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MICROSCOPIC AND MOLECULAR IDENTIFICATION OF *Anaplasma ovis* IN SMALL RUMINANTS IN DUHOK PROVINCE IN KURDISTAN REGION OF IRAQ

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ABSTRACT

In last ten years, there has been a developing enthusiasm for microscopic organisms from the genus *Anaplasma*, particularly the species *A. ovis*. It is associated with the pathogenic action of these microscopic organisms in livestock. *Anaplasma ovis* is a tick-borne obligate intracellular rickettsial bacterium that causes anaplasmosis in domestic and wild small ruminants.

The samples of the present study were collected from small ruminants from inside seven distinct regions (Akre, Simele, Zummar, Feshchapoor, Deraboon, Bajed Kandal,Karoda)of Duhok province, 389 (goats 75 and sheep 314) during the period of April and May 2018, blood sample were taken and thin smear was formed, after Giemsa's staining the slide is observed under microscope.

In this study used Giemsa stain for microscopic examination out of 389 animals 250 were found positive for *Anaplasma ovis* infection with a prevalence rate of 64.26 % and 139 of them were negative with a prevalence rate of 35.73 %.

According to the species of animals, the highest prevalence of *A. ovis* infection in animals by using microscopic examination was 67.83 %, 213 positive sample from total 314 blood samples from sheep and lowest prevalence was 49.33 %, 37 positive sample from total 75 blood samples from goats.

PCR analysis of 100 blood samples obtained from total 250 positive blood samples after DNA extraction and measure of concentration and purity we used 2 primers that target major surface protein 4 (MSP4) in *A. ovis* genomic DNA.

The results of PCR test with major surface protein 4 primer was 83 samples positive from total 100 samples, According to the species of animals, the highest prevalence of *A. ovis* was 83.7 %, 72 positive sample from total 86 blood samples from sheep and lowest prevalence was 78.5 %, 11 positive sample from total 14 blood samples from goats.

INTRODUCTION

Anaplasma ovis is a tick-borne mandatory intraery-throcytic bacterium of sheep, goats and wild ruminants (Friedhoff, 1997; Yabsley *et al.*, 2005; de la Fuente *et al.*,2006; 2007).

The spread of the microbes is for the most part through vectors, all the more explicitly ticks. The greater significant types are *Rhipicephalus*, *Amblyomma*, *Dermacentor* and *Ixodes* (Rymaszewska and Grenda, 2008).

The intense period of the malady is portrayed by fever, dynamic paleness,icterus, weight reduction, milk yield diminishing, and some time death (Splitter *et al.*, 1956; Yasini *et al.*, 2012). The infection with *A. ovis* may incline animals to different irresistible or parasitic infections that bother the state of the creature and can prompt its demise (Kocan *et al.*, 2004).

A long time ago the identification of *A. ovis* has been founded by use minuscule assessment of Giemsa-recolored blood spreads (Ndung'u *et al.*, 1995), considering the reality that it is a moderately modest examination not requesting as cutting edge and costly hardware with respect to serology and PCR. Be that as it may, blood smear assessment is a somewhat harsh strategy requiring experienced work force (Renneker *et al.*, 2013; Ybanez *et al.*, 2013), and about 0.2 % of the RBCs may infected (Shompole et al., 1989).

Nucleic acid based atomic apparatuses, for example, PCR utilizing the 16S ribosomal RNA quality (Liu *et al* .,2005). likewise, significant superficial protein 4 (MSP4) quality (de la Fuente *et al* ., 2007). besides, the opposite line blotching technique (Bekker *et al* ., 2002). have been demonstrated to be of extraordinary

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analytic incentive in the distinguishing proof of *A. ovis* infection.

MATERIALS AND METHODS

Blood samples collection

The samples was collected from different sex of animals about 337 samples from females and 52 blood samples from males,all blood samples we took from small ruminants 314 samples from sheep and 75 samples from goats.

The animals were set up for blood testing by shaving and sterilizing the site of infusion. Three to five ml of jugular vein blood was gathered from every creature utilizing dispensable needles. The blood was kept in K2EDTA tubes, marked and put away at - 20 ^{0}C in the high examination research center in the college of veterinary medicine, University of Duhok.

Blood tests were acquired from clinically speculated males and females sheep and goats from various ages of one to more than 3 years old creatures inside seven distinct regions (Akre, Simele, Zummar, Feshchapoor, Deraboon, Bajed Kandal,Karoda) and diverse Sub regions within towns,The considered creatures were Kurdish and blended varieties, number of creatures, age of animals.

