Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 361 - 370
Received 05 March 2021; Accepted 01 April 2021.

Molecular Identification of Three Species of Hard Ticks (Acari: Ixodidae) Collected on Al-Najaf Province, Iraq

Maallh T. Al-Husseini[1*]
Faculty of Science, University of Kufa, Kufa, Iraq
Email: mallaha.alhusseini@uokufa.edu.iq

ABSTRACT

The present study was included molecular study of three species that grouped into Hard ticks (Acari: Ixodidae) based on cytochrome oxidase I (COI) and 18S rRNA genes collected from three local areas' in An Najaf Province. Present results of study were showed that there were three abundant species of hard ticks identified as *Boophilusannulatus, Rhipicephalussanguineus*, and *Hyalommadromedarii* at prevalence ratio (48.1, 38.9 and 12.9) percentage respectively. Total number of hard ticks samples was (499) that infested on sheep and goat among them the females number were (228) more than males number were (155) whereas nymph number were lower (66). By using cytochrome oxidase I (COI) and 18S rRNA gene markers specific PCR assay present study was differentiated among*B. annulatus, R. sanguineus* and *H. dromedarii* which abundant in An Najaf province. The results of gel electrophoresis based on specific PCR assay were detected that amplified segment size were 780 bp in18S rRNAgene and 700 bp in cytochrome oxidase I (COI) for species *R. sanguineus* and *H.dromedarii* respectively.

Keywords
Boophilusannulatus; Rhipicephalussanguineus;Hyalommadromedarii; hard ticks; COI;

Introduction

Hard ticks are one of the most medically important groups because they are vectors many important diseases such as Lyme disease, Boutonneuse fever and Tularemia [1]. Hard ticks are common ectoparasites that sucked blood from many of vertebrates. Since Hard ticks are obligatory ectoparasites on enormous hosts and they also cause significant effect to their domestic animals such as blood loss and reduce body weight [2].The hard tick species found in Iraq were recorded in previous studies such as the study [3] showed there are 13 species based on (morphological keys and mentioned that *Hyalommaanatolicum*and *Rhipicephalusturanicus* were the most dominant hard tick species in middle and south of Iraq. Related to An Najaf Province-Iraq, presence of infections with hard ticks on sheep in high numbers and increased during different seasons [4] .Study of [4] found that *H. anatolicum* is one of the common ticks that affect cattle in An Najaf province. More recently, study [5] from Duhok province- Iraq who recorded six species of hard ticks infested on sheep including *Rhipicephalusannulatus, R.sanguineus, R. turanicus, Hyalommaanatolicum*. *H.marginatum* and *H.asiaticumasiaticum*. The present study was attempted to detect some predominant hard tick species in An Najaf province from sheep via molecular tools by using specific PCR assay depending on mitochondrial gene markers and 18S rRNA genes to differentiate between morphologically indefinite hard tick species.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 361 - 370
Received 05 March 2021; Accepted 01 April 2021.

Materials and Methods:

1. Sample collection and Identification :

Samples of hard ticks were collected from different body parts of sheep and goat in An Najaf province –Iraq. Hard ticks were removed from the sheep using forceps and kept in ethanol 70% and stored at freezing for further molecular data analysis. All samples were sorted to females, males, nymphs and full feeding adults. Later using light microscope hard ticks were classified morphologically into genera and species according to morphological keys [6, 7 and 8] .Study areas were selected from three local collection as showing in the figure (1).

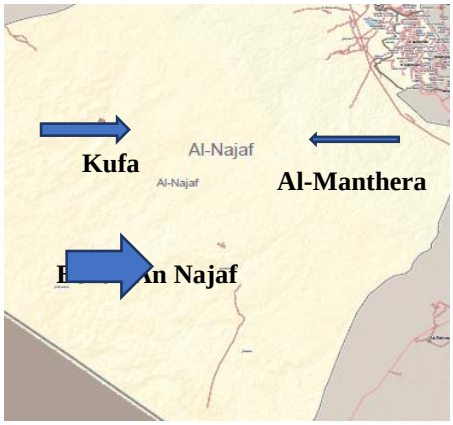

Figure 1. Study areas of three local collections of An Najaf province-Iraq.

