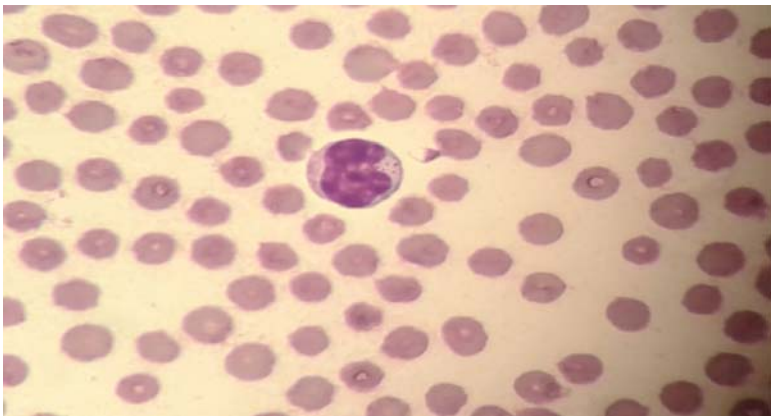
following conditions: two minutes incubation at 94° C for initial denaturation, followed by 40 cycles of 30 second at 94 ° C for denaturation, 30 second at 54° C for annealing and one minutes at 72 ° C for extension,finally the addition extension step for 5 minutes to complete DNA extension and holding at 4 ° C . The PCR product of 345bp were analyzed by 1.5% agarose gel electrophoresis.

The amplicon of positive samples were send to Bioneer company (unite) in Korea for DNA sequencing by AB DNA sequencing system. The phylogenetic analysis were accomplished dependence on NCBI blast alignment identification (www. website)and unweight pair group method with arithmetic mean tree (UPGMA tree) by using MEGA7 software.

Results and discussion:

During one year, 130 blood samples were collected from tick infestedation cows In in Al-Diwaniyah. 63.8% (83/130) gave positive results for microscopic examination of Diff-quick stained blood smears for presence of Anaplasma like inclusion bodies (figure 1)

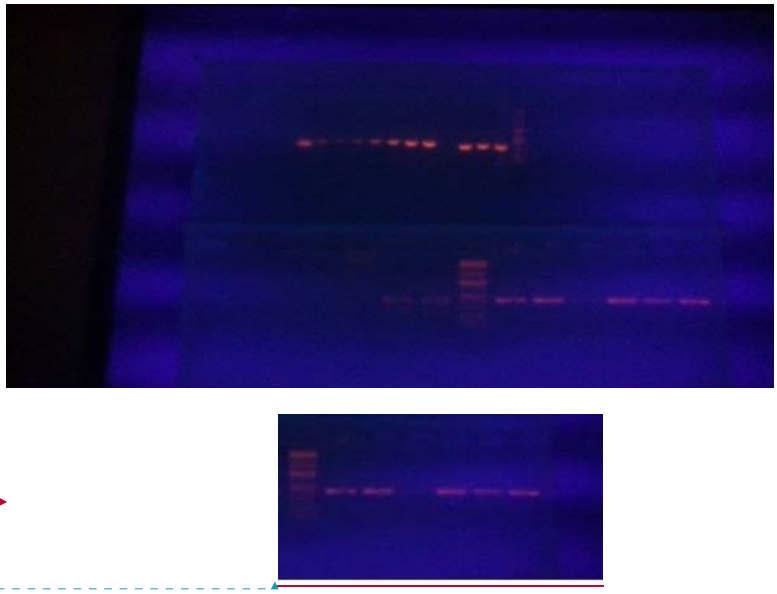
منسّق:متوسط



منسّق:الخط: ١٢ نقطة، خط اللغة العربية وغيرها: ١٢ نقطة

Figure1: Blood smear of cow show the *Anaplasma spp.* stained by Diff-quick stain (100×).

Products of anticipated size (345bp) for the 16S rDNA gene parcial sequence of Anaplasma were obtained in 28.9% (42of 83) by convential PCR of blood samples collected from different region of Al-Diwaniyiah city(Figure .2)



منسّق:الخط: (افتراضي) +برنامج نصي معقد لعناوين (New Times Roman)، ١٤ نقطة، خط اللغة العربية وغيرها: +برنامج نصي معقد لعناوين (Roman New Times)، ١٤ نقطة

منسّق:متوسط

Fig. 2: Electrophoresis of 345bp 16SrRNA gene Anaplama detected from cow in Iraq, Marker: Line 1 (100bp), lane 2, 3 5, 6 and 7 represnted amplification product 345bp .