Molecular identification

DNA Extraction DNA was extracted from aliquots (200 µL) of the whole blood samples with the genomic DNA extraction Kit from blood (Ge Net Bio, Korea) according to the manufacturer's instructions. DNAs were eluted into 200 µL Buffer AE and couriered to Scientific Research Center, college of veterinary medicine, University of Duhok and frozen at −20°C until PCRs were performed.

Polymerase Chain Reaction (PCR)

Two primers (foreword and revers) was used that target the major surface protein 4 (MSP4) in *Anaplasma ovis* genomic DNA for identifying of *Anaplasma ovis* which were depicted by

(Ali Yousefi *et al*., 2017) as shown in (Table 1).

Table (1) : Forward and Reverse oligonucleotide primers sequences used to amplify the MSP4 genes of *Anaplasma ovis* according to Ali Yousefi *et al*., 2017

Primer	Primer Type (MSP4gene)	Sequence (5'- 3')	Size
M-OV F	HQ456350.1	TGAAGGGAGCGGGGTCATGGG	114-134
M-OV R	HQ456350.1	GGTAATTGCAGCCAGGGACTCT	438-460

Components of PCR

The PCR was performed in 20mL reaction mixture containing 10 mm Tris–HCl (PH 9.0), 30 mm KC 1.5 mm MgCl2, 250 mm each dNTP, 0.5 mm each sense and antisense primers, 1 IU Taq DNA polymerase and 2mL of DNA in automated Eppendorf Mastercycler, (Germany) for 35 cycles. After an initial denaturation step of 5 min at 95^{0}C, each cycle consisted of a denaturing step of 30 s at 95^{0}C, anannealing for 30 s at 56^{0}C and an extension step of 30 s at 72^{0}C. Finally, PCR was completed with the additional extension step for 7 min. The PCR products were analyzed on 1.5% agarose gel, 85 V well be used as avoltage source in 1x TAE buffer for 40 min and visualized using ethidiumbromide and UV transilluminator.

Statistical analysis

The data were analyzed to be better understand and to ensure high accuracy with efficient data conditioning the IBM SPSS Statistics software version 26 are used. From the descriptive statistic the frequency is used. This tool contains (Mean, Median, Mode, Stander Deviation, variance, Range, Minimum, Maximum, Skewness, Kurtoses). Also, the Bar Charts with the calculation of Conversion Matrix that contain the specificity and sensitivity are used and $p<0.05$ was considered as statistically significant.

RESULTS

In this study used Giemsa stain for microscopic examination out of 389 animals 250 were found positive for *Anaplasma ovis* infection with infection rate of 64.26 % and 139 of them were negative 35.73 %.

Morphological characteristics, *Anaplasma* inclusion bodies appeared as one uniform dark staining dote like circular bodies on the periphery to the infected goat erythrocytes, The presence of *Anaplasma ovis* in RBC can illustrate in (Figure 1).

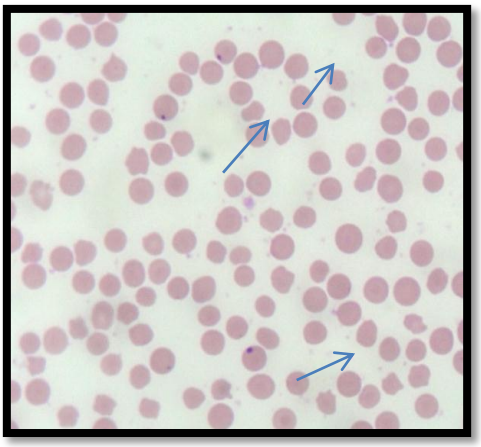

Fig. (1): *Anaplasma ovis* inclusions in erythrocytes of sheep stained with Giemsa by light microscope lens utilizing 100x oil emersion

Results by using microscopic examination highest prevalence was observed in Akre region with 69.23 %, 45 positive sample from total 65 sample and the lowest was reported in Derabon region with 53.57 %, 15 positive sample from total 28 sample as shown in (Table 2), With regard to gender of animals, the infection rate of *A.ovis* was 67.65 %, 225 positive sample from 337 in females and 48.07 %, 25 positive sample from total 52 sample in males as shown in (Table 3),and according to the species of animals, the highest prevalence of *A. ovis* infection was 67.83 %, 213 positive sample from total 314 blood samples from sheep and lowest prevalence was 49.33 %, 37 positive sample from total 75 blood samples from goat as shown in (Table 4).

