MORPHOLOGCAL AND MOLECULAR STUDY OF HARD TICKS SPECIES THAT INFESTED SMALL RUMINANTS IN DUHOK GOVERNORATE, KURDISTAN REGION, IRAQ

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ABSTRACT

Ticks are harmful ectoparasite that feed on human and animal blood and causing many diseases through the world. They infested many hosts including: mammals, reptiles and birds. Ticks are important vector and they have the ability to transmit a variety of pathogenic agent to humans and animals. Ticks are divided into two major groups which are hard tick (Ixodidae) and soft tick (Argasidae). Because there was no such study done on identification of tick species by PCR technique in Kurdistan and particularly in Duhok Governorate, therefore present study was done to identify tick species by using molecular study by using of 16S rRNA and DNA sequencing. About 1000 ticks were collected from both sheep and goat respectively (500 and 500), form Duhok Governorate including: Barwaria, Zakho, Sumeil, Mangeshik, Sersin, Shekhan and Akre, Iraqi Kurdistan, between May and June 2016, between April and June 2017. The results of present study three genera of tick were detected in small ruminants by microscopic identification including: *Rhipicephalus* spp., *Hyalomma* spp. and *Boophilus* spp. Distribution of tick among sheep and goat according to the gender, the rate of infection in female was higher than in male in both species Ewe and Doe was 32.6% and 31.11% respectively as compared to male in both species (Ram and Buck) was 21.15% and 15.11% respectively. The distribution of

gender of tick in was higher in male ticks than female tick with ratio 2:1. Distribution of identified ticks in present study including (*Rhipicephalus*, *Hyalomma* and *Boophilus*) respectively, in Barwaria were (82.6%, 13.3%, and 4.1) respectively, in Zaxo were (48.3%, 42.5% and 10.3%), in Sumel were (47%, 42.7% and 10.3%), in Mangeshik were (73,2%, 26.8%, and 0%), in Sersink were (61.5%, 38.5% and 0%), in Shekhan were (78.8%, 11.8% and 9.4%) and in Akre were (60%, 34% and 6%). On molecular study, 60 samples from 150 were positive with size 460 bp after 16S rRNA amplification and have got clear bands on agarose gel 1% and electrophoresis and 20 PCR positive products were sent to Humanizing Genomics, Macrogen Company (Korea) using primer 16S_rRNA gene for sequencing both forward and Reverse. Six species of tick under two genera were founded including: *Rhipicephalus* and *Hyalomma* were identified which including: *Hyalomma anatolicum, H. marginatum, R. annulatus, R. sanguineus* and *R. turanicus*. *H. asiaticum asiaticum* for the first time was recorded in Kurdistan, and especially in Duhok city. Moreover, all sequences were submitted to NCBI using BankIt software and we obtained accession number. Phylogenetic tree was constructed based on 16S rRNA for both samples: 16S rRNA (MN594483) and (MN594490) were identical 100% to reference sequences respectively: (KU664367.1 and HM176656.1) and other sequences were identical 99% to the references sequence. In conclusion the present study is the first study for identification of tick species among sheep and goats in Duhok Governorate, Iraqi Kurdistan by sequencing analysis.

INTRODUCTION

Ticks are harmful ectoparasite that feed on human and animal blood and causing many diseases through the world. They infested many hosts including: mammals, reptiles and birds (1, 2, 3). Ticks are important vector and they have the ability to transmit a variety of pathogenic agent to humans and animals (4). Ticks are divided into two major groups which are hard tick (Ixodidae) and soft tick (Argasidae) (5). Nowadays, There are about 877-878 tick species; most of these are under the two famous families including: *Ixodidae* and *Argasidae* (6). Hard ticks are distributed around the world with their hosts ranging from wild to domestic vertebrate, except for fish. Classifications and phylogenetic inferences for Ixodidae have traditionally been depend on the morphological, biological and ecological features, often suggesting host specificity as the main factor (7, 8). Today, Molecular tools and DNA marker are widely used for the

identification of tick species, such as ITS, 16S rDNA, 18S rDNA and 28S rDNA (9). For the first time the molecular study by using 16S rRNA gene was used for identification of tick species that infested sheep and goats in Duhok Governorate, Kurdistan region, Iraq and sequencing.