DNA sequencing and phylogenetic analysis

Only ten PCR products were selected according ~~regions that~~regions that give an amplicon of the expected size for Anaplasma 16S rDNA partial gene from different region of Al-Diwaniyiah city were studied.

The ~~sequence analysis~~sequence analysis ~~revealed that~~revealed that all these bacterial isolates belong to the Anaplasma genus .Four of these were included as *A. phagocytophilum*, two as *A. marginale* and four ~~were~~ as Anaplasma sp. All sequences of targeting ~~gene obtained~~gene has been obtained from ten isolates were deposited in gene bank ~~with~~, gen bank recorded the publishing our sequence data as accession numbers ~~elucidated~~ in table 1.

جدول منسّق

Table 1: Accession numbers of Anaplasma species in current study.

Accession numbers	Anaplasma species
MG195905.1	*A. phagocytophilum*
MG195906.1	*A. phagocytophilum*
MH155431.1	*A. phagocytophilum*
MH155432.1	*A. phagocytophilum*
MH155593.1	*A. marginale*
MH155594.1	*A. marginale*
MH155603.1	Anaplasma sp.
MH155604.1	Anaplasma sp.
MH155605.1	Anaplasma sp.
MH155606.1	Anaplasma sp.

Comparative analysis of 16S rDNA nucleotide sequences from samples in ~~Iraq~~ our country ~~samples~~ with the number of *A. phagocytophillum* isolates that ~~present~~ recording in the gene bank database were shown in figure 3.

The sequences of Anaplasma *16S rDNA* partial gene were compared ~~to~~ with other published sequnces from the same genbank website (~~present in Gene Bank~~). The phylogenetic analysis revealed there were a ~~closed~~ closely related to NCBI-Blast *A. phagocytophillum 16S rDNA* gene ……(KC854154.1), whereas other NCBI-Blast *A. phagocytophillum 16S rDNA*gene Human, dog, goat, and cat host were show different out of tree at total genetic change (0.005%) (Figure 3).