Table (2): Infection rate of *A.ovis* in sheep and goats by using Microscopic examination in different districts and sub districts of Duhok province

Area	No. of samples examined	No. of positive samples	Percentage of positive samples %
Akre	65	45	69.23
Bajed Kandal	52	33	63.46
Derabon	28	15	53.57
Feshckapor	100	67	67
Karoda	20	12	60
Simele	24	15	62.5
Zummar	100	63	63
Total	389	250	64.26

Table (3): Infection rate of *A.ovis* in small ruminants by using Microscopic examination according to the sex of animal

Sex of animal	No. of samples examined	No. of positive samples	Percentage %
Male	52	25	48.07
Female	337	225	67.65
Total	389	250	64.26

Table (4) : Infection rate of *A.ovis* in sheep and goats by using Microscopic examination

Species of animal	No. of samples examined	No. of positive samples	Percentage %
Sheep	314	213	67.83
Goats	75	37	49.33
Total	389	250	64.26

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polymerase chain (PCR):

PCR analysis of 100 blood samples obtained from total 250 positive blood samples by microscopic examination after DNA extraction and measured of concentration and purity used 2 primers that target major surface protein 4 (MSP4) in *Anaplasma ovis* genomic DNA. The polymerase chain reaction outcome was pass through an agarose gel (Figure 2).

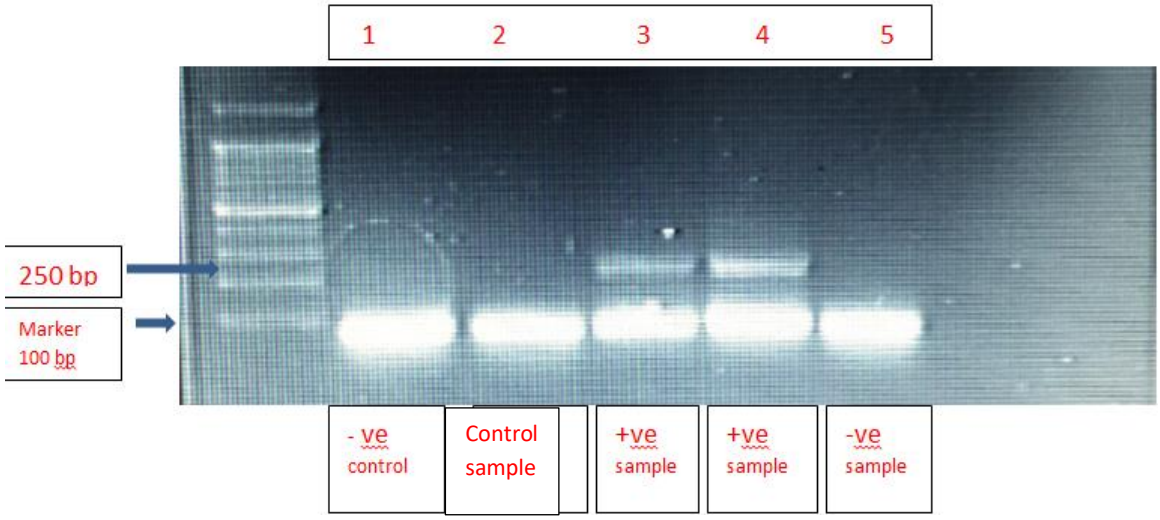

Fig. (2) : Conventional PCR product of MSP4 gene in *A.ovis* run on 1.5% agarose gel stained with ethidiumbromide,using 100bp DNA marker, 1; control sample, 2;negative sample from sheep,3;positive sample from sheep with 250bp product,4;positive sample from goatwith 250bpproduct, 5;negative sample from goat.

The results of conventional PCR revealed that *Anaplasma ovis* infection was 83/100 (83 %) compared to microscopic examination which was 250/389 (64.26%) as shown in (Table 5).

Table 5: Infection rate of *A.ovis* in small ruminants by using microscopic examination and PCR

Test	No. of samples examined	No. of positive samples	Percentage of positive samples %	No. of negative samples	Percentage of negative samples %
Microscopic examination	389	250	64.26	139	35.73
PCR	100	83	83	17	17

According to area of sample collection the results of PCR test ,The highest prevalence was observed in Akre region with 88.8 %, 16 positive samples from total 18 samples and the lowest was reported in Bajedkandal region with 76.9 %, 10 positive samples from total 13 samples as shown in (Table 6).

