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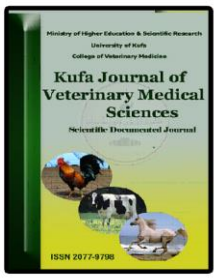

Molecular detection of haemotropic mycoplasma infection in sheep

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Abstract:
This study was the first report about hemotropic mycoplasmoses in sheep at middle of Iraq as well as the first trail to investigate this infection among ticks .EDTA blood samples were collected from four hundred (400) Awassi sheep during February – July \2016 ,two hundard (200) of sheep clinicallyhad signs of anemia ,weakness (infected group)and another 200 sheep that clinically healthy(healthy group) ,all examined sheep were ranked according to gender(male and female) and to age periods (less than and more than one year) and each sub group included 100 sheep , that tested by PCR assay by using specific *Mycoplasma ovis* primer with 1341 bp that detect infection rates 17.5% which significantly higher in infected group 25.5% than healthy group 7.5% and the yearling sheep was more susceptible to infection 22.5% than adult 12.5% and the infection was higher in female 20.4% than in male 14.5% , the *Mycoplasma ovis* infection was recorded with higher percentage in July 24.7% while no infection *in* February will be showed .
All blood samples of positivity infected sheep (70) were submitted into blood indices analysis which exhibited significant reduction in all blood parameters (RBCs ,Hb ,PCV ,MCH ,MCHC, platelets) when compare with group of negativity PCR healthy sheep as control group .
The trail to investigate about *Mycoplasma ovis* in (40) ticks picked up from examined sheep from tail and ear sites with standard parasitological methods by using the same primer of *Mycoplasma ovis* by PCR , this technique was gave negative result .
The conclusion include *Mycoplasma ovis* infection was registered in Awassi sheep with increasable rate in emaciated ,anemic sheep and so in yearling ,female and warm months of year with haemolytic macrocytic anemia.
Keywords: *Mycoplasma ovis* , Awassi sheep ,Ticks ,PCR ,Blood.

الكشف الجزيئي عن الاصابة بالمايكوبلازما الدموية في الاغنام

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الخلاصة :
تمثل هذه الدراسة أول تقرير حول المايكوبلازما الدموية في اغنام العواسي في وسط العراق وكذلك المحاولة الأولى للتحري عن المسبب (*Mycoplasma ovis*)في القراد، تم جمع عينات الدم في انابيب تحوي مانع التخثر من أربعمائة (400) من الاغنام خلال الفترة من شباط – تموز \ 2016)، والتي تضمنت 200 من اغنام العواسي التي تعاني من علامات الأغنام فقر الدم والضعف (المجموعة المصابة) ومائتين 200 من الأغنام التي تبدو سليمة سريريا (المجموعة السليمة)، وقسمت الاغنام في المجموعتين بالتساوي حسب الجنس(200 ذكور و 200 اناث)وحسب الفئات العمرية (200 أقل من سنة و200 أكثر من سنة) ، وتم التحري عن

المايكوبلازما الدموية في عينات دم الاغنام وعينات القراد كلا على حدة باستخدام تقنية تفاعل السلسلة المتبلمرة PCR باستخدام البادئ الخاص بالمايكوبلازما الدموية والذي يحوي على 1341 قاعدة ناتروجينية والتي اظهرت وجود الاصابة في الاغنام المفحوصة بنسبة 17.5٪ وكانت نسبة الاصابة أعلى بكثير في المجموعة المصابة 25.5٪ عما عليه في المجموعة السليمة 7.5٪ وان الأغنام بعمر سنة فما دون أكثر عرضة الإصابة 22.5٪ من البالغات 12.5٪ والاصابة في الإناث 20.4٪ اعلى منها في الذكور 14.5٪، وقد سجلت اعلى نسبة للاصابة في شهر تموز 24.7٪، في حين لم تسجل أي إصابة في شهر شباط.وكانت نتيجة التحليل للصورة الدمية للاغنام المصابة انخفاض ملحوظ في كل المعايير المدروسة والتي اظهرت اصابة الاغنام بفقر الدم التحللي من نوع كبير الخلايا عند المقارنة مع مجموعة من الحيوانات السليمة والت كانت سالبة لفحص السلسلة المتبلمرة .

بينما محاولة التحري عن *Mycoplasma ovis* في عينات القراد(40) والتي جمعت من مناطق الذيل والاذان للنعاج المفحوصة حيث اظهرت نتيجة سالبة لفحص السلسلة المتبلمرة .