MATERIAL AND METHOD

Ticks collection

About 1000 ticks were collected from both sheep and goat respectively (500 and 500), fromDuhok Governorate including: Barwaria, Zakho, Sumeil, Mangeshik, Sersin, Shekhan and Akre, Iraqi Kurdistan, between May and June 2016, between April and June 2017.

Microscopic examination

All ticks were examined at lab under dissecting microscope with the aid of morphological key, then grouped into pools according to genus and were preserved in 70% of ethanol (10, 11)

Extraction of DNA from ticks

One tick from each genus washed with different concentrations of ethanol (10, 30 , 50, and 70 percent) for one hour per concentration, then twice in PBS. Tick was crushed with portal homogenizer by using of 0.5 ml PBS, centrifuged and preserved at 18ºC till DNA extracted. Extraction of whole genome of tick was done by using special tissue kit, DNA-Sorb-AM nucleic acid extraction kit (AmpliSens ®, Russia). The purity and quality of tick DNA samples was evaluated by Nanodrop Spectrophotometer and running of samples on gel electrophoresis 1% of Agarose gel (12).

Molecular Identification of hard ticks

In this study, one pair of primerwas used: the 16S rRNA gene fragment of size approximately 460 bp), was able to catch different hard tick spp., foreword 5'-CCG GTC TGA ACT CAG ATC AAG T-3' and reverse 5'-GCT CAA TGA TTT TTT AAA TTG CTG T-3'(13). PCR reaction were performed of green master mix (2X) (Promega, USA or or GeNet Bio master mix). The PCR reactions were conducted at a final 25µl rate. There was a 12.5 µl of GeNet Bio master

mix, 1 µl from both forward and reverse primers, 2µl of Template DNA, 10 pmol/µl of each of forward and reverse and complete the volume to 25µl with added of 9.5µl nuclease-free water. According to Mangold *et al.,* (1997), the cycler state of PCR was defined as outlined in table 1. Eventually, for 1:40 min, 10µl of PCR products were visualized under UV on 1% agarose gel with 85 volt

Table 1. PCR conditions of 16SrRNA gene.

Sr. No.	Step	Temperature (ºC)	Time	Number of cycles
1	Initial Denaturation	95	5 Min	1
2	Denaturation	95	30 Sec	35
3	Annealing	55	30 Sec	
4	Extension	72	30 Sec	
5	Final Extension	72	5 Min	1

RESULTS

Microscopic Results

During this study 1000 ticks were isolated from both sheep (500 ticks) and goats (500 ticks) from Duhok Governorate, Iraqi, Kurdistan and depend on the microscopic identification of tick, three genus were founded including Rhipicephlus spp (*R. turinus,* and *R. sanguineus*), Hyalomma (*H. analoticum and Hyalomma marginatum*) spp. and *Boophilus microplus*. Rhipicephalus spp were more prevalent on sheep and goat, then followed by Hyalomma spp., Boophilus spp and bout 150 ticks were including: 78 engorged females) were remained unidentified, because was difficult to identify morphologically under the dissecting microscope as in figures (1-8)