2. Extraction of genomic DNA of hard ticks:

Genomic DNA was extracted from whole body of hard tick preserved in ethanol using genomic DNA Mini kit (Tissue) provided from Geneaid Company according to the manufacturer's modified DNA extraction protocol. 1.1 ml of double distilled water (ddH2O) was added to prepare the proteinase K (by vortex to dissolve and obtain concentrate 11mg/ml) and stored at 4 0C until needed.

3. DNA amplification:

Present study based on the mitochondrial cytochrome oxidase C subunit I (COI) was first amplified by the universal primers LCO1490 Forward (GGTCAACAAATCATAAAGATATTGG) and HCO2198 Reverse (TAAACTTCAGGGTGACCAAAAAATCA)[9] and then the primers (18SF: CATTAAATCAGTTATGGTTCC) and (18SR CGCCGCAATACGAATGC) targeted the 18S rRNA [10] .PCR reaction components were according to the following protocol : 12.5µl. of 2X Master ready mix (provided from KAPA2G Biosystems company) 2.5µl volume each of forward and reverse primer at concentration 10 pmol/ µl , 5 µl of template DNA and the reaction was completed with ddH2O to final volume of 25 µl . PCR tubes were carried out on SURE Cycler G8800 PCR apparatus according the thermocycling reaction condition as recommended each primer recommended by [10 and 11]as showing in Table (1and 2). The amplified PCR products were stained by Ethidium Bromide and then were analyzed on 1.5 % agarose gel and tested under UV imager.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 361 - 370
Received 05 March 2021; Accepted 01 April 2021.

Table 1.PCR conditions of mitochondrial cytochrome oxidase C subunitI (COI) gene

Sr.No.	step	Temperature (ºC)	Time	Cycles No.
1	Initial Denaturation	94	30 sec.	1
2	Denaturation	94	30 sec.	40
3	Annealing	40	30 sec.	40
4	Extension	72	60 sec.	40
5	Final Extension	72	10 Min.	1

Table 2.PCR condition of the 18S rRNA gene

Sr.No.	step	Temperature (ºC)	Time	Cycles No.
1	Initial Denaturation	94	3 min.	1
2	Denaturation	94	30 sec.	35
3	Annealing	45	50 sec.	35
4	Extension	72	30 sec.	35
5	Final Extension	72	10 Min.	1

Results

1. Morphological Characters:-

During present study a total number (449) of hard ticks were collected from three local collections of An Najaf province-Iraq that infested on sheep and goat among them the females number were (228) more than males number were (155) whereas nymph number was lower as (66) as showing in table (3).Whereas Al-Manathera as rural local region recorded a maximum percentage was (84.85)% followed by Behar An Najaf was (11.13)% and Kufa city as (4) % (Table 4).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 361 - 370
Received 05 March 2021; Accepted 01 April 2021.

Table 3. three species of hard tick collected from local regions of An Najaf province-Iraq

Local collection	Tick species	Males	Females	Nymph	Total
1.Al-Manathera	*Rhipicephalussanguineus,* *Boophilusannulatus* *Hyalommadromedarii*	50 70 20	70 86 30	30 20 5	150 176 55
2.Behar An Najaf	*Rhipicephalussanguineus,* *Boophilusannulatus* *Hyalommadromedarii*	7 5 1	8 20 2	3 4 0	18 29 3
3.Kufa	*Rhipicephalussanguineus,* *Boophilusannulatus* *Hyalommadromedarii*	0 2 0	7 5 0	0 4 0	7 11 0
Total		155	228	66	449

Table4. The percentages of hard tick collected on each local region of An Najaf province-Iraq

Local Collection	% Percentage of Tick
1.Al-Manathera	84.85
2.Behar An Najaf	11.13
3.Kufa	4

Present study based on morphological and molecular characteristics revealed that there were three species of hard ticks identified as *Boophilusannulatus,Rhipicephalussanguineus*, and *Hyalommadromedarii* at prevalence ratio (48.1, 38.9 and 12.9) % respectively as showed in (Fig.2,3,4 and,5).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 361 - 370
Received 05 March 2021; Accepted 01 April 2021.