خلاصة البحث تسجيل الاصابة بالمايكوبلازما الدموية الغنمية *Mycoplasma ovis*ولاول مرة في اغنام العواسي في محافظات الفرات الاوسط \العراق مع زيادة معدلات الاصابة في الاغنام التي تعاني من الهزال و فقر الدم التحللي المرافق لهذه الاصابة وفي الاعمار اليافعة وفي الاناث اكثر مماعليه في الاغنام البالغة و الذكور.

الكلمات المفتاحية: المايكوبلازما الدموية الغنمية ، تفاعل السلسلة المتبلمرة ،اغنام العواسي ، القراد ، الدم

Introduction :

Haemotropic mycoplasmas were previously known as *Haemobartonella* and *Eperythrozoon*, but recently are reclassified within the genus *Mycoplasma* according to 16S rRNA gene sequencing (1).

Haemoplasmas are parasites of RBCs of mammals, including human , cats, dogs, cattle, sheep, goats, pigs and rodents .Symptoms are not specific, but generally include anemia, pallor mucosa , anorexia, weight loss and depression. So, in acute stage of the disease constant fever, can be seen often (2).

Mycoplasma ovis (*Eperythrozoon ovis*) is a haemotropic mycoplasma that attaches to the red blood cells of sheep , it was first described by Neitz in 1934,it infect sheep and may cause mild to severe anaemia which is reversible, combined with reticulocytosis, anisocytosis, macrocytosis and polychromasia with a high lethality rate in lambs, whereas adults are usually chronically infected but show no clinical signs, with a low lethality rate (3).

Mycoplasma ovis infection is transmitted by blood-feeding arthropods and the most important way is insect bites such as ticks, so the other ways have

been described including intrauterine transmission, direct transfer of bacteria during the animal fight and transfer to newborns by milk and has been reported in domestic small ruminants as sheep and goats (4, 5) , in white-tailed deer (*Odocoileus virginianus*) and reindeer (*Rangifer tarandus*) (6 , 7). *Mycoplasma ovis* was detected for the first time by PCR in Germany in sheep by Neimark *et al* (5), it has also been reported in Hungary (8), Japan (9) and the USA (10).

Mycoplasma ovis are difficult to grow, the common methods of diagnosis are cytology, and microscopic examinations but the observation of organisms on erythrocytes in staining blood smears is known to be unreliable and electron microscopy are used to identify these pathogens , so, the most reliable and definitive method for the detection of haemoplasmas by polymerase chain reaction (PCR) (11).

Materials and methods:

EDTA anticoagulated blood samples (5 ml)were collected with sterile processing by jugular

venipuncture from a total of (400) sheep during six months(February \ 2016 –July\ 2016) included 200 sheep which clinically showed emaciation ,pale mucous membrane, weakness as well as 200 sheep which that clinically healthy ,one ml of blood directly examine by Hemo-analyzer (Horiba-France) to detect CBC blood indices and other (4 ml) anti-coagulated blood stored at freeze for PCR test .

Testing sheep in both sex(200 male and 200 female) were ranked in two age groups: less and more than one year(200 sheep in each group).

Collection of ticks :

A total of 40 ticks were collected from sheep (10 from each sheep sub -group) from tail and ear areas of body sheep . Ticks were stored in 70% ethanol at 4°C. , and identified under the stereo microscope according to the taxonomic keys and classification criteria (such as capitulum, palp, festoon, cervical canal, anal shield, accessorial shield, and scutum colour) (12).

DNA of tick extraction:

Ticks were processed in groups as five ticks from each animal group were examined in one pooled sample, and each tick group was washed three times in sterile phosphate-buffered saline and then stored by freezing . The frozen ticks were cut into pieces with ethanol-flamed scissors in 1.5-ml Eppendorf tubes. The samples were subjected to three cycles of freezing and thawing. Digestion with proteinase K (200 mg/ml) was performed by incubation at 56°C overnight. After centrifugation at maximum speed for 10 min, the supernatant was transferred into a fresh tube. DNA was isolated by phenol-chloroform extraction and ethanol precipitation. DNA pellets were washed once with ice-cold 70% ethanol, air dried, and re-suspended in 200 ml of Tris-HCl (13)

Polymerase chain reaction(PCR):

DNA was extracted from whole blood and other extracted from tick using the Wizard® Genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions and stored at -20 °C until used. The PCR assay for *Mycoplasma ovis* DNA detection was performed using the protocol described by Grazziotin *et al.* (2011), with a set of primers which characterization was summarized in table (1) .