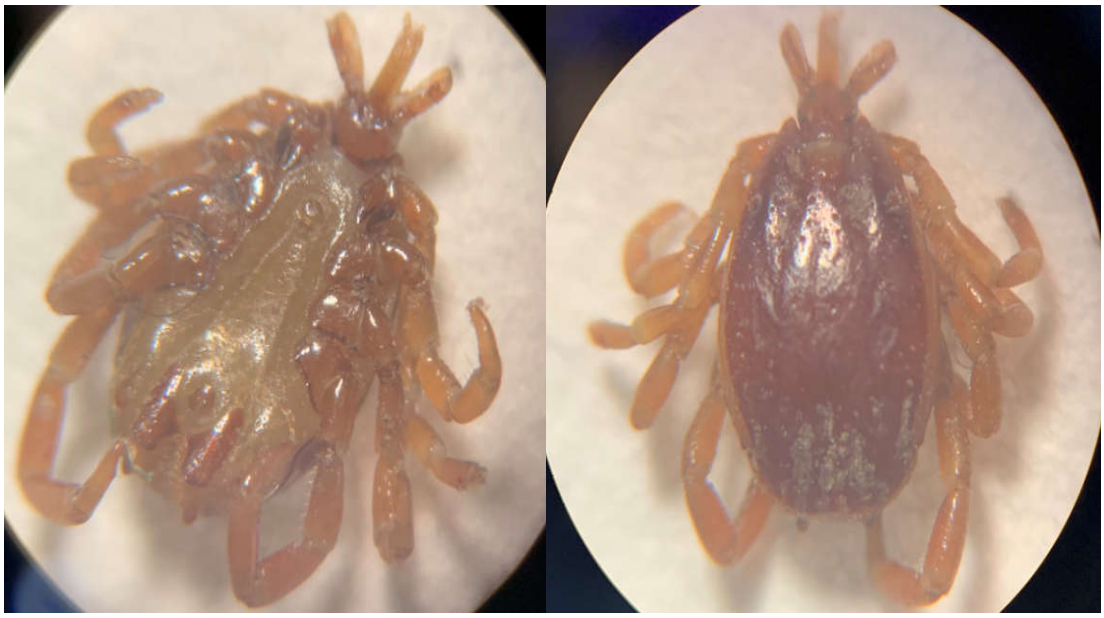

Figure1. *Hyalomma analoticum analoticum* Male (Ventral and Dorsal Views)

Figure2. *Hyalomma analoticum analoticum* Female (Ventral and Dorsal Views)

Figure3. *Hyalomma marginatum* (Ventral and Dorsal Views)

Figure4. *Rhipicephalus turanicus* Male (Ventral and Dorsal Views)

Figure5. *Rhipicephalus turanicus* Female (Ventral and Dorsal Views)

Figure6. *Rhipicephalus sangiuneus* Male (Ventral and Dorsal Views)

Figure7. *Boophilus microplus* Male (Ventral and Dorsal Views)

Figure8. *Boophilus microplus* Female (Ventral and Dorsal Views)

Table 2: Shows the distribution of hard tick among small ruminants in both sexes. The rate of infection in female was higher than in male in both species Ewe and Doe was 32.6% and 31.11% respectively as compared to male in both species (Ram and Buck) was 21.15% and 15.11% respectively.

Table2.: The distribution of hard tick among small ruminants from different area in Duhok Governorate, Iraqi Kurdistan:

Gender of Animals	No. of Animal Affected	%Positive case (%)	Positive from total infected cases (%)
Ewe n=250	108	(43)%	(32.63)%
Ram n=80	70	(87.5)%	(21.15)%
Doe n=200	103	(51.5)%	(31.11)%
Buck n=130	50	(38.46)%	(15.11)%
Total n= 660	331	(50.1)%	(100)%

Table 3: Shows the distribution the gender of tick in Duhok Governorate, Iraqi Kurdistan was higher in male ticks than female tick with ratio 2:1

Table3. Ratio between male and female of hard tics:

No. of Tick spp.	Male No.	Male (%)	Female No.	Female (%)	Total No. (%)	Ratio between Male and Female
R. turincus	200	(71.17)%	81	(28.8)%	(281)%	2:1
R. sangiuneus	180	(65.45)%	95	(34.5)%	(275)%	2:1
H.analoticum	95	(70.37)%	40	(29.6)5	(135)%	2:1
H. marginatum	75	(70.09)%	32	(29)%	(107)%	2:1
Boophilus spp	52	(100)%	0	(0)%	(52)%	2:1
Total:	602	(70.82)%	248	(29.17)%	(850)%	2:1

Table 4:Shows the distribution of identified ticks in present study including (Rhipicephalus, Hyalomma and Boophilus) respectively, in Barwaria were (82.6%, 13.3%, and 4.1) respecively, in Zaxo were (48.3%, 42.5% and 10.3%) r, in Sumel were (47%, 42.7% and 10.3%), in Mangeshik were (73,2%, 26.8%, and 0%), in Sersink were(61.5%, 38.5% and 0%), in Shekhan were (78.8%, 11.8% and 9.4%) and in Akre were (60%, 34% and 6%).