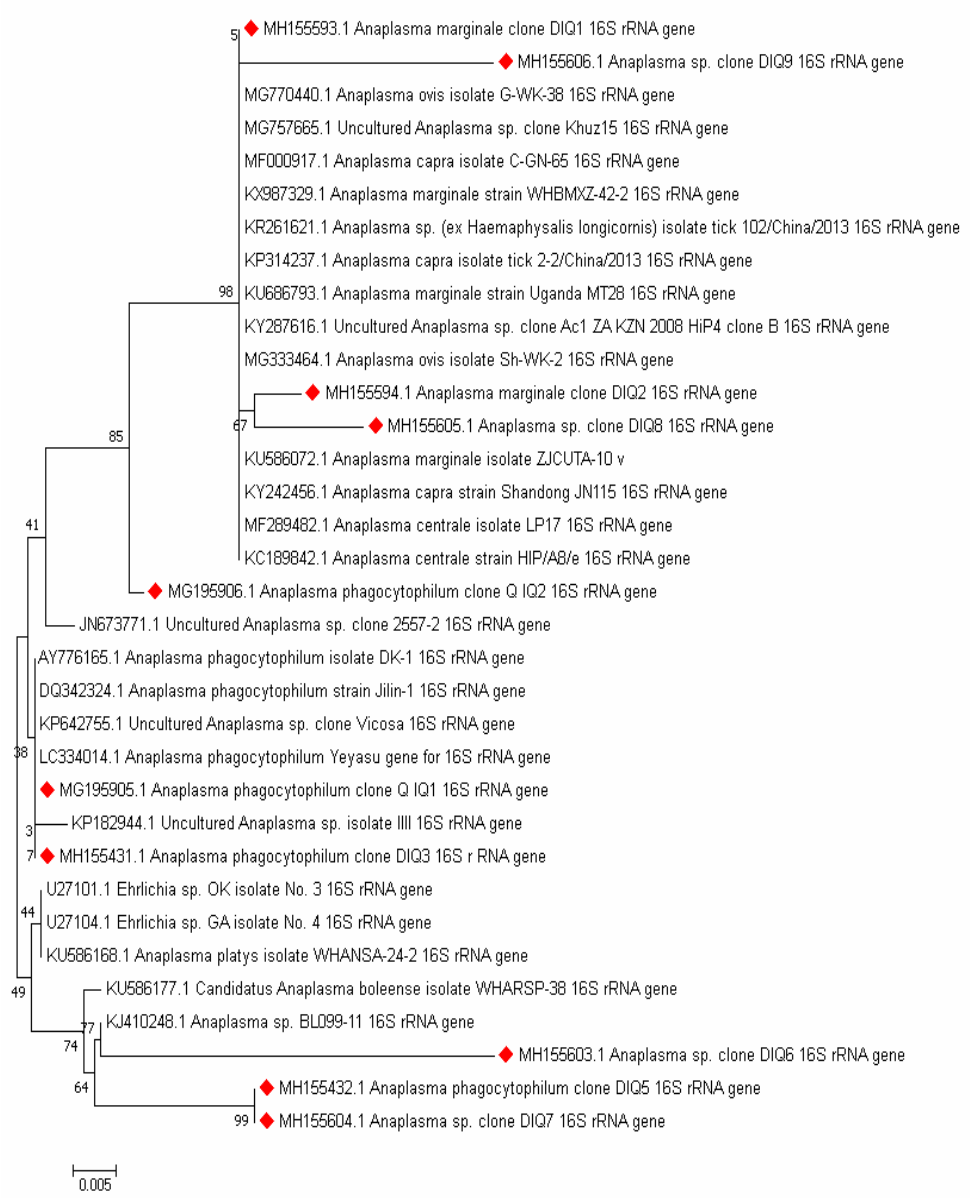
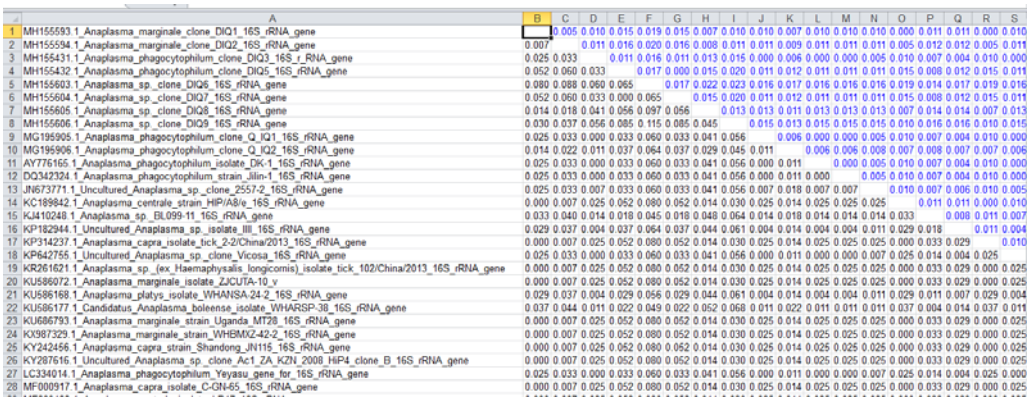