Table(1): The *Mycoplasma ovis* primers used in the study

Primers	Sequence		Product size
Mycoplasma ovis	F	5,-ATGCAAGTCGAACGAGTAGA-3	1341 bp
	R	5-TGATACTTTCTATAGTTTG-3	

Statistical analysis :

The results of the present study were analyzed statistically by using two way ANOVA test by the Microsoft SPSS program (version) software 2010.

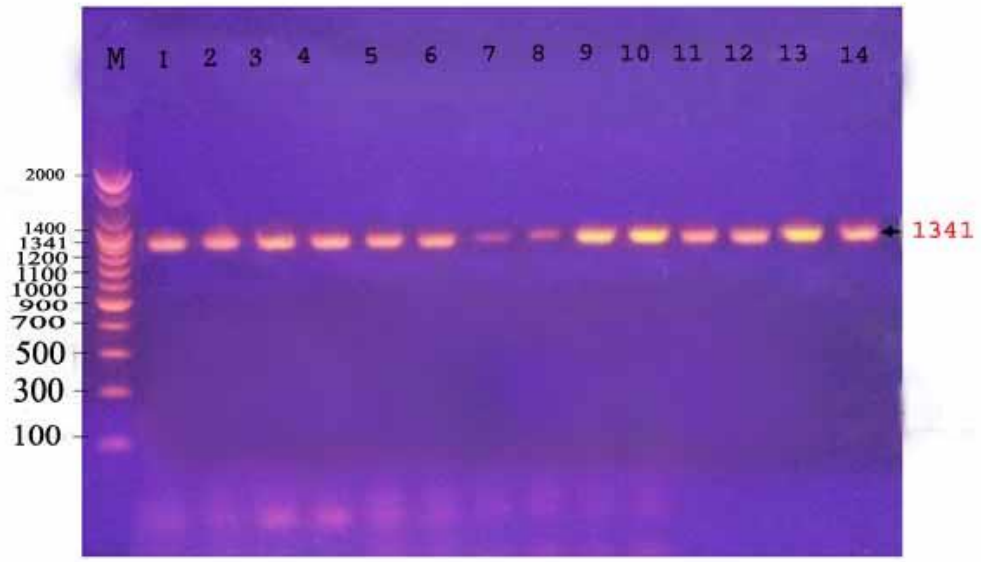
Results:

By using specific *Mycoplasma ovis* primer ,there was 17.5% (70\400) of Awassi sheep in Al-Diwaniyah city were positive(figure 1) and the high infection rate appeared in infected group 25%(55\200) , so hemotropic mycoplasmoses recorded in clinically healthy sheep group 7.5%(15\200) table (2)

Table(2): Infection rate of *Mycoplasma ovis* infection in examined Awassi sheep

Sheep group	Examined sheep	+M.ovis PCR	%
Infected	200	55	25.5 a
Healthy	200	15	7.5 b
Total	400	70	17.5

The different small letters refers to significant variations at (p≤ 0.05)



Figure(1): Agarose gel electrophoresis image that show the PCR product analysis of 16S rRNA gene in Mycoplasma ovis positive isolates. Where M: marker (100-2000 bp), lane (1-14) positive *Mycoplasma genus* at (1341bp) PCR product.

The hemotropic mycoplasmas infection was in high rate in yearling sheep 22.5% (45\200) rather than in adult sheep significantly 12.5%(25\200),however , the infection rate in infected group was higher in yearling sheep than in adult but in healthy group the infection was higher significantly in adult 10%(10\100) than in yearling sheep 5%(5\100) table (3).

Table(3): *Mycoplasma ovis* infection rate and age relationship of examined Awassi sheep

Sheep group	Age period			Age period		
Infected= 200	Less than one year			More than one year		
	Examined	+PCR	%	Examined	+PCR	%
	100	40	40 a A	100	15	15b A
Healthy=200	100	5	5 aA	100	10	10bB
Total	200	45	22.5a	200	25	12.5b

The differences in small letters horizontally and differences in capital letters vertically refer to presence of significant value at ($p \leq 0.05$).

In table (4) , there was a significant affect of gender on *Mycoplasma ovis infection* , the female was highly infected 20.5%(41\200) when compare with male 14.5%(29\200) , but in healthy group , gender was not affected significantly on infection rate which showed as 9% and 6% in male and female respectively .

Table(4): Gender affection on Mycoplasma ovis infection rate in examined Awassi sheep.

Sheep group	Gender			Gender		
Infected= 200	Male			Female		
	Examined	+PCR	%	Examined	+PCR	%
	100	20	20 a A	100	35	35b A
Healthy=200	100	9	9 aA	100	6	6 aB
Total	200	29	14.5 a	200	41	20.5b

The differences in small letters horizontally and differences in capital letters vertically refer to presence of significant value at ($p \leq 0.05$).