Table4. Distribution of species of hard ticks among Duhok Governorate:

Area for collection of ticks	No. Rhipicephalus spp. (%)	*No. Hyalomma Spp.* (%)	No. Boophilus spp (%)	Total No.
Barwaria	180 (82.6)%	29 (13.3)%	9 (4.1)%	218
Zaxo	42 (48.3)%	37 (42.5)%	8 (9.2)%	87
Sumel	55 (47)%	50 (42.7)%	12 (10.3)%	117
Mangeshik	30 (73.2)%	11 (26.8)%	0 (0)%	41
Sersink	32 (61.5)5	20 (38.5)%	0 (0)%	52
Shekhan	67 (78.8)%	10 (11.8)%	8 (9.4)%	85
Akre	150 (60)%	85 (34)%	15 (6)%	250

PCR Results

Pure DNA which extracted from 150 ticks were amplified by PCR. 60 of total 150 samples showed distinct band with molecular weight approximately 460 bp as shown in Figure (9) and Table (5)

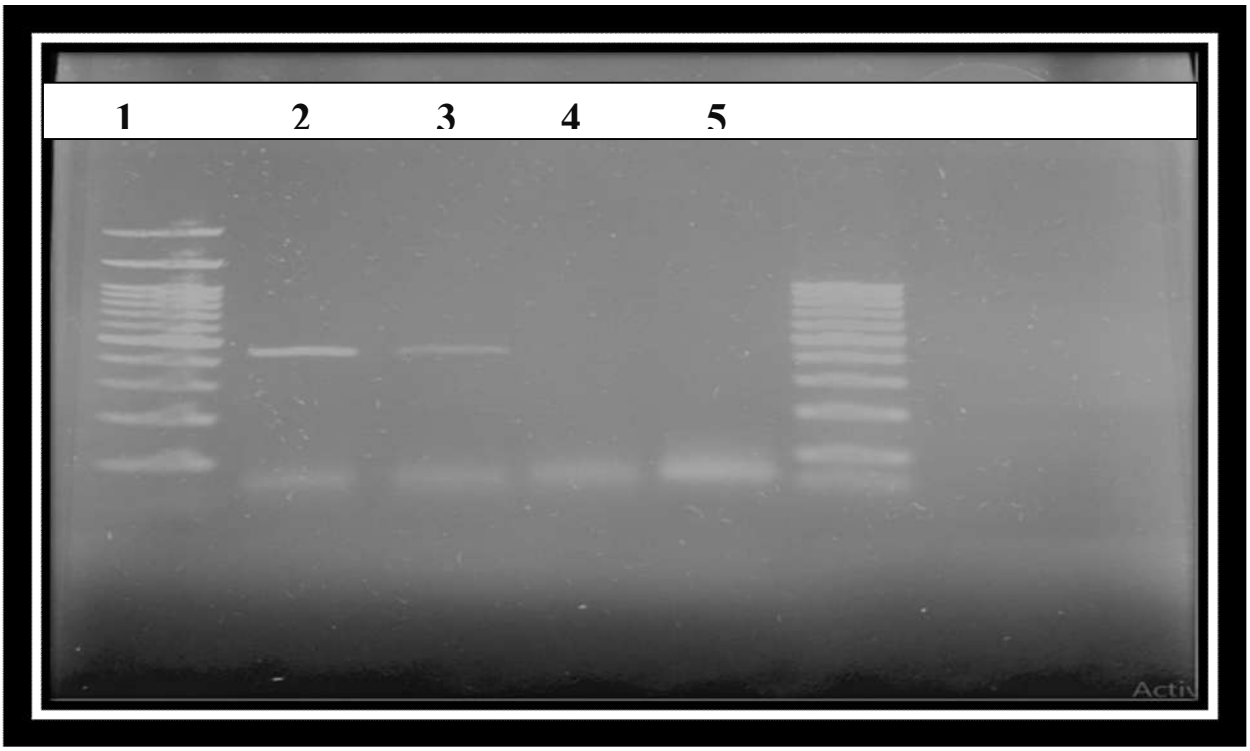

Figure 9: PCR amplification of 16S rRNA gene. Lane 1, 100 bp molecular sizemarker ; lane 2-5 PCR products on Agarose 1.5%.

Table5. PCR results of Hard Tick spp. in Duhok Governorate, Iraqi Kurdistan

Sample size	Gene	Size Bp	Positive cases No.	(%)
150	16S rRNA	460	(60)	(40)

Sequencing of 16S rRNA gene fragment

The sequences of twenty PCR prudcts were submitted to GenBank (accession number from genBank as following: MN594483.1, MN594484.1, MN594485.1, MN594486, MN594487, MN594488, MN594489, MN594490, MN594491, MN594492.1, MN594493.1 and MN594494.1) as in Table (3). The similarity of the sequence with homologous sequences deposited in GenBank was calculated using the "BLAST" tool on (NCBI) website. During this study, six species under two genera of the hard ticks were identified by molecular study and sequencing including: three species were under the genus *Hyalomma* and three species were under the genus *Rhipiciphalus* that infect small ruminants in Duhok Governorate from these species a new species under the Hylomma genra *(Hyalomma asiatium asiaticum)* with accession number (MN594484), was recently reported in Duhok Governorat, Iraqi Kurdistan were identify as Table No. (6)