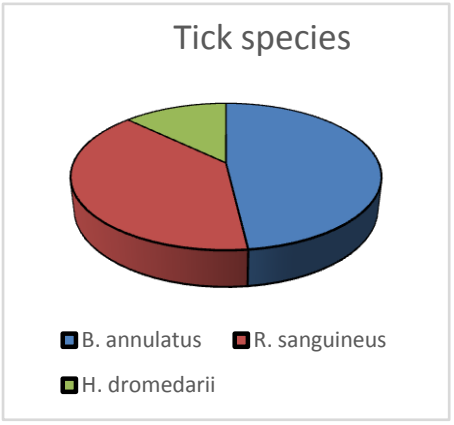

Figure 2.prevalence of three hard tick species collected from An Najaf Province –Iraq.

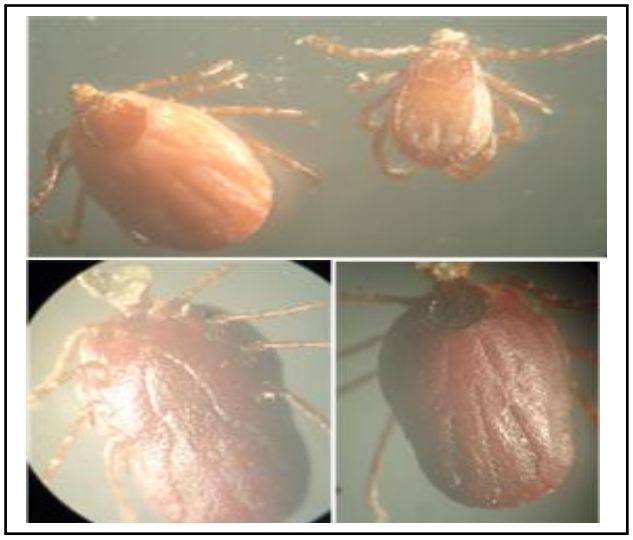

Figure 3. the nymph with male (upper) and adult female (lower) of the cattle tick *Boophilusannulatus*Photographed by light microscope(40X).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 361 - 370
Received 05 March 2021; Accepted 01 April 2021.

Figure 4.the adult female (upper) and male (Dorsal and Ventral view) of the brown dog tick *R. sanguineus* Photographed by light microscope(40X).

Figure 5. the nymph with male (upper) and adult female (lower) (Dorsal and Ventral view) of the camel tick *Hyalommadromedarii*Photographed by light microscope (40X).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 361 - 370
Received 05 March 2021; Accepted 01 April 2021.

2. Molecular Characters:-

Present study based on PCR diagnosis was indicated that there was two species of *R. sanguineus* and *H.dromedarii* were analyzed by using both two specific primers of DNA markers (COI) and 18 rRNA genes the products PCR amplified fragments size were 700 and 780 bp respectively as showed in Fig. (6 and 7).

