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Passer 3 (2021) 180- 186

http://passer.garmian.edu.krd/

Occurrence of Anaplasmosis (*Anaplasma Marginale*) in cattle in Sulaimani province, Kurdistan region of Iraq

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Received 08 July 2021; revised 10 July 2021;
accepted 11 August 2021; available online 19 August 2021

doi:10.24271/psr.30

ABSTRACT

The goal of this study was to determine both the incidence of anaplasmosis (*Anaplasma Marginale*) and phylogenetic relationship between *A. marginale* isolates from cattle in Sulaimani province, Kurdistan Region- Iraq during (March 10th to April 10th 2021) and those from other *Anaplasma* spp. A total of two isolates were tested for the major surface protein (msp4) gene for this purpose. Eighty blood samples of cattle (51 males and 29 females) were examined using both microscopic examination and PCR tests. Overall results were 23/80 (28.7 5%) and 8/80 (10 %) using microscopic examination and PCR assay, respectively. Age and sex were not significant factors in the appearance of infection, since no statistically significant difference in infection rate has been observed among sex and age group of cattle (P value >0.05). The results also revealed that the accuracies of traditional method and PCR assays in the diagnosis of the disease were 81 %, and 100 respectively. There was moderate correlation (0.43) between both techniques by the Kappa (k) test. However, The PCR technique recorded the highest sensitivity (100%) and specify (100%) for *A. marginale* detection. In conclusion, by the findings of the present study, it has been confirmed for the first time that *A. marginale* is the causative agent of anaplasmosis of cattle in the study areas and the best technique for the detection of either acute or chronic cases in cattle was the PCR assay.

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Keywords: Anaplasma marginale, Blood smear, PCR, Sulaimani province.

1. Introduction

Anaplasmosis is a tick-borne disease of cattle and other ruminants caused by the intracellular rickettsia *Anaplasma marginale*, in both tropical and subtropical areas of the world, and also causing great economic losses by decreasing livestock production [1]. There are four recognized *Anaplasma* species in cattle, including *Anaplasma marginale*, *A. centrale*, *A. phagocytophilum*, and *A. bovis*. Among these species, *A. marginale* is responsible for almost all outbreaks of clinical disease [2,3]. They are transmitted either mechanically or biologically through arthropod vectors [4,3]. Besides, carrier animals are the source of infection [5]. Infection with *Anaplasma* spp. is characterized by fever, hemolytic anemia, jaundice, anorexia, pale mucous membranes, decreased milk production, weight loss, hyperexcitability, reproductive problems and death in some cases [6,7,8,9,10]. Haemoglobinuria and haemoglobinaemia have not been recorded during acute phase of the infection, and this may assist in the differential diagnosis of anaplasmosis from babesiosis, which is often endemic in most tropical and subtropical countries. Recent studies reported cattle infection with *A. marginale* in the Asian countries, including Iraq [11,12,13,14,15,16], Iran [17], Turkey [18], Saudi Arabia [19,20], and Egypt[21]. Seasonality plays a role in the intensity of the disease transmission, for example [13] showed that the incidence rate of the disease was peaked in both spring and summer. Conventional microscopic examination, serological, and molecular diagnostic techniques are used for the diagnosis of the disease [22,23,24,3,25]. Traditional Microscopic examination of giemsa-stained blood smears is easy to perform, inexpensive, and considered as a "gold standard" test for confirming the acute clinical cases. However, it is labor intensive and tedious for large numbers of specimens, less sensitive, and is not recommended for routine examination of carrier animals due to the bacteremia in the infected erythrocytes [26,27]. Thus, molecular methods, with a high degree of sensitivity and specificity, have been developed to identify various *Anaplasma* spp. in persistently infected cattle. The

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Peer-reviewed under the responsibility of the University of Garmian.

incidence rate of anaplasmosis caused by of *A. marginale* needs to be determined in cattle in the various areas of Kurdistan Region, therefore the study was carried out to assess the spread of *A. marginale* in cattle during spring (March 10th to April 10th) using giemsa-stain and molecular characterization and to compare the results with conventional microscopy technique in Sulaimani province, Kurdistan Region- Iraq.