The infection were recorded in all months of the study in different rates except February , the higher infection rate 24.7% (21\85) in July, in April 18.82% , in May 18% , in March 14.66% and 13.33% in June (table 5).

Table(5):Relationship between *Mycoplasma ovis* infection and months of study in examined Awassi sheep

Months\2016	Examined sheep	+PCR *M.ovis*	%
February	25	-	0
March	75	11	14.66 a
April	85	16	18.82 b
May	100	18	18 b
June	30	4	13.33 a
July	85	21	24.7 c
Total	400	70	17.5

The different small letters refers to significant variations at ($p \leq 0.05$)

The results of blood indices were summarized in table (6) including the seventy (70) blood samples of positivity PCR sheep that compare with twenty (20) negativity PCR (10 blood sample from each group) that revealed significant decrease values of RBCs (6.04±0.6),Hb (6.98±0.18) ,PCV (25.46±1.87) ,MCV(29.6±0.33) ,MCH(7.1±1.9),MCHC(21.08± 4.4) when compared with the same blood indices in control group .

These results indicate a hemolytic macrocytic anemia was recorded in sheep gave positive to *Mycoplasma ovis* infection by PCR assay .

Table(6): Blood indices ofsheep with *Mycoplasma ovis* infection in examined Awassi sheep groups .

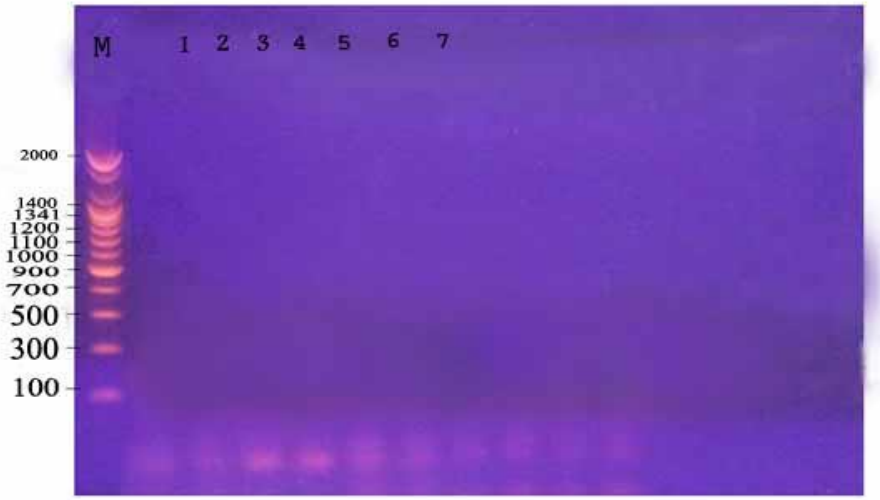
Blood parameters	+PCR sheep M±SE	- PCR sheep (control) M±SE
RBCs $\times 10^6$ corpuscles\ml	6.04±0.6 a	9.9±1.5 b

Hb g\dl	6.98±0.18 a	8.8±1.58 b
PCV %	25.46±1.87 a	32.55±3.66 b
MCV (fl)	29.6±0.33 a	31.56±2.76 b
MCH pg\ cell	7.1±1.9 a	8.2±0.71 b
MCHC g\dl	21.08±4.4 a	23.8±2.91 b
Platelets × 10^{3}\ml	290±133.4 a	230.8±112.8 b

The different small letters horizontally refers to significant variations at (p≤ 0.05)

There were two genus of tick collected *Rhipicephalus spp* and *Hyalomma spp* according to parasitological key .

While trail to investigate about *Mycoplasma ovis* DNA in ticks by PCR that collected from examined sheep exhibited negative result (figure 2).In spite from high concentration of DNA product as well as highly purity was produced within DNA extraction processing from homogenized tick samples .



Figure(2): Agarose gel electrophoresis image that show no the PCR product analysis of 16S rRNA gene in Mycoplasma ovis (negative isolates). Where M: marker (100-2000bp), lane (1-7) negative *Mycoplasma ovis* product.

Discussion:

This study is demonstrating the first record of ovine haemotropic mycoplasma infection (*Mycoplasma ovis* 17.5%) in Awassi sheep in Al-Diwaniyah province\Iraq by using PCR assay ,that confirms the global spread of *Mycoplasma ovis* infection in ovine as first detection of infection in Germany (5), in Hungary (4), in USA (10) and in Tunisia 6.28% (14) ,so ,in Argentina 81% (15) and 8.5% in some areas in Hungary (16).