Table 6. Distribution of species of hard ticks and the GenBank accession number:

Study Ticks	Accession No.	Species	Similarity (%)	References Accession No.	Country
1.Hyalomma	MN594484	*Hyalomma asiaticum asiaticum*	(99)%	JX051079.1	China, Mongolia
	MN594488	*Hyalomma anatolicum*	(99)%	HM176656.1	India
	MN594494	*Hyalomma marginatum*	(99)%	L34307.1	U.S.A
2.Rhipicephalus	MN594491	*Rhipicephalus annulatus*	(99)%	MF946466.1	India
	MN594492	*Rhipicephalus sanguineus*	(99)%	KX553960.1	French
	MN594493	*Rhipicephalus turanicus*	(100)%	KY583065.1	China

Phylogenetic Tree and Analysis

During this analysis, MEGA 7 technology was used to construct phylogenetic relationships and Neighbor-Joining tree depends on the alignment of 16S rRNA sequences to evaluate the phylogenetic relationship species status of two types of ticks in this sample. Phylogenetic tree as in figure (10): is divided into two ancestors: first ancestor was divided into two clades, in which the first clad was arranged as cluster, which included which *R. turanicus* (MN594493, MN594483, MN594486, MN594485 and MN594487), *R. sangiuneus* (MN594492 and MN594489) and using *annulatus* as out group. In the second ancestor, also there were two clades, first clade was used as out group was *H asiaticum asiaticum* (MN594494), while second clade was grouped as cluster which included *H. marginatum* (MN594494) and *H. analoticum*

analoticum (MN594490 and (MN594488) they were closely identical to each other therefore, they clustered with a bootstrap value of 99.