2. Material and Methods

2. 1. Epidemiological Data

The study was undertaken in the Sulaimani province, Kurdistan Region, Iraq during spring (10th March – 10th April 2021). The climate of Sulaimani is variable according to its seasons; temperature in spring is relatively moderate (20° - 35° Celsius). The clinical manifestations of anaplasmosis were noted on some animals during sampling such as, weakness, high temperature, and increased respiration rate. In addition, tick infestations were seen on some examined animals. Full objective information obtained which was arranged in a sheet of questionnaire form including data of sex, age, flock, size, morbidity and mortality rates.

2. 2. Samples Collection

A total of 80 blood samples (51 male, 29 female) Arabic, local, Simmental and crossbred cattle reared in open management system were examined for anaplasmosis from various area of Sulaimani province (Tanjaro, Kalar, Smud, Said Sadiq), Kurdistan Region, Iraq. All samples obtained from two age group, less than 1 year (n= 34) and more than 1 year (n=46). Almost 6ml of blood samples from Jugular vein of cattle randomly were collected; 2ml of the blood were put in tube containing EDTA. The samples were stored and transfer to laboratory in a special designed cool box. Giemsa's stain was done for the blood samples of EDTA tubes to determine of anaplasmosis microscopically. Then the blood samples were stored at - 70 ºC for PCR test. Ethical standards were considered during sampling.

2. 3. Microscopic Examination (ME)

After preparation of peripheral blood smears on glass slides, the slides were dried and fixed with methanol for about 5 minutes, then stained by 10% Giemsa stain for 30 minutes and examined under Oil immersion lens (100 x magnifications). However, *Anaplasma* was identified on the basis of its morphology [28,29,30,25].

2. 4. DNA extraction

The DNA was extracted used (200 μl) of the whole blood samples with DNA extraction Kit (ADD BIO INC, DAEGEON Republic of Korea) according to the manufacturer's instructions. The DNAs were eluted into 100 μl Elution buffer at room temperature where they were frozen at −20°C until PCRs performed.

2. 5. PCR amplification and sequencing

For the detection of *Anaplasma* spp. (*A. ovis* and *A. marginale*) PCR method was used. One pair of oligonucleotide primers was designed based on the msp4 gene sequence of *Anaplasma* spp. Primers for the PCR were forward strand primer 5'- TTGTTTACAGGGGGCCTGTC- 3' and reverse strand primer 5'- GAACAGGAATCTTGCTCCAAG-3' were provided by (Macrogen Co. Republic Korea). The major surface protein (msp4) gene amplified by using PCR Premix (2X). The reactions were administered in 0.2 ml PCR tube supported the subsequent specifications: 10 µl supreme script PCR premix, 5.µl DNA, 1.µl forward (10 pmol), 1.µl reverse primers (10 pmol), and three µl ultra-pure water to form up a final volume of 20.µl. The conventional PCR machine (Hercuvan, UK) was programmed as followed: initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 30s; annealing at 60 °C for 40s, and extension at 72 °C for 45s and a final extension at 72 °C for 10-min.the obtained PCR products visualized under UV illumination after electrophoresis on 1% agarose gels stained with safe dye (Urex.Poland) under 100 volt for 60 minutes. The expected PCR product for A. Marginale was 831 bp. PCR products of two positive samples were used for sequencing (Macrogen, Republic korea), and were submitted to the national center for bioinformatics and information (Genbank). The Sequences were assembled by the MEGA.7 software. The msp4 sequences were trimmed and aligned using the ClustaW Multiple sequence alignment tool [31] The Neighbor joining method was used to infer the phylogenetic and evolutionary tree [32]. With The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) [33]. The evolutionary distances were computed using the Kimura 2-parameter method [34].

2. 6. Statistical analysis

The results of Anaplasma-infection diagnostic using conventional and PCR assay were divided into four types: True positive, false positive, True negative, and false negative. The sensitivity of each of the two techniques was calculated as [True positive ÷ (True positive + false negative)] X 100. The specificity of the tests was calculated as [True negative ÷ (True negative + false positive)] X 100. Chi-square was used to compare the differences between both age groups and sex of animals. After, all of data were achieved and then the statistical analyses of data were done by a computerized version 22.0 of the Statistical Package for Social Sciences (SPSS) software (by IBM, USA).