The variations in infection rates among many countries belong to many factors like climate condition variations , transmitter animal carrier available , vectors distribution ,so mixed rearing of goat and sheep, physiological and nutritional status of host (17) .

However ,the infection rate in clinically ill-thrift and anemic sheep

25.5% higher than in clinically healthy 7.5% which in agreement with many results by Suzki et al (18) ,and by Rjeibi et al(14) whom find the infection with *Mycoplasma ovis* was recorded in anemic and animals have ill thrift syndrome with high rate so occurred in many animals which do not develop clinical sings with less than infection ,so, similar findings by Brun-Hansen et al(19), (20) and (21) that the infection is higher rate in animal suffering anorexia ,jaundice and weight loss (acute form) than of sheep have hidden(chronic) form of disease (without apparent clinical sings) and that confirm by (22) whom showedthat the disease is manifest with depression followed by the development of anemia, exercise intolerance and ill-thrift. .

The present study is showing the yearling sheep with high infection rate than in adult and that in similar to two separated reports in Japan by Tagawa et al(9) and (18) which supported by findings by Honrok et al (9) whom record high *Mycoplasma ovis* infection in yearling sheep than in adult .

Innate immunity (natural resistance) that associated with *Mycoplasma ovis* rasises ,so age –related and or acquired immunity to haemotropic mycoplasma will be developing (23) that illustrate the infection rate is decline in aged sheep while it higher in younger animals as well as in adult sheep which could be exposure many times for causative agent that motivate for immune response to prevent infection or at least reduce severity of haemotropic mycoplasma infection that illustrate many infected adult sheep apparently healthy (without marked clinical sings) .

However ,the gender have a significant affection on infection rate with higher infection in female than in male is contrast with result that sex is not affecting on infection rate by Abdullah et al (24) in goat,however ,there is no research is available about sex and *Mycoplasma ovis* infection in sheep . Female could be express for specific stressor factors associated with pregnancy ,lactation that accompanied with another factors like nutritional deficiency ,external and internal parasite infestation and these factors can affected on activity on immune system against infection and lead to increase host susceptibility for infection .

In July ,higher infection rate documented but no-infection in February appeared that is a compatible with findings in Hungary in which high infection in July and August during 2006 (4) .

The infection with high rate occurrence in most months of year due to distribution of many transmitter vectors like mosquitoes ,ticks ,sheep ked(25) and that vectors multiply and become active when the availability of favorable climatic conditions particularly in warm moths of years , while, Suzuki et al (18) was pointed that infection could be detected at all time in years in Japan which represent warm and sever cold months and they attributed that to distributed vectors in warm months as well as many infected animals became permanent carrier and bearing *Mycoplasma ovis* (26) that give positive result to diagnosed test during cold moths when vector hidden .

Macrocytic anemia with significant decline in CBC indexes which accompanied with infection in Awassi sheep that obtained in present study was compatible with many reports that revealed the hemogram changeable associated with *Mycoplasma ovis* infection in sheep ,so in other small ruminant as (27),(8) ,(5) ,(9) as well as report by Stoffregen et al (7) in reindeer and these hemogram results

are occurring due to sever destruction of RBCs by many mechanisms like the organism secrete free radicles or stimulate auto-antibodies that stimulate host cells lysis (28),(29) or increase the permeability of cells membrane which all promote RBCs death (30),so phagocytosed the infected RBCs which bearing these causative agent on their cell membrane without penetrating it cell membrane that lead to haemolytic anemia and reduction most CBC pictures.

The negative result in the first trainer to detect *Mycoplasma ovis* in ticks , in which this trains was designed according to many positivity studies applicated to detect some blood parasite in ticks like studies by Hilpertshauser et al (31) and (32) and the selection of tick according to papers that confirm that blood feeding arthropods transmitted *Mycoplasma ovis* like as ticks and mosquitos (5), (33) .

However ,in conventional PCR the negative result is not considered definitive and false negative result could be appeared due to presence of inhibitory substances in complex biological samples which their provoke a significant reduction or even blockage of the amplification activity of DNA polymerases that affect on sensitivity degree of PCR ,as well as the skilled personnel to carry out test and results analysis affect on final results (34) ,(35).

On other hand ,dose of mycoplasmas in transmitted vectors depend on time of vector feeding ,stage of infection in host ,as well as these arthropods are mechanical vectors ,therefore, they are not always carrying mycoplasmas at each time .Moreover, conventional PCR type(that used in present research) is need higher DNA product concentration which more than one thousand that can RT-PCR could be detection (36).

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