According to the species of animals, the highest infection rate of *A. ovis* when using conventional PCR test was (72/86) 83.7 % of blood samples from sheep and lowest infection rate was (11/ 14) 78.5 % of blood samples from goat as shown in (Table 7).

Table (6): Infection rate of *A.ovis* in small ruminants by using PCR test in different Districts of Duhok province

Area	No. of samples examined	No. of positive samples by PCR	Percentage %
Akre	18	16	88.8
Bajed Kandal	13	10	76.9
Derabon	6	5	83.3
Feshckapor	27	22	81.4
Karoda	5	4	80
Simele	6	5	83.3
Zummar	25	21	84
Total	100	83	83

Table (7) : Infection rate of *A.ovis* in small ruminants by using PCR test according to the species of animal

Species of animal	No. of samples examined	No. of positive samples	Percentage %
Sheep	86	72	83.7
Goats	14	11	78.5
Total	100	83	83

Data of results was analyzed by using IBM SPSS statistics software version 26 to get high accuracy and to be better understand for both two tests, microscopic examination as shown in (Table 8) and PCR test as shown in (Table 9).

Table (8): SPSS statistics analysis results of 389 samples by using microscopic examination

		Area	Species	Age	Sex	Weight	Breed	Temperature	Microscopic Examinaton
N	Valid	389	389	389	389	389	389	389	389
	Missing	0	0	0	0	0	0	0	0
Mean		4.11	1.80	2.79	1.13	82.11	2.09	39.86	1.36
Std. Error of Mean		.110	.020	.024	.017	.777	.052	.055	.024
Median		4.00	2.00	3.00	1.00	80.00	2.00	40.20	1.00
Mode		4a	2	3	1	75	1	41	1
Std. Deviation		2.173	.401	.473	.335	15.333	1.027	1.084	.481
Variance		4.723	.161	.223	.112	235.107	1.054	1.176	.231
Skewness		.034	-1.502	-2.195	2.228	-.289	.183	-.290	.586
Std. Error of Skewness		.124	.124	.124	.124	.124	.124	.124	.124
Kurtosis		-1.328	.257	4.137	2.981	1.921	-1.454	-1.435	-1.665
Std. Error of Kurtosis		.247	.247	.247	.247	.247	.247	.247	.247
Range		6	1	2	1	105	3	4	1
Minimum		1	1	1	1	20	1	38	1
Maximum		7	2	3	2	125	4	42	2
Sum		1597	700	1085	439	31940	812	15504	529

a. Multiple modes exist. The smallest value is shown

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Table (9) : SPSS Statistics results of 100 samples by using PCR test

		Area	Species	Age	Sex	Weight	Breed	Temperature	DNA Conc	DNA Purety	PCR Test
N	Valid	100	100	100	100	100	100	100	100	100	100
	Missing	0	0	0	0	0	0	0	0	0	0
Mean		4.06	1.85	1.92	1.15	84.50	1.70	40.56	15.5110	1.9001	1.17
Std. Error of Mean		.219	.036	.027	.036	1.394	.087	.050	.84970	.03814	.038
Median		4.00	2.00	2.00	1.00	82.50	1.00	40.50	14.1500	1.8600	1.00
Mode		4	2	2	1	75	1	41	6.50[a]	1.74	1
Std. Deviation		2.187	.359	.273	.359	13.935	.870	.496	8.49701	.38145	.378
Variance		4.784	.129	.074	.129	194.192	.758	.246	72.199	.146	.143
Skewness		.046	-1.990	-3.144	1.990	.608	1.191	-.532	.465	.632	1.784
Std. Error of Skewness		.241	.241	.241	.241	.241	.241	.241	.241	.241	.241
Kurtosis		-1.322	2.001	8.043	2.001	.550	.761	.600	-.801	.619	1.206
Std. Error of Kurtosis		.478	.478	.478	.478	.478	.478	.478	.478	.478	.478
Range		6	1	1	1	65	3	3	31.80	1.81	1
Minimum		1	1	1	1	55	1	39	2.40	1.09	1
Maximum		7	2	2	2	120	4	42	34.20	2.90	2
Sum		406	185	192	115	8450	170	4056	1551.10	190.01	117

a. Multiple modes exist. The smallest value is shown

It's clear that from table (8) the Range of the Weight feature which are the differences between the Minimum value (55) and Maximum value (120) is equal to (65).that give Variance between (the Mean (84.50), Median (82.50), Mode (75)) and (the Stander Deviation is (15.333)) equal to (235.107).