3. Results

Out of 80 samples, 23 (28.75 %) were found positive for the A. marginale; while, 8 (10 %) samples were detected positive by both conventional microscopic using Giemsa staining method and PCR tests respectively (Table 1).

Table 1: Incidence of A. marginale in cattle using light-microscope and PCR techniques.

Tests	N0. Of samples examined	Positive No. (%)	Negative No. (%)
Microscopic examination	80	23 (28.75)	57 (71.25)
PCR	80	8 (10)	72 (90)

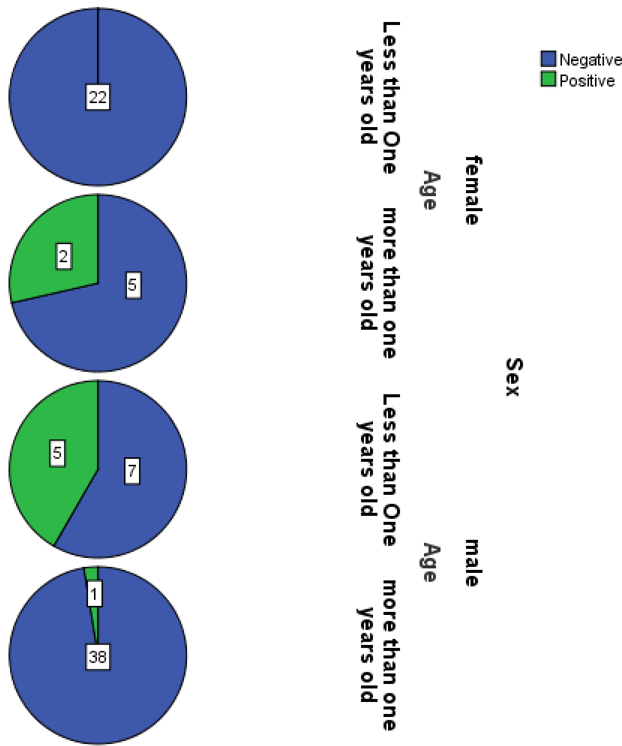

Figure 1: Odd ratio 0.6, male animals has half fold greater chance to infected with anaplasma; Odd ratio 2.48, animals less than one years old has 2.5gold greater chance to infect with anaplasma; Age and sex were not significant factors in the appearance of infection, since no statistically significant difference (P>0.05).

The infection rate was studied between males and females cattle, the results observed that the rate statistically non-significant (P>0.05). Meanwhile, the rate was higher in males than females. Out of 51 male animals 6 (11.8 %) were positive. While, in females out of 29 animals 2 (6.9 %) were positive using PCR method (Table 2).

Table 2: The infection rate of A. marginale according to sex of the animals using PCR

Sex	No. of examined animals	PCR test	
		Negative No. (%)	Positive No. (%)
Male	51	45 (88.2)	6 (11.8)
Female	29	27 (93.1)	2 (6.9)
Total	80	72 (90)	8 (10)

The rate of anaplasmosis related to the sex in the animals shows no significant differences (P>0.05).

The incidence rate was higher in the age group < 1 year than the age group > 1 year: 5 (14.7 %) compared to 3 (6.5 %). Hence, there were no statistically significant in both ages (Table 3).

Table 3: The infection rate of anaplasmosis according to age of the aborted animals.

Age	No. of examined animals	PCR test	
		Negative No. (%)	Positive No. (%)
Under one year	34	29 (85.3)	5 (14.7)
Above one year	46	43 (93.5)	3 (6.5)
Total	80	72 (90)	8 (10)

The rate of anaplasmosis related to the age in the animals illustrations no significant differences (P>0.05).

The results revealed that the accuracies of direct microscopic examination using Giemsa-stained smears and PCR assays in the diagnosis of the disease were 81 %, and 100% respectively. The PCR technique recorded the highest sensitivity (100%) for diagnosis of anaplasmosis (Table 4).

Table 4: Performance values of both Traditional and PCR techniques of anaplasama investigation among cattle.