Figure 3 : Phylogenetic tree of Anaplasma sp.in current study based on the 16S rRNA partial gene sequence. A neighbor joining tree was conducted using MEGA 7. Software. Alignment of 345 bp partial 16S rRNA sequence was used in this tree. The sequences labeled with red color were obtained in current study.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	MH155593.1_Anaplasma_marginale_clone_DIQ1_16S_rRNA_gene		0.005	0.010	0.015	0.019	0.015	0.007	0.010	0.010	0.007	0.010	0.010	0.010	0.000	0.011	0.011	0.000	0.010
2	MH155594.1_Anaplasma_marginale_clone_DIQ2_16S_rRNA_gene	0.007		0.011	0.016	0.020	0.016	0.008	0.011	0.011	0.009	0.011	0.011	0.011	0.005	0.012	0.012	0.005	0.011
3	MH155431.1_Anaplasma_phagocytophilum_clone_DIQ3_16S_r_RNA_gene	0.025	0.033		0.011	0.016	0.011	0.013	0.015	0.000	0.006	0.000	0.000	0.005	0.010	0.007	0.004	0.010	0.000
4	MH155432.1_Anaplasma_phagocytophilum_clone_DIQ5_16S_rRNA_gene	0.052	0.060	0.033		0.017	0.000	0.015	0.020	0.011	0.012	0.011	0.011	0.011	0.015	0.008	0.012	0.015	0.011
5	MH155603.1_Anaplasma_sp._clone_DIQ6_16S_rRNA_gene	0.080	0.088	0.060	0.065		0.017	0.022	0.023	0.016	0.017	0.016	0.016	0.016	0.019	0.014	0.017	0.019	0.016
6	MH155604.1_Anaplasma_sp._clone_DIQ7_16S_rRNA_gene	0.052	0.060	0.033	0.000	0.065		0.015	0.020	0.011	0.012	0.011	0.011	0.011	0.015	0.008	0.012	0.015	0.011
7	MH155605.1_Anaplasma_sp._clone_DIQ8_16S_rRNA_gene	0.014	0.018	0.041	0.056	0.097	0.056		0.013	0.013	0.011	0.013	0.013	0.013	0.007	0.014	0.014	0.007	0.013
8	MH155606.1_Anaplasma_sp._clone_DIQ9_16S_rRNA_gene	0.030	0.037	0.056	0.085	0.115	0.085	0.045		0.015	0.013	0.015	0.015	0.015	0.010	0.016	0.016	0.010	0.015
9	MG195905.1_Anaplasma_phagocytophilum_clone_Q_IQ1_16S_rRNA_gene	0.025	0.033	0.000	0.033	0.060	0.033	0.041	0.056		0.006	0.000	0.000	0.005	0.010	0.007	0.004	0.010	0.000
10	MG195906.1_Anaplasma_phagocytophilum_clone_Q_IQ2_16S_rRNA_gene	0.014	0.022	0.011	0.037	0.064	0.037	0.029	0.045	0.011		0.006	0.006	0.008	0.007	0.008	0.007	0.007	0.006
11	AY776165.1_Anaplasma_phagocytophilum_isolate_DK-1_16S_rRNA_gene	0.025	0.033	0.000	0.033	0.060	0.033	0.041	0.056	0.000	0.011		0.000	0.005	0.010	0.007	0.004	0.010	0.000
12	DQ342324.1_Anaplasma_phagocytophilum_strain_Jilin-1_16S_rRNA_gene	0.025	0.033	0.000	0.033	0.060	0.033	0.041	0.056	0.000	0.011	0.000		0.005	0.010	0.007	0.004	0.010	0.000
13	JN673771.1_Uncultured_Anaplasma_sp._clone_2557-2_16S_rRNA_gene	0.025	0.033	0.007	0.033	0.060	0.033	0.041	0.056	0.007	0.018	0.007	0.007		0.010	0.007	0.006	0.010	0.005
14	KC189842.1_Anaplasma_centrale_strain_HIP/A8/e_16S_rRNA_gene	0.000	0.007	0.025	0.052	0.080	0.052	0.014	0.030	0.025	0.014	0.025	0.025	0.025		0.011	0.011	0.000	0.010
15	KJ410248.1_Anaplasma_sp._BL099-11_16S_rRNA_gene	0.033	0.040	0.014	0.018	0.045	0.018	0.048	0.064	0.014	0.018	0.014	0.014	0.014	0.033		0.008	0.011	0.007
16	KP182944.1_Uncultured_Anaplasma_sp._isolate_IIII_16S_rRNA_gene	0.029	0.037	0.004	0.037	0.064	0.037	0.044	0.061	0.004	0.014	0.004	0.004	0.011	0.029	0.018		0.011	0.004
17	KP314237.1_Anaplasma_capra_isolate_tick_2-2/China/2013_16S_rRNA_gene	0.000	0.007	0.025	0.052	0.080	0.052	0.014	0.030	0.025	0.014	0.025	0.025	0.025	0.000	0.033	0.029		0.010
18	KP642755.1_Uncultured_Anaplasma_sp._clone_Vicosa_16S_rRNA_gene	0.025	0.033	0.000	0.033	0.060	0.033	0.041	0.056	0.000	0.011	0.000	0.000	0.007	0.025	0.014	0.004	0.025	
19	KR261621.1_Anaplasma_sp._(ex_Haemaphysalis_longicornis)_isolate_tick_102/China/2013_16S_rRNA_gene	0.000	0.007	0.025	0.052	0.080	0.052	0.014	0.030	0.025	0.014	0.025	0.025	0.025	0.000	0.033	0.029	0.000	0.025
20	KU586072.1_Anaplasma_marginale_isolate_ZJCUTA-10_v	0.000	0.007	0.025	0.052	0.080	0.052	0.014	0.030	0.025	0.014	0.025	0.025	0.025	0.000	0.033	0.029	0.000	0.025
21	KU586168.1_Anaplasma_platys_isolate_WHANSA-24-2_16S_rRNA_gene	0.029	0.037	0.004	0.029	0.056	0.029	0.044	0.061	0.004	0.014	0.004	0.004	0.011	0.029	0.011	0.007	0.029	0.004
22	KU586177.1_Candidatus_Anaplasma_boleense_isolate_WHARSP-38_16S_rRNA_gene	0.037	0.044	0.011	0.022	0.049	0.022	0.052	0.068	0.011	0.022	0.011	0.011	0.011	0.037	0.004	0.014	0.037	0.011
23	KU686793.1_Anaplasma_marginale_strain_Uganda_MT28_16S_rRNA_gene	0.000	0.007	0.025	0.052	0.080	0.052	0.014	0.030	0.025	0.014	0.025	0.025	0.025	0.000	0.033	0.029	0.000	0.025
24	KX987329.1_Anaplasma_marginale_strain_WHBMXZ-42-2_16S_rRNA_gene	0.000	0.007	0.025	0.052	0.080	0.052	0.014	0.030	0.025	0.014	0.025	0.025	0.025	0.000	0.033	0.029	0.000	0.025
25	KY242456.1_Anaplasma_capra_strain_Shandong_JN115_16S_rRNA_gene	0.000	0.007	0.025	0.052	0.080	0.052	0.014	0.030	0.025	0.014	0.025	0.025	0.025	0.000	0.033	0.029	0.000	0.025
26	KY287616.1_Uncultured_Anaplasma_sp._clone_Ac1_ZA_KZN_2008_HIP4_clone_B_16S_rRNA_gene	0.000	0.007	0.025	0.052	0.080	0.052	0.014	0.030	0.025	0.014	0.025	0.025	0.025	0.000	0.033	0.029	0.000	0.025
27	LC334014.1_Anaplasma_phagocytophilum_Yeyasu_gene_for_16S_rRNA_gene	0.025	0.033	0.000	0.033	0.060	0.033	0.041	0.056	0.000	0.011	0.000	0.000	0.007	0.025	0.014	0.004	0.025	0.000
28	MF000917.1_Anaplasma_capra_isolate_C-GN-65_16S_rRNA_gene	0.000	0.007	0.025	0.052	0.080	0.052	0.014	0.030	0.025	0.014	0.025	0.025	0.025	0.000	0.033	0.029	0.000	0.025