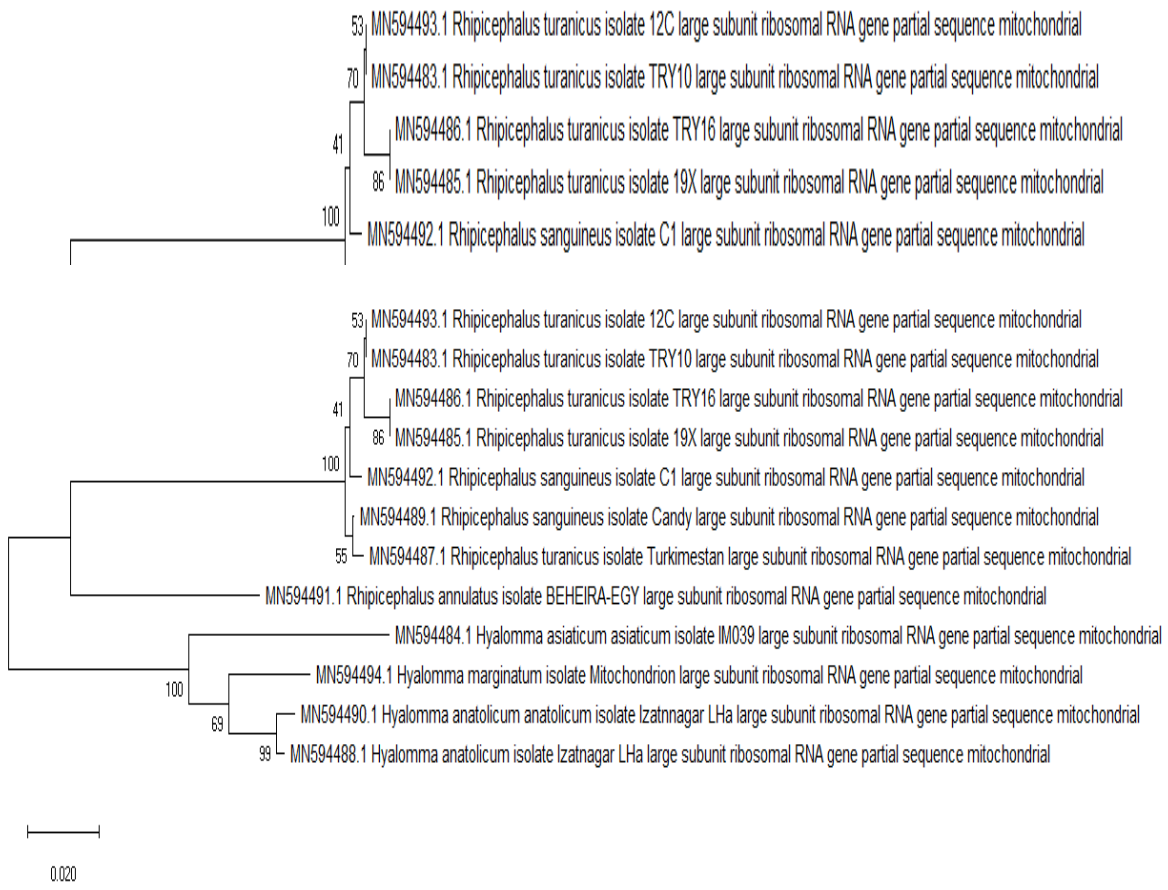

Figure 10:.Phylogenetic Tree among Tick species infested small ruminants in Duhok Governorate, Iraqi Kurdistan

DISCUSSION

The microscopical examination of hard ticks revealed the presence of six species of hard tick were collected from sheep and goats which including *Rhipicephlus spp (R. turinus,* and *R. sanguineus), Hyalomma (H. analoticum* and *Hyalomma marginatum) spp. and Boophilus microplus* in this study the distribution of Rhipicephalus spp. came at first. There were several studies that support all these species in Kurdistan, Iraqi region by (14, 15). There was another

article support same species that found in Yazad Province, Iran by (16). A study was disagree with this results done by AL-Fatlawi *et al.,* (17), they recorded that Hyalomma spp. were more predominant in south of Iraq.

Hyalomma anatolicum and Rhipicephalus turanicus and Rhipicephalus sanguineus were recorded by Kadum *et al.,* (18) in middle and south of Iraq and recorded in north of Iraq by (19) and *Omer et al., (*15) they reported some species of hard ticks were included *anatolicum anatolicum, marginatum marginatum,* and *Rhipecephalus appendiculatus.*

The Data showed that the infection rate of infesting with hard ticks was higher in male than in the female in both (Ram and Buck) was 32.6% and 31.11% respectively, as compared to male in both species (Ewe and Doe) was 21.15% and 15.11% . This obtained results from this study had light similarity with the result of work done by Bukbuka *et al.* (20) in Northeastern Nigeria was (17.2%) in male of sheep and (17.1 %) in female of sheep.