Tests	Performance values (%)				
	Sensitivity	Specificity	PPV	NPV	Accuracy
Microscopic examination	100	35	79	100	81
PCR	100	100	100	100	100

PPV = positive predictive value, NPV = negative predictive value

The microscopic examination test of the disease gave the high rate of false-positive results (18.8%), compared to PCR (0.0%) method. On the other hand, the false positives were (0.0 %) using both assays (Table 5).

Table 5: Results of A. marginale detection techniques

Tests	True positive No. (%)	True negative No. (%)	False-positive No. (%)	False-negative No. (%)
Microscopic examination	8 (10.0)	57 (71.3)	15 (18.8)	0.0 (0.0)
PCR	8 (10.0)	72 (90.0)	0.0 (0.0)	0.0 (0.0)

By Kappa test there was moderate correlation (0.43) between both techniques and there were significant (p value 0.00) btween both of them

3. 1. Morphology of A. marginale

The microscopic examination of the Giemsa-stained blood smears of infected erythrocytes revealed that anaplasma inclusion bodies appeared as one uniform dark staining dote like circular bodies on the periphery to the infected cattle erythrocytes (Figure 2).

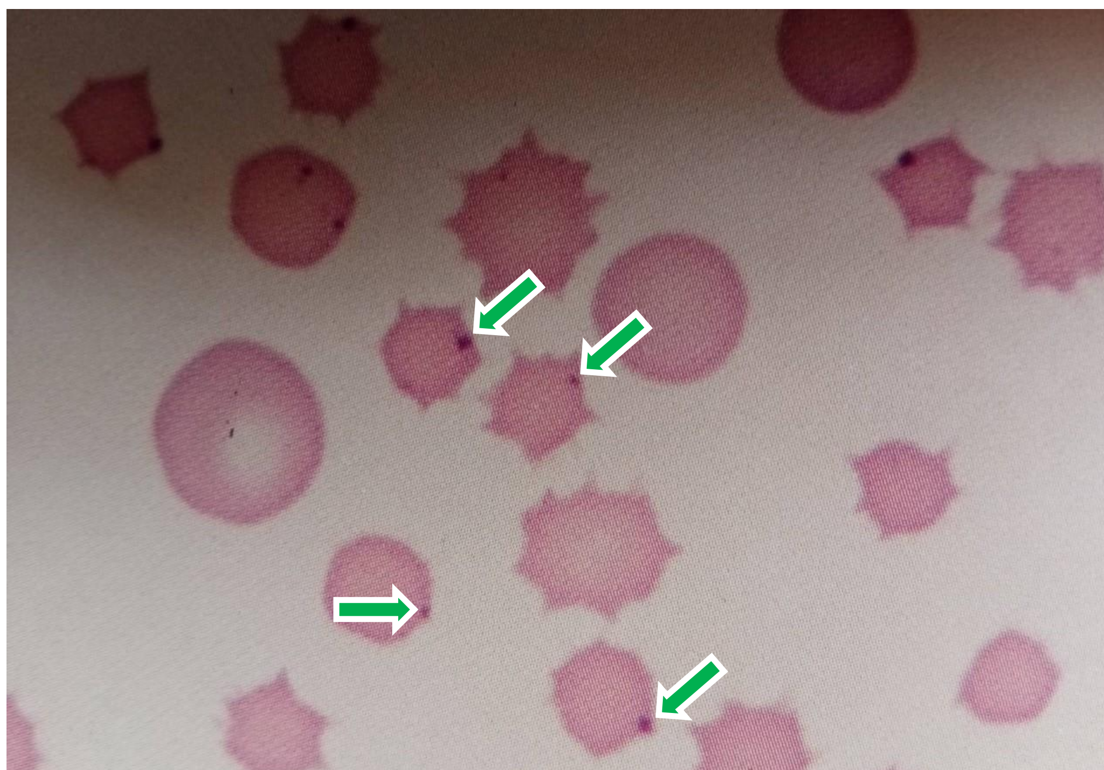

Figure 2: Intracellular inclusion bodies peripheral location A. marginale in the blood of naturally infected cattle, giemsa staine X1000 (arrows).

3. 2. Identification of Anaplasma marginale

PCR test of the DNA isolated from blood samples showed that 8 out of the total 80 blood samples were *Anaplasma* spp. Positive and revealed an expected PCR product of 831 bp. For determination *A. marginale* the PCR products were sent for sequencing , the sequences data were submitted to GenBank with accession numbers (MZO98220, and MZ098221) (Figure 3).