Figure : Comparison of the sequences obtained of 16S rRNA partial fragments of Anaplasma genotypes (1-10) with published sequnces of Anaplasma spp. for comparison purpose.

Discussion

The using of PCR assay coupled with sequencing has allowed for the identification of uncultivable bacteria such as *A. phagocytophilum* that have been impossible or difficult to identify based on convential microbiological techniques.

Despite small numbers were used in this study the PCR demonstrated that Anaplasma infection are highly prevalent (28.9%) among cattle population in Al-Diwaniya, 40% of these were *A. phagocytophilum* , compared with study conducted in Iran (Noaman and Shayan 2009)in which *A. phagocytophilum* infection was 1.33% (2/150) and this difference due to nature of specimens and detection technique and this require application of effective tick control program because anaplasmosis influences on animal health and production.

Sequence alignment of ten detected Anaplasma(table 1)in current study revealed 95-99% similarity with some strains in Genbankand this highly confirmed for bacterium identification in Iraq. On the other hand, the difference between two local strain was as much

The average infection rates of

The remaining four Anaplasma spp. reported in current study were not identical 100% and reveal two of them (MH155603.1 and MH155604.1) closely related to *A. phagocytophilum*while other two(MH155605.1 and MH155606.1) were closely related to *A. marginale*while in Mongolian gazelle China appear to be most closely related to *A. ovis* and *A. central* (

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