In this analysis, there was another difference was the ratio of male to female was 2:1 and the number of males was dominant, the percentage was higher in male than female as follow respectively (70.82%) and (29.17%) and these ratio were different from results were done by Kadir *et al.,* (21) in Kurdistan, Iraq Region and this results may be due to the changes of the climate in Duhok governorate and the season of the collection of ticks. These agree with the results of Salim abadi *et al*., (22), they found a relative frequencies tick sex were 57% male ticks and 34% female tick.

For the first time PCR assay in Kurdistan, Iraq and in Duhok especially is used for the identification of tick species. Two markers were used in this study: the first one was ribosomal Ribonucleic acid 16S rRNA for the identification of tick species and the second one was 18S rRNA for detection of piroplasms in both tick and blood.

During this study used Ribonucleic acid 16S rRNA for the identification of tick species and sequencing of it as a good marker for identification of hard tick species to solve morphological tick identification problems and sometimes morphological identification of ticks is not sufficient to detect the species. This is study is agreed with studies done by (23, 24)

With regard to tick species, 16S rDNA has been used and has been successful in constructing phylogeny of species of hard tick and 16S rRNA is helpful in building of the phylogenetic tree of hard tick species, but a problem associated with 16S rDNA is that using this gene alone is not sufficient to obtain full tree resolution so that the best way to solve it is accompanied by another gene such as 12S rDNA (24, 25).

Overall 150 hard ticks (male and female and engorged female) were evaluated by using S16 rRNA with PCR assay and only 65 samples from which were gave distinct bands, 20 samples were sent to Korea for sequencing. Sequenced samples in this study were showed that there was six species of hard tick under two genera among small ruminants in Duhok Governorate in 2016-2018 including: *H. asiaticum asiaticum* this species was isolated and sequenced for the first time in Kurdistan, Iraq and in Duhok Province, Iraqmainl, and there was no such study recorded this species, *H. anatolicum, H. marginatum, R. annulatus, R. sanguineus* and *R. turanicus*. Therefore, the use of 16S rRNA is a good marker in identification of these hard tick species in this study. Same species of hard ticks were recorded in Mali, West Africa by (26). A similar study that support that for the first time *H. asiaticum asiaticum* was reported in south of Iraq.

Phylogenetic analysis and tree allows genetic connections between closely related species to be resolved and has become a useful tool in several fields of biological research (27). Phylogenetic tree of the present study was constructed based on 16S rRNA sequences and there were deletion , transition and transvertion in some nucleotide of sequenced samples and these were effect on the length of nucloetide, the 16S rRNA sequence of two samples were similar 100% to the sequences of dereference within the GenBank respectively (MN594483 and MN594490), while the rest sequences were identical 99% to the sequences reference this is the first recorded tick in Kurdistan, Iraq *H. asiaticum asiaticum* with accession number MN594484 was similar 99% to China sequence with accession number (JK051079), was differ in one nucleotide (0.1%) and was as out group of cluster of Hyalomma. But there was no such article supported this type of tick here and this study was used molecular study and sequences analysis for the first time in Kurdistan and Duhok particularly for identification of tick species.

دراسة شكلية و جزئية لانواع القراد الصلب التي تصيب المجترات الصغيرة في محافظة دهوك /كوردستان العراق

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الخلاصة

تمت خلال الدراسة الحالية دراسة شكلية لتصنيف القراد والبايروبلازم الناشئ عن القراد في الدم وايضاً في القراد. جُمعت حوالي ١٠٠٠ من القراد بواقع ٥٠٠ في كل من الأغنام والماعز على التوالي، مع ١٠٠ عينة من الدم من الحيوانات وايضاً الغدد اللمفية المتضخمة في المناطق حول محافظة دهوك (برواري، زاخو، سميل، سرسنك، شيخان، وعقرة) للفترة من مايس الى حزيران ٢٠١٦، ومن نيسان الى حزيران ٢٠١٧،