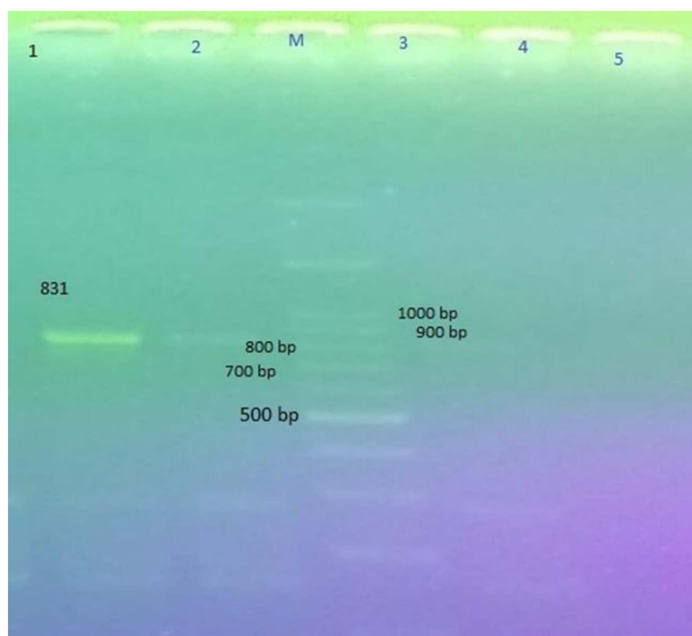

Figure 3: Amplification of msp4 gene of A. marginale; Lane M: 100 bp ladder (Genet bio); Lanes 1, 2: PCR positive for A. marginali size 831 bp; Lane 3-4: Negative sample

3. 3. Phylogenetic tree and sequence analysis of MSP 4 gene

The obtained nucleotide and amino acid sequences of two *A. marginale* partial MSP 4 genes during this study were compared with different *Anaplasma* spp. reference strain in Genbank by multiple alignments with the ClustalW enclosed in MEGA.7 software. The two nucleotide sequences of detected *A. marginale* displayed a limited diversity, as they are closely associated each another with the identity 99.61% whereas amino acid identity is 99.27%. The nucleotide sequence identities between the two field *A. marginale* isolate partial MSP4 and 12 isolates in different countries retrieved from GenBank ranged between 98.60%and 99.50%. The highest identity (99.50%) was found between the two isolates and AMCRO1 (accession no. MN202185, isolated from Croatia, in 2019). The lowest identity (98.60%) was found between the two current isolates and Aizwal (accession no. MH373245, isolated from India, in 2018). (Table: 2). Phylogenetic analysis of the MSP4 nucleotide sequences by neighbor-joining separated the *Anaplasma* spp. into 4 clade: *A. marginale*, *A. ovis*, *A. central*, and *A. phagocytophilum*. The phylogenetic analysis of the partial MSP4 gene sequence *Anaplasma* spp. and isolate in this study showed that two field sequences belonged to (*A. marginale*) and were clustered with other *A. marginale* in different countries (Figure 4). However, two field amino acid sequences it's to create divergence when compared with three *Anaplasma* spp. (*A. phagocytophilum*, *A. ovis and A. centrale*) ranged around (3%, 5%, and 12%) respectively (Table 6).