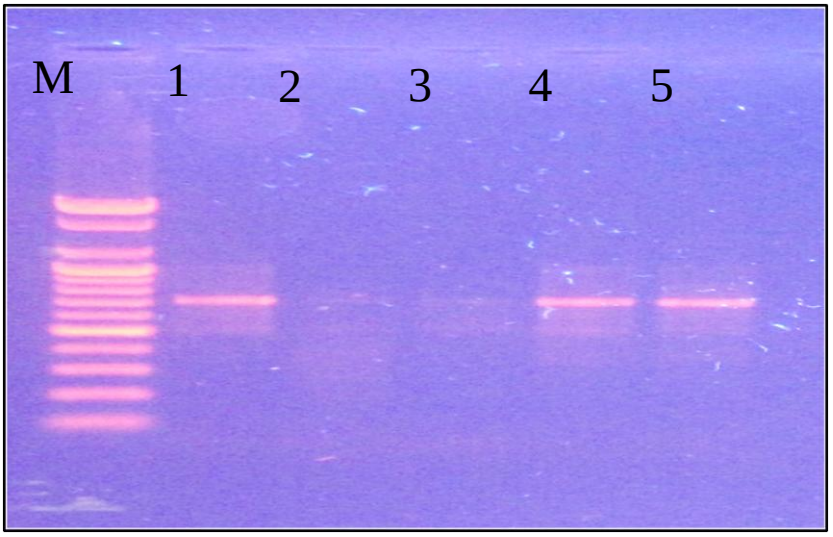

Figure 6. Molecular identification of tick species (*H.dromedarii*and *R. sanguineus*) by PCR products of the mitochondrial cytochrome oxidase I (COI) detected in 1.5% Agarose gel Electrophoresis.(lane M: Marker 100bp DNA Ladder, lanes :(1,4 and 5) positive 700 bp amplicon of COI gene of *H.dromedarii*, lane 2 : control negative ,lane 3: negative result represented of *R. sanguineus*.

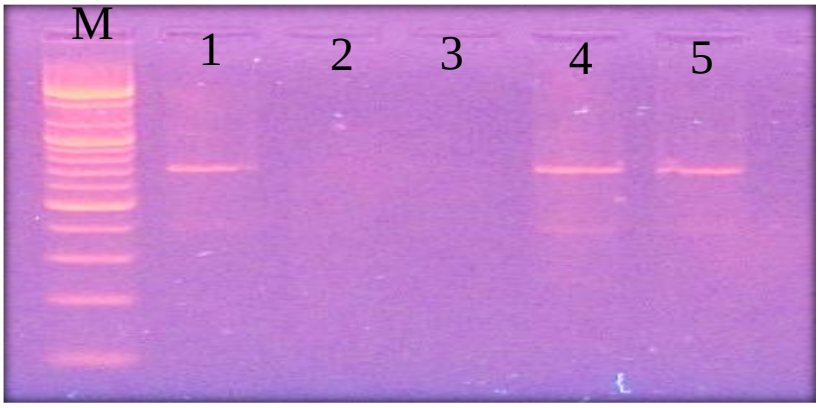

Figure 7. Molecular identification of tick species (*R. sanguineus* and *H.dromedarii*) by PCR products of 18S rRNA gene detected in 1.5% Agarose gel Electrophoresis. (Lane M: Marker 100bp DNA Ladder, lanes :(1, 4 and 5) positive 780 bp amplicon of 18S rRNA gene of *R. sanguineus*, lane 2: control negative, lane 3: negative result represented of*H.dromedarii*.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 361 - 370
Received 05 March 2021; Accepted 01 April 2021.