أظهرت نتائج الدراسة الحالية وخلال فترة الدراسة ثلاثة أنواع جينية من القراد قد تم تحديدها في المجترات الصغيرة من خلال الفحص المجهري وتضمنت أنواع من كل من أسماء علمية *Rhipicephalus, Hyalomma* و*Boophilus* . درس انتشار القراد بين الأغنام والماعز اعتماداً على الجنس، حيث كانت نسبة الإصابة بين الاناث اعلى منها في الذكور وفي كُلٍ من النِعاج والمِعَازٌ حيث كانت: ٣٢.٦% و٣١.١١% على التوالي، فيما كانت في ذكور الاغنام والماعز (الكبش والتيس): ٢١.١٥% و١٥.١١% على التوالي. فيما لوحظ ان توزيع جنس القراد اعلى منه في الذكور مقارنة بالإناث وبنسبة ٢:١. من ناحية أخرى كان توزيع القراد المُشخص في الدراسة الحالية مُتضمنة أسماء علمية (*Rhipicephalus*، *Hyalomma* و*Boophilus*) على التوالي: في منطقة برواري كانت (٨٢.٦%، ١٣.٣%، و٤.١%)، زاخو (٤٨.٣%، ٤٢.٥%،و١٠.٣%)، سميل (٤٧%، ٤٢.٧%، و١٠.٣%)، مانكيش (٧٣.٢%، ٢٦.٨%، و٠%)، سرسنك (٦١.٥%، ٣٨.٥%،و٠%)، شيخان (٧٨.٨%. ١١.٨%، و٩.٤%) وفي عقرة كانت (٦٠%، ٣٤%، و٦%). وفيما يتعلق بالدراسة الجزيئية ٦٠ عينة من مجموع ١٥٠ عينة كانت موجبة مع حجم ٤٦٠ قاعدة نيتروجينية (base pair) بعد تضخيم الحامض النووي الريبي الرايبوسومي (16S rRNA) وفقد تم الحصول على حُزم واضحة من خلال استخدام الاكاروز ١% وجهاز الترحيل الكهربائي (Electrophoresis)، وتم ارسال ٢٠ عينة موجبة لتقنية تفاعل البوليميريز المتعدد الى Humanizing Genomics, Macrogen Company (Korea) باستخدام جين (16S rRNA) لمعرفة التسلسل الجيني. اظهرت النتائج وجود ستة أنواع من القراد تحت هذه الاجناس: *Rhipicephalus* و*Hyalomma* حيث شُخصت الأنواع: *H. anatolicum, H. marginatum, R.annulatus, R. sanguineus and R. turanicus* . ولأول مرة في كوردستان وخاصة في محافظة دهوك تم تسجيل الجنس: *H. asiaticum asiaticum*. إضافة الى ان كل التسلسلات الجينية رفعت الى المركز الوطني لمعلومات التكنولوجيا الحيوية NCBI باستخدام برنامج ال BankIt وتم الحصول على رقم الانضمام من قبل بنك الجينات ومن ثم شجرة النشوء والتطور تم انشاؤها بالاعتماد على (16S rRNA) في كلتا العينتين 16S rRNA

(MN594483)و 16S rRNA (MN594490) كانت مطابقة ١٠٠%لمصادر التاية وحُددت الأرقام التسلسلية الجينية كالآتي: (KU664367.1 and HM176656.1) على التوالي. اما بقية التسلسلات الجينية كانت مطابقة ٩٩% لمصادر التسلسلات الجينية. واخيراً اظهرت نتائج الدراسة الجزيئية للبابيروبلازم في الدم ٢٥% موجبة.

كاستنتاج عتبر الدراسة الحالية هي اول دراسة لتصنيف أنواع القراد بين الأغنام والماعز في محافظة دهوك، وفي كوردستان العراق من خلال تفاعل البلمرة المتعدد وفحص التسلسل الجيني.

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