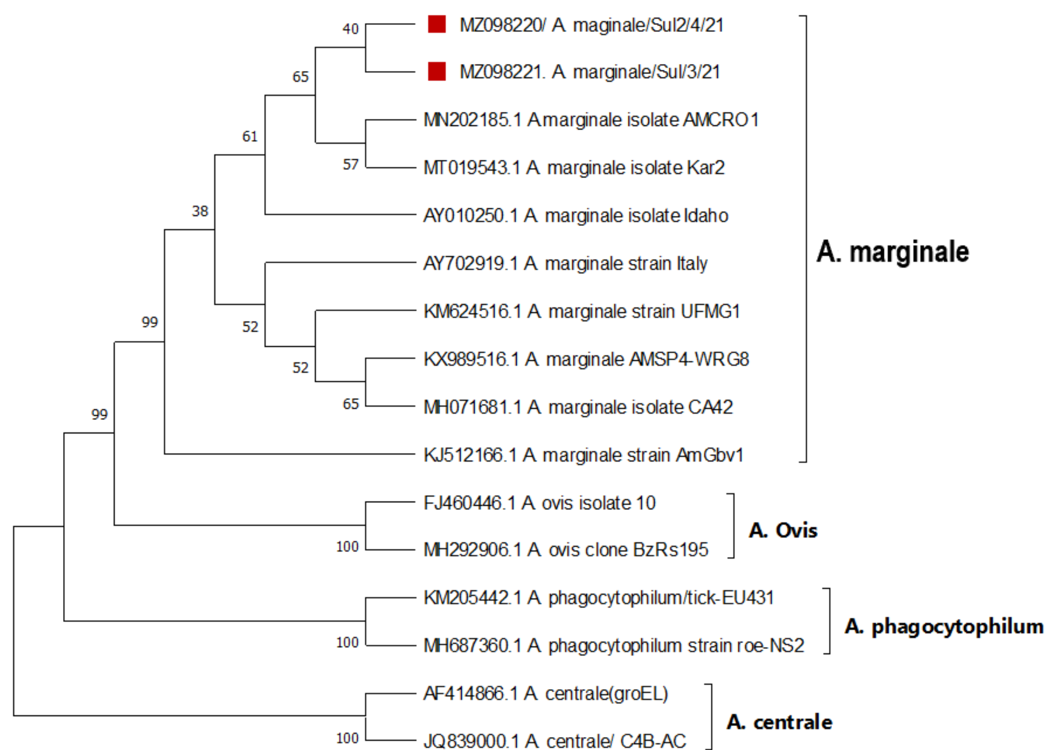

Figure 4: Phylogenetic tree generated based on major surface proteins 4 (MSP4) sequence data of Anaplasma species in this study and similar sequences from GenBank database constructed using Neighbor-joining method. MEGA7

Table 6: Identities between Field isolates and different Anaplasma spp. Related isolates obtained from sequence comparison

Accession No.	Countries/strain	*Anaplasma spp.*	DNA Identities	a.a Identities
AF428090	Israel	*A.centrale*	83.45	88.32%
FJ460446.	Greece/	*A.ovis*	90.63	95.26%
MK423171	Iraq	*A. phagocytophilum*	59.00	97.08%
EU106082	Nigeria/Zaria	*A.marginale*	99.08	98.46%
KJ512166	Tunisia/AmGbv1	*A.marginale*	99.00	98.88%
JN564650.1	Mexic/Mex-14-010	*A.marginale*	98.66	97.81%
AY010250	USA/Idaho	*A.marginale*	99.27	98.54%
EF067340.	Spain/_Va-48	*A. marginale*	99.27	98.91%
HM063432	Hunagry/HU-2009	*A. marginale*	99.03	98.91%
KX781292.	Turkey/Ams1	*A. marginale*	98.66	98.81%
MN202185	Croatia/AMCRO1	*A. marginale*	99.51	98.91%
MH373245	India/Aizwal	*A.marginale*	98.60	97.08

4. Discussions

Yet, while very little information is available on the prevalence of anaplasmosis in Sualimani province, Iraq. Therefore, by the findings of the current study, it has been confirmed for the first time that *A. marginale* is also one of the main causes of anaplasmosis in cattle in the study areas. Clinical symptoms seldom aid in identification of the disease. Generally, laboratory examinations are important for diagnosis including confirmatory