Discussions

Present study was appeared that there were three species of hard ticks were collected from sheep and coats were *B. annulatus,R.sanguineus,* and *H. dromedarii*at prevalence rate (48.1, 38.9 and 12.9) % respectively in An Najaf province. For more accurate present study used two markers the first one is mitochondrial cytochrome oxidase subunit I gene(COI) for aid in identification of species *H. dromedarii* and the second one was ribosomal Ribonucleic acid 18S rRNA for detection second species of *R. sanguineus*.Since the 18S rRNA is the best used for genera level identification while the (COI) is the most useful marker for tick detection at species level [12, 13 and 14]. Present Results of the Molecular study was showed that PCR assay was able to detect two species easily *R. sanguineus* and *H. dromedarii*by marker genes, although difficulty distinguished between two species *R. sanguineus*and*B.annulatus* but, both morphological and molecular were required to accomplish correct identification. Molecular study will become standard technique for tick identification supporting morphological identification. In present study we utilized two DNA markers CO1 and 18S rRNA revealed that documented length of amplified fragments were 700 and 780 bp respectively. Previous molecular study [11] also mentioned that the specific PCR assay aid in identification of *Rhipicephalus*sp by the fragment amplified was 780 bp in 18S rRNA gene in Canidae in Iraq and the study was represented the first evidence of 18S rRNA. According to former studies [15] the species *R. sanguineus* regarded as complex includes species with very similar morphology which can ready be misidentified.The Species *Boophilusannulatus* recently it has renamed as a subgenus of the *Rhipicephalus* genus [16].Since based on molecular and morphological studies some *Rhipicephalus* were found to be more closely related to *R.* (*Boophilus)* species. Morphological and molecular data have provided important evidence supporting the genus *Rhipicephalus* as paraphyletic to the genus*Boophilus* [17 and 18]. The two species *R.* (*Boophilus) annulatus* and *R. sanguineus* are inhibited arid and temperate climates of Mediterranean regions to Middle East parts and several investigators indicate that*Rhipicephalus* (*Boophilus)* represent a cryptic species [19]. Iraq particularly in meddle and northern part describe with very hot summer may be temperature reaches up to 45 °C. [20]. present study was distinguished *R. sanguineus* from *R.* (*Boophilus) annulatus* via morphological and molecular approaches.

In most region of Iraq the dominant tick genera responsible for infestation of sheep belong to *Rhipicephalus*spp. and*Hyalomma*spp.Study of [21] observed four species of hard ticks were *H. anatolicumanatolicum, Hyalommamarginatum, Rhipicephalusturanicus,* and*Rhipicephalussanguineus* collected from infested sheep in Sulaimani governorate - Iraq. The study of [21] indicated that the highest prevalence was detected in *Boophilus* sp. Followed by *Hyalomma*spp and *Rhipicephalus*spp and these findings are agreed with present study. More recently investigate [5 and 26] found six species in north of Iraq that grouped into genera of *Rhipicephalus*spp. and *Hyalomma* sp. also were recorded. Study of [22] also demonstrated eight main species of hard ticks including (*R. turanicus, R. sanguineus, H.anatolicumanatolicum,H.marginatummarginatum, H. anaotolicumexcavatum. B.annulatus,H.turanicum* and *H. detritumdetritum* were previously recorded from sheep and goats in Erbil northern of Iraq the species *B.annulatus,* was the most prevalence tick species at rate (39.8) % .Comparative studies between present study with other previous studies carried out in different regions of Iraq and other neighboring countries indicated that the main three genera *Hyalomma, Boophilus,* and *Rhipicephalus* were observed. In the middle and southern parts of Iraq previous studies indicated that hard ticks infested sheep and goat were also three genera

B.annulatus and *R. sanguineus* [23]. Study of [4] mentionedthat*Hyalommaanatolicum* was dominant ticks that affect cattle in An Najaf province. The most abundant tick species in borderline of Iraq –Iran was *R. sanguineus*at percentage (60.05 %) collected from sheep [24].
The hard ticks *Hyalomma*are inhabited regions of long dry seasons in Asia whereas the *H. dromedarii*most associated with camels and mainly occurred in areas in Egypt and Iran where adaptation to widely-distributed domestic hosts preferred the camels [9]. Present study was recorded *H. dromedarii*from sheep in An Najaf province.

Hard ticks are transmitters for many of pathogens in Iraq to domestic animals the study [25] have been demonstrated that five tick species were infected with *Theileriaequi* from equids in Erbil province transmitted by *H. marginatummarginatum, H. anatolicumexacavatum, B. microplus, B.annulatus*and *R. turanicus*. Study of [27] detected the presence of *Coxiellaburnetti* by Trans-PCR in *H. anatolicumanatolicum* and *Rsanguineus* collected in southeast Iran. Since hard ticks are regarded as, vectors for pathogenic agents might be transported to the humans and domestic animals and they needed further studies in the future.