In table (9) the Range of the Weight feature which are the differences between the Minimum value (20) and Maximum value (125) is equal to (55).that give beggar Variance between (the Mean (82.11), Median (80), Mode (75)) and (the Stander Deviation is (13.935)) equal to (194.192).

The statistical observe of the Weight feature can be one of the most important point that give the reason behind the most accurate results in the microscopic examination and PCR test.

DISCUSSION

In most countries, little attention is paid to *A. ovis* infection, There are only few recent reports on anaplasmosis of sheep. Some information of Ovine and Caprine anaplasmosis in Africa, American, Asia and Europe was reported (Jansson Lagerkvist, 2017). In Iraq, the investigations of *A. ovis* infections in sheep and goat have been studied (Al-rabiaa Ali 2016).

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.

The results of current study were agreed with a few past investigations that have been done in Iraq , and more centered around nearby reproducing goats and sheep (Alsaad KM,2009 ; Naqid IA, Zangana,2011; Renneker S *et al*., 2013), It is also agreed with (Roger *et al*., 2008). Who expressed that the anaplasmosis is mainly recorded in animals in pre-winter and winter seasons.

In the present study as indicated by the species of animals, the infection rate of *A. ovis* was 67.83 % in examined sheep and infection rate was 49.33 % in examined goats. The results disagreement with the study of (Bahzad. H. salih Mustafa,2011) which demonstrated that the predominance of *A .ovis* among sheep which was 4.8% in Sulaimani province-Iraq by using microscopic examination because he examined

all animals but we tested just the suspected animals that have a clinical signs of infected with *A. ovis*.

The results of the present study were concurrence with (Renneker, Abdo *et al*.,2013,) results that shows (62.6%) of tested animals were infected with *A. ovis* in Kurdistan region of Iraq.

Other studies in Iraq like Alsaad *et al*., (2009) which found the infection rate of *A. ovis* in local goats in Mosul region Iraq was 24.74% using direct microscopic examination of Giemsa stained blood smears. It has additionally revealed by (Al-Amerey and Hasso, 2002) that found the infection rate of *A. ovis* in Baghdad was 32.2% using direct microscopic examination of Giemsa stained blood smears. those results was lower than the results of the present study because that may be of many factors such as age ,species, breed of animals and climate differentiation that affected on the presence of ticks which act as a vector of transmission of *A. ovis*.

The results of the current study according to the age of animals, the highest infection rate of *A. ovis* was 72.64 % in age group over 2 years and the lowest was 18.18 % in age group under 1 year .

The results of the present study were completely in line with the findings of (Razmi *et al.*, 2006; Naqid *et al.*, 2011). who reported that adult animals were more susceptible to anaplasmosis than younger animal.

The results of the current study also agreement with (Al.Amery and Hasso, 2002) that reported the oldest group have the opportunity for presenting to vector was more the more youthful animals. The ages under eight months have most reduced rate than the age over four years of age (Shompole *et al*., 1989). In addition, the lower exposure to ticks because of the nonattendance of lambs grazing and the defensive impact of colostral antibodies that can last as long as a quarter of a year among young animals (Friedhoff, 1997).

PCR-based methods permit identification of parasites at low parasitaemia while segregating different types of co-infecting agents (Shayan and Rahbar ,2005). It has been appeared in a few investigations that molecular methodologies are considerably more sensitive and more accurate than microscopic assessment (Schnittger *et al*., 2004).

The current results by using conventional PCR was 83 % (83/100) of animals infected with *Anaplasma ovis*. This results disagreement with Renneker *et al*.,(2013) ;Razmi *et al*., (2006) that announced the infection rate of *A. ovis* in sheep from Kurdistan was 66.65 % of animals because the differentiation in technique that used.

In different nations, comparable outcomes have been accounted for in Iran, with a predominance of 80.3% (Razmi *et al*., 2006) it is agreement with the current results, and in Portugal and Iraq with 82.5% and 66.6% commonness, separately (Renneker *et al*., 2013). A lower predominance (27.5%) in goats has been accounted for in China (Zhang *et al*., 2013). In Sudan and Turkey with 41.6% and 31.4%, separately, in sheep (Renneker *et al*., 2013). In Cyprus (51%) (Chochlakis *et al*., 2009). In Italy (57%) (Torina *et al*., 2008).

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