diagnosis [10]. The conventional microscopic examination is one of the most common methods used for diagnosis of the disease among cattle, which is based on morphological structures inside erythrocytes. In the current study, when the animals examined by microscopy *Anaplasma*-like inclusion bodies were also found at the margin of infected erythrocytes. These results were in agreement with [10]. In the current study, out of 80 blood samples 23 were positive microscopically, from which 8 specimens were found positive by PCR. This means that 15 specimens were *Anaplasma* false-positive. Therefore the high incidence rate in the current study area by light microscopy might be due to miss diagnosis of the organism with other structure such as, Howell-Jolly bodies, Heinz bodies or staining artifacts, which are often seen in Giemsa stained blood smears [35,36]. Meanwhile, diagnostic method microscopically for pre-symptomatic and chronic cases needs expertise for reading of the slides. For instance, in case of low amount of bacteremia (<0.1%) finding of the organism can be difficult because of their extremely low ratio in the stained blood smears. Therefore, performing of this conventional method for diagnosis of carrier animals is not preferable and requires more time to identify the forms of anaplasma in the RBCs [37,38,39,36]. In addition, good smear preparation, high amount of bacteremia, good staining smears as well as a well-trained microscopist need to proper identification of *Anaplasma* by light microscopic [35]. Thus the PCR test was applied in the current study because of its accuracy in sensitivity (100%) and specificity (100%) as well as the fact that it is considered as a potent reliability test for detection of the disease in cattle. Results illustrated that PCR was found more sensitive (100 %) and specific (100%) than microscopic examination. These results were also in agreement with the results of other researchers [40,35,25] who mentioned, that the high degrees of sensitivity and specificity of PCR assay. according to reports phylogenetic research of *Anaplasma* spp. utilizing msp has three key molecular targets *msp*1α, msp4, and msp5 [41] msp4 have been used for phylogenetic characterization of *A. marginale* isolates from Sulaimani/Iraq. In the present study, *msp4* phylogeny indicating four main clades were *A. marginale, A.* (*A. phagocytophilum*, *A. ovis and A. centrale*), the genetic distance difference observed between *A. marginale* and other three clade ranged around (3%, 5%, and 12%) respectively.

The disease' prevalent varied among researchers depending on the type of methods used in the same area and in different areas as well as depending on the geographic location, size and type of sample obtained. The disease' prevalence is lower in Kurdistan Region of Iraq (10 % in cattle) in comparison to different location of Iraq and other countries those used PCR and other techniques. In Isfahan, Iran the prevalent rate was 50 % using PCR assay [17]. In Tunisia, [42] recorded the prevalence rate among cattle was 24.7 % . while, in India, the rate was 45.2% % using PCR assay [43]. However, in Wassit, Iraq the rate was 75% [44]. In north-eastern Uganda, the rate was 87.7% using PCR [45]. In Kruger National Park, South Africa the prevalence rate was also 17.3% [46]. In Egypt [21] reported that prevalence rate was 18 % in cattle. On contrast to our results, according to the results of other studies, the prevalence of anaplasmosis among cattle was 0.98-3.4 % in Sudia Arabia [19,20]. In Turkey, the rate of the disease was 2.8 % [18]. Other research has shown similar prevalence rates, for instance, the rate of the disease in Erbil,Iraq the rate was 9. 09% in cattle [13]. The differences in the results of PCR test between this study and previous studies may be attributed to the variation in the number of samples many factors such as climatic conditions, breeds' susceptibility, vectors' distribution, breeding system, immunization, prophylactic strategy and systems of treatment. However, season is one of the most common considerable differences because in spring and summer highest rate of the disease was recorded [47]. In the finding of current study, age and sex were not significant factors in the appearance of infection, since no statistically significant difference in infection rate has been observed among sex and age group of cattle. Bovine anaplasmosis can be seen at any age group in cattle, however, the severity of disease and death rate increase with the advance in age, with clinical anaplasmosis being more commonly in cattle older than 1 year of age [48,44]. Findings of the current study showed that there was moderate correlation (0.43) between Microscopy and PCR techniques; Meanwhile, there was significant (P value 0.00) between both of them, which was determined by Kappa (k) test. These result was in agreement with [35].

5. Conclusions

The current work was carried out in Sulaimani/Iraq as the first molecular detection of *Anaplasma* species based on the sequencing of msp4 gene fragment. The findings of the present study showed that giemsa-staining method could be a suitable technique to determine only acute cases of bovine anaplasmosis. Age and sex were not significant factors in the appearance of infection, since no statistically significant difference in infection rate has been observed among sex and age group of cattle (P value >0.05). However, PCR technique accepted as an accurate tool to determine *Anaplasma* even in asymptomatic carrier animals which is quite necessary for carrying out of control programs successfully. Therefore, more research is needed, including a large number of isolates from around Iraq, to truly define the genetic diversity of *A. marginale* isolates.

Conflict of interests

None.

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