References

[1]Mathison,A.B. and Pritt,B. S. (2014). Laboratory Identification of Arthropod Ectoparasites. Clinical Microbiol. Reviews. Vol: 27 pp. 48–67.

[2]Alsaad,K.M. ;Al-Obaidib,Q.T. and Esmaeel,S.A.(2009). Haematological and biochemical study on the effect of some common parasites in negative goat in Mosual area. Iraqi J.Vet. Sci. Vol: 23:101-106pp.

[3]Shubber, H.W.K.; Al-Hassani, N.A.W. and Mohammad,M.K. (2014). Ixodid Ticks diversity in the middle and south of Iraq. International .J.R.C. Research .Vol:5.Issue, No. (9). 1518-1523pp.

[4]Al-Fatlawi, M.A.A.; Ali, M.J. and Albayati, H.H. (2018).Morphological and Phylogenetic study of Hyalommaanatolicum in Al-Najaf-Iraq. Iraqi J. of Veterin.Scie.Vol:32 (No.2):pp.261-266.

[5] Ismael, S.S. and Omer, L.T. (2020).Morphological and molecular study of hard ticks species that infested small ruminants in Duhok governorate, Kurdistan region, Iraq. Bas.J.Vet.Res.Vol:19, No.1, pp.88-108.

[6]Keirans, J.E. and Litwak, T.R. (1989).Pictorial key to the adults of Hard ticks Family Ixodidae (Ixodida: Ixodidae) East of the Mississippi River .J.Med. Entomol.Vol:26 No. (5).435-448pp.

[7] Barker, S.C. and Walker, (2014). Ticks of Australia: The species that infest domestic animals and humans. Magnolia Press Auckland. New Zealand Zootaxa3816:Vol. (1): 1-144 pp.

[8] Walker, A.R.; Bouattour, A.; Camicas,J.L.; Estrada-Peña, A.; Horak,I.G.; Latif,A.A.; Pegram, R.G. and Preston, P.M. (2003). Ticks of Domestic Animals in Africa: a Guide to Identification of Species. a textbook. University of Edinburgh, Atalanta, Houten, the Netherlands.1-227pp.

[9]Fallatah, S. A.; Ghallab,E.H. and Khater, E.I.(2019).Phylogenetic diversity and DNA barcoding of the camel tick Hyalommadromedarii (Acari: Ixodidae) of the Eastern region of Saudi Arabia. Tropical Biomedicine Vol.: 36 No. (2): 390–401pp.

[10]Abdullah, H.H.A.M.; El-Molla, A.; Salib, F.A.; Allam, N.A.T.; Ghazy, A.A. and Abdel-Shafy, S. (2016). Morphological and molecular identification of the brown dog tick Rhipicephalussanguineus and the camel tick Hyalommadromedarii (Acari: Ixodidae) vectors of Rickettsioses in Egypt. Veterinary World.Vol. 9 No. (10): 1087-1101pp.

[11]Hadi, H.D.; Hadi, A.F. and Jassim, S.Y. (2020).Molecular diagnosis of Ixodidae from Canidae in Iraq.Plant Archives.Vol:20 No. (2).353-357pp.

[12]Dobson, S.J. and Barker, S.C. (1999). Phylogeny of the hard ticks (Ixodidae) inferred from 18S rRNA indicates that the genus Aponomma is paraphyletic. Mol. Ecol. Resour. Vol: 11. 288-295pp.

[13]Guglielmone, A.A.; Venzal, J.M.; Gonzáleh, Z.;Acuña, D.;Nava, S.;Hinojosa, A. and Mangold, A.J. (2006) .The phylogenetic position of Ixodesstilesi Neumann 1911 (Acari: Ixodidae): Morphological and preliminary molecular evidences from 16S rDNA sequences. Syst. Parasitol.Vol: 65. 1-11pp.

[14]Erster, O.;Roth, A.;Wolkomirsky, R.;Leibovich, B. and Shkap, V. (2013). Comparative analysis of mitochondrial markers from four species of Rhipicephalus (Acari: Ixodidae). Vet. Parasitol.Vol:198. 364-370pp.

[15]Balinandi, S.; Chitimia-Dobler, L. ;Grandi, G.; Nakayiki, T. ; Kabasa, T.; Bbira,J.; Lutwama,J.J.; Bakkes,D.K. ; Malmberg,M. and Mugisha, L.(2020). Morphological and molecular identification of ixodid tick species (Acari: Ixodidae) infesting cattle in Uganda. Parasit. Research.

[16] Murrell, A. and Barker, S.C. (2003) .Synonymy of Boophiluscurtice, 1891 with Rhipicephaluskoch, 1844 (Acari: Ixodidae). Sys. Parasitol. Vol. 56. 169-172pp.

[17]Barker, S.C. and Murrell, A. (2004). A Systematics and evolution of ticks with a list of valid genus and species names. Parasitol.Vol: 129.S15-S36 pp.

[18] Ali, A.; Parizi, L.F.; Ferreira, B.R. and Junior, I.D.S. (2016). A revision of two distinct species of Rhipicephalus: R. microplus and R. australis. Ciência Rural, Santa Maria.Vol.:46. No. (7) 1240-1248 p.

[19] Ali, A.; Parizi, L.F.; Ferreira, B.R. and Junior, I.D.S.(2016). A revision of two distinct species of Rhipicephalus: R. microplus and R. australis. Ciência Rural, Santa Maria.Vol.:46. No. (7) 1240-1248 p.

[20]Kadir, M.A.; Zangana, I.K. and Mustafa, B.H. S. (2012). A study on epidemiology of hard tick (Ixodidae) in sheep in Sulaimani governorate – Iraq. Iraqi J. of Veterinary Sci. Vol: 26.No. (3). 95-103pp.

[21] Mustafa, B.H.; and Faraj, H. (2013).Resistance of Hard ticks (Ixodidae) with some acaricide in cattle (naturally infestation) in Sulaimani governorate fields –Kurdistan regional-Iraq.J.Agr.Sci.Tech.Vol:3 No. (11): 927-934pp.

[22] Ameen, K.A. (2012). Occurrence of hard tick and its possible role in the transmission of pathogenic agents. Thesis submitted to University of Mosul, Iraq.122pp.

[23] Al-Husseini, M.A.T. (2019). Morphological study of the cattle ticks (Acari: Ixodidae) infesting on sheep in An Najaf province-Iraq. IOP Conf. Ser.: Mater. Sci. Eng. 571 .012061.

[24] Banafshi,O. ; Hanafi-Bojd,A.A. ;Karimi,M.; Faghihi,F. ;Beik-Mohammadi, M.;Gholami, S. ;Javaherizadeh, S.;Edalat, H.;Vatandoost, H. and Telmadarraiy, Z.(2018). Tick Ectoparasites of Animals in Borderline of Iran-Iraq and their role on disease Transmission. J. Arthropod-Borne. Dis. Vol. 12, No. (3): 252–261pp.

[25]Aziz, K .J. &AL-Barwary,L.T.O.(2020). Molecular identification of Theileriaequi and Babesiacaballi from ixodid ticks infesting equids in Erbil province, northern of Iraq. Adv. Anim. Vet. Sci.Vol: 8(12). 1286-1293pp

[26] Ismael, S.S. &Omer, L.T. (2021). Molecular identification of new circulating Hyalommaasiaticumasiaticum from sheep and goat in Duhokgovernorate-Iraq. Iraqi J. of Vete. Sci.Vol:35, No.1, 79-83 pp.

[27]Fard, S.R. &Khalili,M.(2011). PCR-Detection of Coxiellaburnetii in Ticks Collected from Sheep and Goats in Southeast Iran. Iran J. Arthropod-Borne Dis.Vol. 5(1): 1–6pp.